Picosecond time resolved spectroscopy used as a tool to probe excited state photophysics of biologically and environmentally relevant systems

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Picosecond time resolved spectroscopy used as a tool to probe excited state photophysics of biologically and environmentally relevant systems

by

Pramit Kumar Chowdhury

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Physical Chemistry

Program of Study Committee:
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Iowa State University
Ames, Iowa
2004

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For the Major Program
Dedicated to my parents for their constant support and unconditional love
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I would like to take this opportunity to express my gratitude and appreciation to my research advisor, Dr. Jacob W. Petrich. I have no hesitation in saying that I have had a wonderful time working under his supervision and cherished each and every moment with him. He has always been there for me in times of need for support and guidance. I think I have been really lucky to have been given a chance to work for my advisor.

I would also like to thank Dr. Mark Hargrove for collaborating with us and also allowing us to use his laser when needed. He has been very gracious and helpful in providing us with proteins and their mutants which we have used and will be using in many of our ongoing projects in collaboration with him. Thanks are also due to Dr. Suman Kundu, for working tirelessly to prepare protein samples for us. I have to admit that I have bugged him a lot over the years of my stay; being a perfect gentleman as he is, he has always provided us with samples on time, without even a single word of anger or frustration.

Dr. Anindya Dutta, Dr. Kaustuv Das and Dr. Mintu Halder, postdocs in our lab with whom I have had the opportunity of working with, have been so very helpful throughout my stay out here. Thanks are also due to Kyle Ashby, Jin Wen and Jaehun Park (all former graduate students of our lab), Lindsay Sanders and Prasun Mukherjee (current graduate students), Dr. Ranjan Das and Dr. Govindarajan Krishnamoorthy (present post doctoral associates) and Tessa Calhoun and Erin Campbell (current undergraduates in our group) for maintaining such a cordial work environment in the laboratory.

I am also very grateful to the Chemistry Department Faculty and Staff for being of such a great help throughout my stay out here.
CHAPTER I: INTRODUCTION

The advent of ultrafast spectroscopy and its subsequent refinements has opened up many opportunities for investigating processes taking place on timescales ranging from picoseconds to subpicoseconds and even in the femtosecond region. This thesis focuses on some of the applications of ultrafast spectroscopy we have been carrying out over the past few years, with the aid of different laser based instruments in our laboratory.

1.1 Excited state photophysics of biologically active perylene quinones

Hypericin (Figure I.1) is a photodynamic pigment found in certain members of the genus Hypericum. It is particularly prevalent in H. perforatum (St. John's wort), which is a traditional medicinal plant used in several European countries [1]. Hypericin has generated great interest in the scientific world because of its wide spectrum of light induced biological activities [2-6] which include virucidal activity against several types of virus, including the HIV virus [7-8].

The photodynamic action of hypericin may be attributed to either singlet oxygen generation (referred to as Type II mechanism; see Figure I.2 for details) or to local acidification of surroundings by light induced deprotonation of the pigment [6, 9-10]. Hypericin belongs to a broad class of compounds known as photosensitizers. Upon absorbing light, a photosensitizer is first excited to its short-lived singlet state (for hypericin the first excited singlet state has a lifetime of about 5 ns in all the neat solvents in which it dissolves). This is followed by intersystem crossing to the longer-lived triplet state (the triplet lifetime for hypericin is about 2 μs in the absence of oxygen). Thus, the higher the yield of the triplet state, the more efficient will the photosensitizer be in exerting its action. The triplet state can
can now induce two types of photosensitizing reactions, Type I and Type II [4].

In Type I, The triplet photosensitizer directly reacts with a substrate via electron and/or proton transfers, to generate reactive species (see Figure I.2 for details). Thus it is obvious
that Type I is dependent on the photosensitizer and substrate concentrations and will be favored when the former is bound to or associated with the latter. For a Type II process, energy is transferred directly from the ground state to oxygen (which is a triplet) to generate singlet oxygen ($^1O_2$), which being a highly reactive species will bring about a variety of oxidative processes.

An outstanding question is the relative importance of the Type I and Type II mechanisms. It has been found that the acidity of the hydroxyl groups of hypericin increased upon excitation into the singlet excited state [11-18]. By using isotopic substitution (H/D), comparing hypericin with its methylated analogs (which lack the peri hydroxyl H-atoms) and complementary studies involving both transient absorption and fluorescent upconversion techniques [11-16], we have argued that the major primary photophysical process in organic solvents is excited state H-atom transfer. Hypericin has also been found to acidify its surroundings upon light absorption [10, 17, 18]. We have suggested that the labile protons resulting from the intramolecular H-atom transfer reactions may be important for understanding the light-induced biological activity of hypericin. The role of protons generated on illumination has become significant owing to the growing body of literature implicating changes in pH with inhibition of replication [19], antitumor activity [20-21] and apoptosis [22-24].

Having briefly reviewed the biological importance of hypericin, it is natural to inquire about its behavior in the excited state as a function of pH. However hypericin is very insoluble in water and hence pH-dependent studies cannot be carried out in homogeneous solutions. Chapter III refers to the study of the excited state photophysics as a function of pH in the well characterized AOT reverse micelles [25] in heptane. Firstly, the reverse micelles
mimic a biological membrane which is important since it is essential for hypericin to become associated with a membrane to exert its antiviral effects *in vivo* [10, 14, 26]. Secondly, the polar core in the reverse micelles is known to solubilize fairly large amounts of water [25]. This allows us to vary the pH systematically and to look for corresponding effects in the excited state photophysics of hypericin. Hypericin has very low solubility in heptane which thus precludes its partitioning into the bulk solvent and complicate analysis.

Given that there are two hydroxyl groups peri to a carbonyl in hypericin, another natural question arises as to how many H-atoms are actually transferred in the excited state and that whether inhibiting the process in one side of the molecule will have any effects on the other.
side. It was previously observed that when hypericin was bound to Human serum albumin (HSA), it no longer underwent an excited state H-atom transfer [14]. It was argued on the basis of the assumption that since hypericin binding to HSA involves the interaction of one of two carbonyl groups of hypericin and N₁-H single tryptophan residue (W214), this would naturally impede H-atom transfer on that half of the molecule which led to the non-existence of H-atom transfer on the other half thus leading to the conclusion that the double H-atom transfer is concerted.

Hypomycin B (Figure 1.1) is also a naturally occurring perylene quinone extracted and prepared as described elsewhere [27]. It is different from hypericin in that it has only one intramolecular H-bond as opposed to four in hypericin. The absence or presence of excited state H-atom transfer in hypomycin B would go a long way in explaining the nature of the H-atom transfer in hypericin, that is, whether it is concerted or not. Chapter IV focuses on a comparison between hypomycin-B and hypocrellin-A (another perylene quinone like hypericin), the latter also exhibiting intramolecular excited state H-atom transfer quite similar to that of hypericin.

1.2 Heme Proteins

Mechanistic studies of ligand binding to heme proteins have been carried out by several groups to have a better understanding of the factors affecting protein response and ligand recognition and rebinding [28-55]. Three major technological advances allowed researchers to start work in this field with renewed interest. The advances were, (i) laser photolysis techniques allowed Frauenfelder and his coworkers to observe CO (carbon monoxide) geminate recombination in sperm whale at low and room temperatures [28], (ii) The ability
to express and mutate specific amino acid residues of Mb (Myoglobin) and Hb (Hemoglobin) in *Escherichia coli* and (iii) development of relatively inexpensive computers fast enough to carry out molecular dynamics (MD) simulations using different programs [28].

Myoglobin (Figure 1.3), a small globular protein found abundantly in vertebrate muscle tissue, has been on the hot seat for the past two decades with people trying to unravel the intricacies involved in ligand dissociation and recombination. Mb increases aerobic capacity of cells by storing oxygen during periods of rest and facilitates delivery of oxygen to the mitochondria during aerobic respiration [51]. The heme pocket is not a static structure but can be thought of as a transient and mobile volume defined by the sidechains of the residues in the helices on the distal side of the heme group. Thus, if the volume accessible to the ligand is not constant and does not always include the iron atom, the probability of ligand recombination in a given time should vary widely for different atomic coordinates of the protein [52]. These results thus clearly suggest that ligand recombination after photolysis will not progress along a simple path but might follow a distribution of trajectories on the way of its recombination with the heme. Several studies have revealed that the timecourse of a ligand rebinding to the Fe atom of the heme is indeed nonexponential. Figure 1.4 illustrates the possible pathways that exist for a ligand after photolysis (here the dissociation of O₂ from sperm whale Mb has been outlined).

In a typical laser photolysis experiment, the iron-ligand bond is first broken by a laser pulse (Figure 1.4). This gives rise to free ligand inside the distal pocket. A rapid increase in the fraction of deoxy-Mb reveals the extent of photodissociation following laser flash. The ligand can then either rebind internally (geminately) from within the distal pocket or escape into the solvent, assisted by protein fluctuations that transiently open channels [29], thus
Figure 1.3. (a) Sperm whale myoglobin and (b) soybean Leghemoglobin are monomeric hemoglobins having similar structures. (c) Myoglobin uses distal pocket stabilization of oxygen (green region), whereas the proximal pocket (red region) is designed to prevent high-affinity binding. (d) The leghemoglobin distal pocket prevents stabilization (red region) and relies on a proximal pocket mechanism to increase its oxygen affinity. (Figure adapted from reference 61)
leaving a residual fraction of deoxy-Mb. Recombination of ligands which migrated out of heme pocket into the aqueous phase is not seen on a picosecond or a nanosecond timescales but is essentially a much slower bimolecular reaction occurring on the microsecond to millisecond timescale. The kinetic phenomena can be interpreted in terms of the following scheme [31, 40]:

\[
\begin{align*}
\text{MbX} & \quad \overset{\text{ground state}}{\Rightarrow} \quad (\text{Mb + X})_{\text{pocket}} & \quad \overset{\text{contact pair}}{\Rightarrow} \quad (\text{Mb + X})_{\text{protein}} & \quad \overset{\text{distal pocket}}{\Rightarrow} \quad \text{Mb + X} \\
& \quad \quad \quad \quad \quad \quad \quad \quad \quad (\text{ps}) & \quad \quad \quad \quad \quad \quad \quad \quad \quad (\text{ns}) & \quad \quad \quad \quad \quad \quad \quad \quad \quad (\text{ms})
\end{align*}
\]

**Scheme I**

X-ray crystallographic investigations of carbonmonoxy myoglobin at different temperatures complemented by time-resolved infrared spectroscopy of CO rebinding has also revealed a great deal of information on protein dynamics [51]. Below the temperature of 180K geminate fraction of ligand recombination increases. However at \(\sim\)180K and above, a new kinetic component appears at the longest times which can be attributed to bimolecular ligand recombination, after the photodissociated ligand has migrated out of the protein [29]. The fraction of the longer kinetic component increases with increasing temperature and tends to dominate above 200K. In other words, with increasing temperature, geminate recombination of CO tends towards single exponential behavior and can be described by a single barrier [31]. Lowering the temperature slows down protein motions that permit ligand escape, thus increasing geminate fractions. Below 180K, a dynamic transition occurs in which large scale protein fluctuations become arrested such that ligands essentially remain in the heme pocket after dissociation.
Figure 1.4. Schematic structural interpretation of geminate recombination of O₂ to mammalian myoglobin under physiological conditions (adapted from reference 40). Top row from left: (a) the heme pocket structure of MbO₂ with oxygen stabilized by hydrogen bonding by distal histidine; (b) geminate intermediates with Fe atom moving out of plane as a function of time. Bottom row from left; (a) transient upward movement by distal histidine opening transient channels for ligand escape into the solvent. (b) equilibrium deoxymyoglobin structure showing a water molecule hydrogen bonded to distal histidine.
Thus, along with an increase of the geminate fraction, lowering of temperature induces non-exponentiality in the nature of CO rebinding to Mb. What might be the reason behind this nonexponential character? Protein molecules happen to exist in several conformational substates (CS) [53-55] with different barriers for rebinding. Different substates have the same overall structure, but they differ in details; they perform the same function, but with different rates. A classic account of conformational states and substates can be found in the paper by Ansari et. al. [53]. In this paper, the protein reaction has been described as being similar to an earthquake, with stress being relieved at the focus. This released energy is dissipated in the form of waves and through the propagation of a deformation. The stress is released through a series of equilibrium fluctuations (EF) and functionally important motions (FIM). Thus, due to the presence of many conformational substates which equilibrate slowly enough relative to rebinding, the ligand faces a distribution of barrier heights during recombination, hence giving rise to nonexponential kinetics.

To investigate the existence of nonexponential kinetics at room temperature, the use of NO (nitric oxide) as the ligand has been universally accepted over CO and O₂. This is because NO is extremely reactive towards the ferrous ion, showing little or no quantum mechanical barrier to bond formation and rebinding to native myoglobin almost completely on picosecond timescales [40, 53]. A large percentage of NO molecules rebind within ~300-500 ps at room temperature. Protein relaxation to equilibrium after dissociation is likely to occur on the same timescale as the NO rebinding. Thus study of NO permits a more direct investigation of the transient structural factors affecting ligand rebinding at room temperature. In some cases it has been found that NO rebinding is nonexponential at room temperature. On the other hand for CO, because of the large electronic barrier at room
temperature to ligand binding, one does not even begin to observe CO recombination until about 100 ns subsequent to photodissociation. Thus Mb would be completely relaxed by the time rebinding occurs, and exponential behavior would be expected and is observed [31] (see Figure I.5 for details).

Leghemoglobin (Lb) (Figure I.3), another monomeric heme protein like Mb, is found in the root nodules of leguminous plants. It plays a very important role in nitrogen fixation, where it facilitates diffusion of oxygen while maintaining a low (nanomolar) free oxygen concentration [32, 56-61]. Though soybean Lb (Lba) has a similar 3D structure to that of Mb, the former has an oxygen affinity almost twenty times higher than that of the latter protein. This 20-fold increase in oxygen affinity for Lba is extremely useful for facilitated diffusion while scavenging oxygen to prevent inhibition of nitrogen fixation. As outlined by Kundu and Hargrove [60], one of the causes of the above disparity might be the difference in structures of the proximal heme pockets of Mb and Lba. In Mb proximal pocket, the Ser(F7) facilitates hydrogen bonding network that drives His(F8) into a conformation that destabilizes ligand binding through trans effect while in Lba, apolar Valine at F7 position provides freedom of rotation to Histidine (F8) and thus the proximal heme pocket stabilizes ligand binding [62].

The extent and rate of geminate ligand recombination in heme proteins is affected mainly in two ways, viz, (i) proximal pocket effects [31, 35-38, 41, 63] and (ii) distal pocket effects [28, 33, 36, 45, 51, 60]. The extent to which the above determine ligand rebinding rates has been a subject of extensive amount of research over the years. Though evidence does suggest an overwhelming domination of the role played by amino acid residues in the distal pocket, still a number of papers do suggest otherwise, that is, the proximal effect has a significant
contribution as well. Almost all the attempts made at unraveling the intricacies of the protein-ligand relationship in heme proteins, have focused on Mb (namely sperm whale Mb). However, given the existence of newly discovered bacterial, protist and plant hemoglobins, one would naturally like to look for alternative systems to have a clearer picture of the emerging view of protein dynamics. The study of leghemoglobin is ideal in this respect because, as mentioned before, despite its similarity to Mb in terms of structure, it does display many opposite extremes of reactivity [60, 64].

Chapters V and VI focus on both the proximal and distal pocket contributions to ligand (NO) recombination on the picosecond timescale. Whereas chapter V shows little and in some cases almost no difference in the recombination due to proximal effects, chapter VI, being the first study of its kind on effects of distal mutations in Lba, shows a significant amount of change both in the 40 ps and 400 ps timescales, for ligand recombination.

As a final note to this section, the following two points are worth mentioning: (i) to carry out pump probe studies on the proteins mentioned above, one might excite the sample at higher energy, typically with a wavelength of ~410 nm, which corresponds to the Soret-band excitation; the other way of exciting the samples is known as the Q-state pump, which is of lower energy (typically ~550 nm) as compared to the Soret band. It has been found that differing amounts of energy initially deposited by excitation does not have a significant effect on ligand recombination dynamics (41). This suggests that ligands dissociate with similar kinetic energies in both the cases. Thus, the additional energy \( \frac{(E_{\text{Soret}}-E_0)}{hc} \) (6000 cm\(^{-1}\)) remains in the reaction products, suggesting that a significantly fast intramolecular energy redistribution before the dissociation to deoxy heme occurs. These hotter reaction products then undergo vibrational cooling. (ii) Another question that might be raised is that if
the characteristic time scales of biochemical processes are microseconds to seconds in duration, how will motions on a picosecond timescale contribute to the survival of an organism? By comparing a triple mutant of Mb, Mb-Y(B10)Q(E7)R(E10) (unique among all heme proteins studied till date because of its very slow geminate recombination, ~800 ns) with *Ascaris suum* hemoglobin (in this protein, the geminate recombination is complete within 0.4 ns), with the former mimicking the distal site of the latter [65, 66], it was found that the oxygen dissociation rate for Mb-YQR is almost >200-fold faster that that of *A. suum* hemoglobin, thus suggesting additional factors affecting the dissociation rate. From these
studies, the authors conclude that the overall oxygen dissociation rate is controlled not only by the iron-ligand coordination bond and local hydrogen bonding network, but also by fast fluctuations of internal side chains occurring on a picosecond timescale, which either permit or obstruct access of the ligand to cavities within the protein from where the oxygen escapes.

1.3 Solvation Dynamics

Solvents can affect chemical reactions in many different ways. A solvent can bring about stabilization of reactants and (or) products relative to their gas phase energetics. This influence is considered to be a static one [67]. On the other hand, the solvent can interact with a solute dynamically, that is, bring about time-dependent change in energetics (see Figure 1.6 for details). This latter phenomenon has been popularly referred to as solvation dynamics. Charge-transfer and electron-transfer reactions have been shown to be highly dependent on the solvent [68-81]. A vast amount of work, both experimental [81-100] and theoretical [101-107] has been carried out on the dynamical aspects of solvation and is still a very active area of research.

The basic principle behind a solvation dynamics experiment is to determine how quickly a polar solvent can respond to a solute dipole that is created in the excited state. For such a study, the solute under investigation should have an appreciable increase in dipole moment in its excited state. In the ground state, the solvent molecules are optimally arranged around the solute to lower the energy of the system and are in equilibrium. After interaction with an ultrashort laser pulse, a sudden dipole is created [82]. This gives rise to a non-equilibrium condition where the solvent molecules have not changed with respect to their initial configuration. This non-equilibrium state lies high on the excited state potential energy
surface. As time progresses, the solvent starts reorienting with respect to the newly created
dipole, resulting in the gradual shifting to states of lower energy. As a result, if one monitors
the fluorescence spectrum as a function of time after initial excitation of solute, one finds a
gradual decrease in the emission maxima. This shift is known as the time dependent
fluorescence stokes shift (TDFSS) and the emission spectra at different times are referred to
as the time-resolved emission spectra (TRES). TDFSS provides a direct measure of the
kinetics of solvation occurring at the microscopic level of relevance to chemical systems
[81]. Figure 1.6 provides a scheme of the dynamical aspects of solvation. Decays collected at
the red edge of the emission spectrum of the probe solute are characterized by rise times.
This rise thus corresponds to the formation of a solvated species.

Solvation times can vary from very short timescales, sub picoseconds in water [86, 88],
to very long timescales, nanoseconds, in restricted environments with micelles, glasses and
proteins being examples of the latter [90, 91, 93-95]. One can follow solvation dynamics by
monitoring rotation times of the probe solute; however in organized assemblies where the
probe is present in a rigid environment, for example tightly bound to a protein or attached to
cyclodextrins, the overall motion of the assembly will be imposed on the rotational dynamics
of the probe solute thus inhibiting us from extracting meaningful information of the probe
dynamics. Solvation dynamics however occurs on fast time scales and hence is relatively
independent of the much slower motions of the macromolecular chains [83]. The dynamical
aspects of solvation are thus a very useful tool to probe the local motions of probes in
restricted environments.

Chapter VII refers to the study of solvation dynamics of a new class of liquids, popularly
known as room temperature ionic liquids (RTIL). RTILs have been a center of interest for
"green chemistry" for the past decade and have generated great interest in both industry and academia [108]. They possess a wide liquidus range, in some cases in excess of 400 °C [109]. It has been shown that the ionic liquids can be used as substitutes for commonly used highly volatile and/or toxic solvents that have been banned. Typically, a RTIL consists of nitrogen- or phosphorus-containing organic cations and large organic or inorganic anions [110, 111]. A great advantage of ionic liquids is that their physical and chemical properties can be varied over a wide range by the selection of suitable cations and anions. This permits the optimization of the ionic reaction medium for a specific application by controlled tuning of the relevant solvent properties. Hence, these liquids have also been termed "designer solvents" [111, 112]. Ionic liquids have negligible vapor pressure which facilitates separation of reaction products by distillation since the problem of formation of azeotropes does not arise. RTILs have also been used as gas chromatographic stationary phases [113], and they have been used in the making of chiral stationary phases by dissolving chiral selectors [115].

Attempts have been made towards defining a polarity parameter for the RTILs [116-118] to help identify the interactions and properties that are important for specific chemical applications. Pande et. al. have studied the solvatochromic probe behavior and effect of water on the emission and absorption spectra of the probes using 1-butyl-3-methylimidazolium hexafluorophosphate as the RTIL [119,120]. Given the wide applicability of RTILs and their tunability to meet the specific requirements for different reactions and processes, one would naturally start to wonder about their dynamic aspects of solvation. Researchers have already begun studying solvation dynamics in different types of RTILs using different probes [121-123] in order to rationalize their unique properties. Chapter VII focuses on the experiments carried out in our laboratory on five different types
of RTILs to provide a comparison and try to correlate solvation dynamics with their corresponding viscosities.

**Dielectric Relaxation in Proteins**

Dielectric relaxation in response to charge separation or transfer is a crucial component of protein electrostatics. In the case of a very fast process such as electron transfer, only the electronic polarizability will contribute to the relaxation at very short times (femtoseconds) while reorientation of polar groups will develop over longer time periods (picoseconds or more). To achieve a better understanding of the protein dynamical response to charge separation processes, several groups have carried out both experimental [94, 105, 125-129] and molecular dynamics simulation studies [124, 127, 130-139]. In spite of the considerable efforts already made towards the understanding of dielectric relaxation processes in proteins, a reliable estimate for the protein’s dielectric response is lacking. The two most widely used models to describe dielectric fluctuations are, namely, (i) a full atomistic description of proteins and (ii) the dielectric continuum description. The first model involves a detailed atomistic computer simulation which obviously is very time consuming and entails enormous computational cost, while the second model oversimplifies the picture with its shortcoming stemming from the fact that a protein is in fact a highly homogeneous dielectric medium. The model we are trying to use is one which would recognize the highly inhomogeneous nature of the protein and which involves a collection of constrained polarizable dipoles [140] embedded in a dielectric continuum solvent to describe the dielectric response of the protein. On the experimental side, we have proposed that the complex of coumarin 153 (C153) with apomyoglobin provides us with a robust system to test the validity of the aforesaid model.
Chapter VIII deals with the exhaustive characterization of the C153 and apomyoglobin complex and is the first step towards achieving our goal of employing the new model system for protein dielectric response.
References


CHAPTER II: EXPERIMENTAL APPARATUS

II.1 Time Correlated Single Photon Counting technique (TCSPC):

Time Correlated Single Photon Counting is the method of choice for fluorescence lifetime measurements. This technique has been widely used by research groups to study emission related processes of various interesting systems. A number of excellent reviews of this technique [1-6] have been published. Here, I would like to give a brief overview of the basic principles and components of this technique and describe the single photon counting apparatus in our laboratory.

A. Basic Principle

A pulsed light source is used to excite an assembly of sample molecules. The fluorescent decay profile of the excited molecules is described by the equation

\[ [S^*] = [S^*]_0 \exp(-t/\tau_S) \]  

where \([S^*]\) and \([S^*]_0\) are the excited state populations of the fluorophore at a certain time 't' and 't = 0' respectively, with \(\tau_S\) being the fluorescent lifetime of the sample. The single photon timing technique records this profile by measuring the time delays between individual fluorescent photons and the excitation pulse. Thus this technique relies on the fact that the probability distribution for emission of a single photon after an excitation event yields the actual intensity against time distribution of all the photons emitted as a result of the excitation [1]. A detector (PMT) detects the emitted photons from the sample. The trigger pulse of the light source and the detector signal are both routed through a set of
discriminators to provide the START and the STOP signals for a very important component of the TCSPC, known as the Time-to-Amplitude Converter (TAC). The output from the TAC is sent through the Analog-to-Digital Converter (ADC) and the counts are stored typically in a Multichannel Analyzer (MCA). Figure II.1 shows a very rough outline of a typical TCSPC setup. Figure II.2 is a detailed outline of the apparatus being used presently in our laboratory.

![Diagram](image)

**Figure II.1.** A schematic outline of a typical TCSPC apparatus adapted from [5].

### B. Light Source

Our laboratory is equipped with a synchronously pumped cavity dumped Coherent 701 Dye Laser. The dye laser is itself pumped by \(~1.2-1.5\) W of 532 nm green, produced after the frequency doubling of the 1064 nm fundamental beam of a mode-locked Coherent Antares 76-S Nd:YAG laser with a repetition rate of 76 MHz. The dye laser, after cavity
dumping has a repetition rate of 3.8 MHz (As will become clear later, this repetition rate would prove to be a very important information for the operating mode of the TAC). The typical pulse width of the dye laser is ~10 ps. The dye laser is tunable from 560-620 nm in the visible range, obtained by changing the micrometer screw reading associated with the birefringent crystal. This visible output can be frequency doubled by focusing into a potassium dihydrogen phosphate crystal (KDP) to generate ultraviolet (UV) excitation source in range of 280-310 nm. The excitation light is subsequently focused into the sample being studied, through an appropriate combination of lenses and excitation polarizer, the latter helping to maintain the proper polarization of the excitation light (please see Figure II.3 for details).

Before ending this section, I would like to mention some regular maintenance procedures which are helpful in keeping the laser running at its optimum performance specifications. It is always a good idea to clean the beam splitter and the output coupler optics of the Antares head, which houses the Nd:YAG rod. A proper way of removing dirt from the surface of the optics is by using Kodak lens cleaning tissues (these have proven to be the best; use of any other brand might lead to unwanted scratches on the optics), in combination with one or two drops of methanol (or acetone) as the cleansing solvent. During this operation, it is very important to remember to either switch the laser off or at least, close the manual shutter to block the 1064 nm fundamental light beam. The fundamental is invisible to the naked eye and is of very high intensity. Daily cleaning of optics (as mentioned above) and the birefringent crystal of the dye laser is also a good practice.

Another aspect of satisfactory laser performance is to optimize both the Antares head and the dye laser optics every time the laser needs to be used. For the Antares head, proper
Figure II.2. Sketch of the detailed arrangement of the TCSPC setup in our laboratory.
optimization of the cavity dumper, output coupler and second harmonic crystal has proven to be more than enough for daily usage. However, if the laser has not been used for a lengthy period of time (a week or more), one might need to optimize the high reflector (also known as the back mirror) at one end of the cavity. It should be kept in mind that the laser power is very sensitive to the position of the back mirror. Hence, this component needs to be handled with utmost care and if possible should only be touched by experienced personnel. Throughout this process of optimization, one needs to keep track of the laser power with the help of a power meter and also monitor the noise as displayed on the LCD screen of the Remote Module of the Antares laser. High values of noise means that the laser is very unstable and working under such a condition might lead to severe damage of the SHG (second harmonic generator). Even before toggling the shutter button of the remote to the on position, it is always a good idea to check on the noise level and bring its value down to 1 or 2 (whichever value is easily achieved by the user) by tweaking the cavity dumper knob (specially the coarse one for larger adjustment steps). Following this routine on a daily basis will definitely lead to satisfactory performance of the laser.

C. Sample Chamber

The sample chamber in our laboratory consists of the following components: (i) sample cuvette holder, the temperature of which is maintained by a Neslab temperature controller (model RTE-111), (ii) emission polarizer after the sample; this polarizer is controlled by a motor which comes in very handy during anisotropy measurements when we can alternate between parallel and perpendicular collections at regular intervals so as to account for the laser instability over long periods of data collection, (iii) a filter mount to
hold both band pass and interference filters; filters are typically used to eliminate unwanted light wavelengths from the sample fluorescence, (iv) lens to focus the emitted light onto the monochromator and (v) shutter and monochromator assembly (monochromator can be replaced from time to time with filters to collect the whole emission band) which in turn is screwed tightly onto the detector (an MCP-PMT in our case) and which brings us to the next topic of our discussion. Figure II.3 details out the different components of the sample chamber.

D. Detector

After a sample is excited with laser light, a portion of the sample molecules will absorb light and move to a higher energy level and subsequently fluoresce. In our laboratory, we collect the fluorescence at right angles to minimize signal distortion by excitation light. The intensity level of light thus collected after the sample is extremely low (will vary according to the sample under investigation) and thus can be treated as single photons [3]. The detector as a result ends up detecting very few photons. Thus one major criterion in detector selection is how sensitive it is to low light levels. Another important factor one has to contend with is the final pulse width of the excitation light after being sent through the detector and TCSPC electronics. The width of the instrument response function is best described to be the result of a combination of three principle factors [1], namely, (i) duration of excitation pulse (ii) transit time spread of photoelectrons in the detector (iii) timing jitter associated with the electronics. Not much can be done with the excitation pulse duration for a commercial laser. Hence the main source of broadening is the detector. One has to be very careful and selective about the detector best suited for the system. The type of detectors
commonly used are, (i) photomultiplier tube (PMT) (ii) micro channel plate photomultiplier tube (MCP-PMT) and (iii) avalanche photodiode (AVD). The following paragraph focuses on our choice of MCP-PMT as the detector.

The basic principle on which the detectors work is ‘the photoelectric effect’. Excellent details of how different kinds of detectors function can be found in a number of books and articles [1-3]. A traditional PMT uses a series of dynodes which are charged metal structures coated with a secondary emitting surface of a semiconductor material [3]. An MCP-PMT on the other hand has thin plates of glass with many microscopic channels (~12.5 microns in diameter; for a comparison, human hair is 60-80 microns in diameter) through
them. In MCP, the electrons have a much shorter distance to travel as compared to a traditional PMT. The time that elapses between the moment an electron is ejected from the cathode and the corresponding bunch of electrons reaching the anode is called Transit Time. Since electrons have to travel shorter path lengths in an MCP-PMT as compared to a traditional PMT, this leads to lesser fluctuation in time intervals between arrival of photons and subsequent anode pulses. As a result there is much less spread in time in the electron bunch moving down the channels in the MCP-PMT. This spread in time is called the Transit Time Spread and has a significant effect on the Full Width at Half Maximum (FWHM) of the instrument response function. The MCP-PMT can thus time events much more accurately leading to narrower instrument function. Avalanche photodiodes have very high quantum efficiency (quantum efficiency is the ratio of anode pulses reaching the detector per photon striking the cathode) but suffer from the disadvantage of pulse widths in the order of ~300-800 ps thus making them less useful for lifetime measurements. The MCP-PMT installed in our system is the Hamamatsu R3809U-50 type. The typical FWHM of our instrument response function is ~130 ps as measured by using a nondairy coffee creamer as the scatterer. It is to be kept in mind that since the MCP is very sensitive, care should always be taken not to expose it to high levels of light; under such a circumstance there is every chance of the MCP-PMT getting burnt. For further help please refer to the MCP manual before beginning to work with the detector.

E. Discriminators

A discriminator is designed to differentiate between varying levels of electrical signals. In other words, if the input signal be below a specified threshold level of the
discriminator, the signal will be completely ignored. Input signals greater than the threshold will be recognized as a result of which the discriminator will produce an output pulse [4]. The output from the MCP-PMT consists of a broad distribution of pulse heights, some of which being generated by dark noise, some by single and rest by multiple photon events [1]. To provide the TAC with constant amplitude pulses independent of pulse shapes, it is important to discriminate between the various pulse heights in order to improve signal-to-noise ratio. Thus pulses have to be sent through discriminators before they can be fed into the TAC. By setting the discriminator threshold level greater than the PMT noise level, but less than the actual signal level, one can eliminate PMT noise and drift on the experiment. In our laboratory we use the TC 455 QUAD CF (constant fraction) Discriminator. For further details concerning the principles of discriminator and TAC operation, we refer the reader to references [1, 4] at the end of the chapter. Figure II.4 is an outline of the different parts of the TCSPC electronics.

F. Time-to Amplitude Converter (TAC)

The time-to amplitude converter often abbreviated as TAC can be viewed as a stopwatch carrying out time correlation between START and STOP pulses. Upon receiving a START signal, the capacitor inside the TAC is charged linearly at a rate set by the constant current source. The charging is discontinued on the acceptance of a STOP pulse. Because the charging current ‘I’ is constant, the voltage developed on the capacitor can be written as

\[ V = \frac{It}{C} \]  
(II.2)
where ‘t’ is the time interval between START and STOP pulses and ‘C’ is the capacitance of the capacitor of the converter. The voltage pulse height is thus proportional to the time interval. Once a START pulse has been accepted, all further START pulse are ignored until the conversion and converter reset process are finished. Similarly, the TAC responds to the first STOP pulse that arrives after the accepted START pulse and ignores all subsequent stop pulses until the next valid START pulse has been accepted. As a result, subsequent START pulses find the TAC dead from the time of acceptance of the last valid START pulse until the end of the TAC reset. This time during which the TAC remains insensitive to the arriving START and STOP signals, is known as the Dead Time (typically in the range of a microsecond). It is important to remember that the TAC can only process one pair of START and STOP pulses in each conversion.

The TAC can be run in two different types of mode, namely, (i) Forward Mode and (ii) Reverse Mode. In forward mode, the excitation light source (in our case the synchronized output of the cavity dumper of the dye laser), acts as the START signal for the TAC while pulses from the detector provide the STOP signal. However at high repetition rates of
excitation source (~ 4 MHz for our laser system), running the TAC in forward mode is a very big disadvantage. At this high repetition rate, which usually means a high rate of TAC START pulses, the TAC gets ‘swamped’ [4] by START pulses but never stopped by a STOP pulse. This makes the TAC always look busy, being unable to accept another START pulse; in other words the TAC remains dead for a long time. To avoid this, the TAC can be run in the reverse mode (as done in our laboratory). In this mode of operation, fluorescence signals from the PMT are routed to the START input of the TAC while trigger signals are sent to the STOP input.

Advantages of using TCSPC in fluorescence lifetime measurements

(i) Temporal Resolution and Lifetime Range: The lifetime range covered by TCSPC can range anywhere from 5 ps to ~ 50 μs (covering a span of over 7 orders of magnitude). In most cases, it is not the TCSPC technique that sets the limits for temporal resolution and lifetime range measurements, but the light source and detector being used. The lower limit of the lifetime range (τ_{min}) can be estimated from the following equation

\[ \tau_{\text{min}} = \frac{1}{10} (\text{FWHM}^2_{\text{Light Source}} + \text{FWHM}^2_{\text{TTS Detector}} + \text{FWHM}^2_{\text{Electronic Jitter}})^{1/2} \]  

where TTS stands for the transit time spread of the detector as described before. The factor 1/10 represents that with proper numerical reconvolution, lifetimes as short as approximately 1/10 of the instrument response function can be resolved.

(ii) Sensitivity: TCSPC measures single photons. This technique requires an excitation source with high repetitive pulse output. Since the process of capturing a photon is repeated several thousands or even million times per second, a sufficiently high number of single photons is processed for the lifetime measurement. Also, since only one photon is
processed at time, the sample excitation pulses are necessarily of low energy, thus causing minimum sample degradation and circumventing nonlinear effects.

(iii) *Noise Statistics*: TCSPC data are governed by Poisson noise statistics. Poissonian noise is given by the square root of the number of counts i.e. the uncertainty in the number of counts is given by $N^{1/2}$, where $N$ is the number of counts in a specific channel of the MCA. This precise knowledge of the noise nature has significant impacts on data analysis and dynamic range. In other words, big data values are ‘noisier’ than smaller data values. Hence, smaller data values can be better viewed and analyzed than with analogue techniques (the TCSPC technique being digital in nature). Also as a single photon can be observed as well as the signal maximum (typically $10^4$), the dynamic range of measurement can be said to be $10^4:1$. This allows display of data on semi-log plot when a multi-exponential with long and short components can be plotted and analyzed with high precision.

(iv) *Robustness*: TCSPC is largely insensitive to fluctuations of excitation pulse intensities, detector output pulses and detector background noise. The background is eliminated to a large extent by setting the discriminator threshold to a certain value, so that only pulses having sufficient intensity will be processed. Also, the constant fraction discriminator evaluates the temporal position of the pulse by the fastest rise of the pulse. Thus pulses of different heights still have the same temporal profile.

II.2. *Pump-Probe Spectroscopy*

Pump-probe spectroscopy is a powerful technique for investigating electronic vibrational properties of a variety of systems. The pump-probe spectroscopy needs at least
two ultrashort light pulses. The "pump" pulse perturbs the sample at time $t = 0$. The second pulse, known as the "probe" pulse, which is much weaker in intensity as compared to the pump pulse and is delayed with respect to the pump, crosses the perturbed sample and acts as a probe. The change in transmission of the weak probe pulse is thus observed as a function of the time delay [2, 7]. In the simplest application of this method, both the pump and probe pulses originate from the same output train of an ultrafast laser and is known as "degenerate" or single color pump-probe spectroscopy. In the "non-degenerate" case (also known as multi color), either two synchronized lasers at different wavelengths or a laser and white light continuum (the one used in our laboratory) are used.

The femtosecond transient absorption apparatus in our lab is home-made and consists of the following four basic components: (i) the Ti:sapphire oscillator (optical resonator), (ii) the amplifier, (iii) the OPA (optical parametric amplifier) assembly and (iv) the detection system. Before delving into the details, let us have a brief overview of a couple of very basic ideas on which the apparatus has been assembled.

(a) **Modelocking**: Modelocking is a technique for producing ultrashort light pulses (in the order of picoseconds and even femtoseconds). For each transverse mode, the laser cavity allows oscillation only at discrete frequencies or longitudinal modes. For a simple plane mirror laser cavity, the frequency separation between any two adjacent modes is given by

$$\Delta v = \frac{c}{2L}$$  \hspace{1cm} (II.4)
where $c$ is the velocity of light and $L$ is the separation distance between the two mirrors. In a simple laser, each of these modes oscillates independently without any fixed relationship between each other. Instead of being independent from each other, if each mode is made to operate with a fixed phase between each other, the laser modes will periodically undergo a constructive interference together, producing an intense burst or pulse of light. Such a laser is said to be modelocked (Figure II.5). The separation time between the pulses is given by

$$\tau = \frac{2L}{c} \quad (\text{II.5})$$

which is exactly the amount of time required for one round trip in the resonating cavity. The duration of each pulse is determined by the number of modes oscillating in phase. Thus if there are $N$ modes oscillating in phase, with a frequency separation $\Delta \nu$, the overall modelocked bandwidth is given by $N\Delta \nu$. It can be shown that the pulse duration is given by the relation

$$\Delta t = \frac{2\pi}{N\Delta \nu} \quad (\text{II.6})$$

Thus wider the bandwidth, shorter will be the pulse. For a pulse with Gaussian temporal shape

$$\Delta t = \frac{0.44}{N\Delta \nu} \quad (\text{II.7})$$
where 0.44 is the time bandwidth product of the pulse and varies depending on the pulse shape while $\Delta t$ is the minimum possible pulse duration. Thus for a Ti:sapphire laser with a bandwidth of 128 THz, $\Delta t$ would be only 3.4 femtoseconds. However, in real life, the actual pulse duration will depend on actual pulse shape and cavity dispersion. The pulses of modelocked lasers are also distinguished by high intensities [9, 10].

There are two different types of modelocking. (1) **Active Modelocking**: This uses an external signal to induce modulation of the intra-cavity light. If the modulation frequency be chosen such that it is the same as the frequency of spacing between adjacent longitudinal modes, then, due to the modulation, for each mode, side bands are generated whose frequency coincides with the frequencies of the two neighboring modes. All modes will be synchronized at a sufficiently strong modulation [10]. Acousto-optical and electro-optical modulators are mostly used for this type of modelocking. Synchronous pumping also belongs to this category of active modelocking. Here the pump source is typically another modelocked laser. This technique requires accurate matching of the cavity lengths of the pump laser and the driven laser [9].

(2) **Passive modelocking**: This technique does not require a signal external to the laser, to produce pulses. A saturable absorber has been used extensively for this type of modelocking. Organic dyes in solution have been used as saturable absorbers. The essential feature of a saturable absorber is that its transmission at the laser wavelength increases nonlinearly with increasing light intensity. Thus, it will encourage the laser to operate in a pulsed mode since loss is lower in this case i.e. it will attenuate continuous wave light [10]. A type of passive modelocking which needs separate mention is the kerr lens modelocking (KLM). For large incident light intensities “I”, the refractive index “n” of a medium depends on the intensity as
Figure II.5. Essentials of modelocking (adapted from reference [8]).
follows:

\[ n(\omega, I) = n_0(\omega) + n_2(\omega) I \]  \hspace{1cm} (II.8)

where \( n_2 \) is the nonlinear refractive index. This intensity-dependent change of the refractive index is caused by the non-linear polarization of the electron shell induced by the electric field of the optical wave and is therefore called the Optical Kerr Effect. Because of the radial intensity variation of a Gaussian beam, the refractive index of the medium shows a radial gradient, under the influence of the beam, with the maximum value of \( n \) at the central axis. This acts as a focusing lens (Figure II.6) and leads to focusing of the incident laser beam (as done in the Ti:sapphire resonator). Since the central part of the pulse profile has the largest intensity, it is focused more strongly than the outer parts which are of lower intensity. If the leading and trailing edges be cut away, the transmitted pulse will be shorter than the incident pulse [11].

\[ \text{Kerr Medium} \]

\[ \text{Phase Fronts} \]

\textbf{Figure II.6.} The self-focusing of an intense laser beam propagating through a Kerr medium is a key step in kerr lens modelocking.
(b) **Group Velocity Dispersion**: In optics, dispersion is a common phenomenon giving rise to separation of wave components into different frequencies. The most commonly seen sequence of dispersion in optics is the separation of white light into a color spectrum by dispersion. For visible light,

\[ n(\lambda_{\text{red}}) < n(\lambda_{\text{yellow}}) < n(\lambda_{\text{blue}}) \]  

(II.9)

from where we have the relation \( \frac{dn}{d\lambda} < 0 \) which means that \( n \) (refractive index) decreases with increasing wavelength (\( \lambda \)). This accounts for the stronger bending of blue as compared to red, resulting in angular spread of colors. Dispersion can be of two types, namely, (i) spatial (angular) and (ii) temporal (chirp) (Figure II.7). An optical pulse can be treated as a wave packet propagating through a dispersive medium, with a central frequency \( \omega_0 \). The wave propagation vector \( k (k = n\omega/c) \) can be expanded with respect to frequency based on the Taylor series as follows:

![Angular Dispersion](image)

![Chirp](image)

**Figure II.7.** Effects of dispersion of light (adapted from ref [8]).
where $\delta \omega = \omega - \omega_0$. The first term $k(\omega_0)$ is the propagation vector for the center of the pulse frequency spectrum [12]. The linear term $\frac{\partial k}{\partial \omega}$ is referred to as the inverse of group velocity ($v_g$) of the light pulse [13]. The third term (quadratic), $\frac{\partial^2 k}{\partial \omega^2}$, describes the change in pulse shape during propagation i.e. the group velocity dispersion (GVD). As a result of GVD, a short pulse will experience a change in its duration while propagating in a dispersive medium. Using the relations $k = \frac{\omega n}{c}$, $\lambda = \frac{2\pi c}{\omega}$ and simple calculus, it can be shown that

$$v_g = c[n - \lambda \frac{\partial n}{\partial \lambda} \lambda_0]^{-1}$$

Group velocity is very important for nonlinear processes like sum frequency generation (upconversion) or second harmonic generation, where the group velocity mismatch between generating and generated pulses can lead to pulse broadening. For frequency doubling, the group velocity mismatch is given by

$$\Delta v_g = [v_g(\lambda_0) - v_g\left(\frac{\lambda_0}{2}\right)]^{-1}$$
In order to keep second harmonic pulse width shorter than or equal to that of the
fundamental, the crystal length should be shorter than

\[ L_c = \tau_p \Delta v_g \]  \hspace{1cm} (II.13)

where \( \tau_p \) is the pulse width of the fundamental. If the crystal be of much greater length than
\( L_c \), then pulse width is given by

\[ \tau_{SH} = \tau_p \left(1 + \frac{L}{L_c}\right) \]  \hspace{1cm} (II.14)

In experiments involving sum frequency generation, the broadening is dependent on the
group velocity mismatch between the two input beams. Coming back to GVD which was
previously expressed as

\[ g = \frac{\partial^2 k}{\partial \omega^2} \]  \hspace{1cm} (II.15)

simple manipulations will lead to

\[ g = \frac{\lambda^3}{2\pi c^2} \frac{\partial^2 n}{\partial \lambda^2} \]  \hspace{1cm} (II.16)
meaning that GVD is proportional to $\frac{\partial^2 n}{\partial \lambda^2}$. GVD brings about pulse broadening by introducing a frequency sweep or "chirp" of the pulse. The lower frequency components of the pulse travel through the medium with a higher velocity than the higher frequencies (assuming normal dispersion) thus introducing a frequency sweep. Pulse broadening due to GVD can be corrected by recombining individual frequency components and eliminating chirp, by using a separated grating pair, prism pair or both. Simple propagation of pulse through a tilted window can cause spatial chirp (Figure II.8). In our Ti:sapphire oscillator, the crystal will introduce a spatial chirp which we have tried to eliminate with the pair of prisms (Figure II.8). One should use low dispersion materials such as fused silica or use reflective optics (which show no GVD) [13].

Figure II.8. GVD and its correction using a prism pair. A similar concept has been used in the Ti:sapphire oscillator of our laboratory.
In the following few pages, I have given a detailed description of the transient absorption apparatus being used by our group.

(i) **The oscillator:** Figure II.10 is a schematic diagram of the oscillator using the Titanium (Ti):sapphire (Ti:Al₂O₃) crystal as the gain medium. The crystal contains about 0.1% titanium added to sapphire (Al₂O₃) to replace aluminum in the crystal lattice. The titanium atom interacts strongly with the host crystal and this combines with the structure of the titanium energy levels to make the range of transition energies exceptionally broad. This gives the Ti:sapphire laser the broadest wavelength range of any solid-state laser (Figure II.9). Power is highest in the 700 to 900 nm region, but the tuning range is much broader, from 660 to 1180 nm [14]. The broad gain bandwidth of the Ti:sapphire crystal means that in addition to a large tuning range it is especially well suited for ultrashort-pulse generation and amplification [15]. The oscillator is pumped by 5 Watts of green from the Millenia V diode based CW green laser purchased from Spectra Physics. Details of how to

![Figure II.9. The absorption and emission spectra of the Ti:sapphire crystal. One can clearly see the broad bandwidth of the laser crystal.](image-url)
optimize the oscillator from scratch have already been outlined in references [16, 17]. To prevent reflections from the amplifier unit getting back into the cavity of the oscillator and disrupting modelocking, the optical isolator is used which ends up separating the seed and the weakly reflected beams.

The main component of the isolator is a Faraday rotator used in combination with a thin film polarizer (TFP) and a half wave plate. Faraday rotation is the rotation of plane polarized light due to magnetic-field-induced circular birefringence in a material. In a nonabsorbing or weakly absorbing medium, a linearly polarized monochromatic light beam passing through the material along the direction of the applied field experiences circular birefringence, which results in rotation of the plane of polarization of the incident beam. The angle of rotation $\theta$ can be expressed as

$$\theta = VBL = \frac{\pi \Delta n L}{\lambda}$$  \hspace{1cm} (II.17)

where $\Delta n$ is the magnitude of circular birefringence (a difference in refractive indices of left and right circularly polarized light), $L$ is the length of the medium traversed, $\lambda$ is the wavelength of light in the vacuum and $B$ is the magnetic field. $V$ is known as the Verdet constant and is a quantitative measure of the Faraday rotation ability of the magneto-optic material [18]. The key property of the Faraday rotator is that it is unidirectional. If the same polarized light beam (which on passing through the rotator had its polarization rotated by angle $\theta$) is reflected back, the beam acquires an additional rotation of $\theta$, for a total of $2\theta$ relative to input polarization. Horizontally polarized pulses from the Ti:sapphire oscillator
Figure II.10. Complete diagram of the Ti:sapphire oscillator. T, Ti:sapphire crystal; PD, fast photodiode; OF, optical fiber connected to a CCD used to look at the modelocked pulse on a computer; P1 and P2, prisms to compensate for GVD; BS, beam splitter; HR, high reflector; M1 and M2, curved mirrors; L, focusing lens; OC, output coupler; TFP, thin film polarizer (the TFP, half wave plate and Faraday rotator constitute the optical isolator); M6, grating, M4 and M5 all make up the stretcher.

pass through the thin film polarizer (TFP) which transmits horizontally (p-polarized) light and reflects vertical (s-polarized) ones and then through the half wave plate into the Faraday rotator. The pulses from the stretcher are retraced back through the rotator where polarization is turned by 45° to give rise to vertically polarized beam which is subsequently reflected by the TFP into the amplifier box. Since the grating has poor diffraction for vertically polarized light, the weak reflected amplified light is effectively filtered by the stretcher (Figure II.10).
The regenerative amplifier: Pulses with high peak power have a tendency to self-focus in condensed media, causing extensive damage. To avoid this, the seed pulse from the oscillator is chirped by a pulse stretcher which broadens the pulse temporally thus reducing peak powers by at least 3 orders of magnitude. The amplifier system consists mainly of a Ti:sapphire crystal (pumped by a Nd:YLF laser operated at a frequency of 1 kHz), a Pockels cell, a fast photodetector and a pulse compressor along with a host of lenses and mirrors. A detailed diagram of the amplifier can be found in reference [17]. The regenerative amplifier operation can be visualized as four simple steps [19], (1) trapping a single pulse from the modelocked seed train in the amplifier resonator, (2) simultaneously Q-switching the amplifier resonator, (3) letting the trapped pulse build up gain by undergoing N round trips, where N>2 and (4) at saturation, cavity dumping the amplified pulse. The compressor has the effect of compressing the previously stretched pulse, after amplification, with the help of a dual grating system. For details about the working of the amplifier, the reader is advised to consult references [16, 17, 20]. The following paragraph focuses on the working principle of a Pockels cell.

The Pockels cell belongs to the broad class of electro-optic phase modulators. It consists of a crystal of electro-optic material such as potassium dihydrogen phosphate (KDP), ammonium dihydrogen phosphate (ADP) or lithium niobate (LiNbO3), with electrodes located at appropriate positions to apply voltage across the crystal. Electro-optic materials have the property that applying a transverse electric field causes a small but significant change in the optical index of refraction (Kerr effect) and hence in the optical phase shift through the crystal [21]. Applying a voltage causes the refractive index to increase for optical fields polarized along one transverse axis, and to decrease for fields
polarized along the orthogonal transverse axis, bringing about an electrically induced birefringence. If the latter be combined with a polarized optical beam, the applied voltage can then produce polarization rotation of the optical signal. This rotation combined with optical polarizers, can then produce amplitude modulation and switching. The change optical phase shift in a Pockels cell is linearly proportional to the voltage applied across or along the crystal.

(iii) **The Optical Parametric Amplifier (OPA) assembly**: The OPA is based on the principle of optical parametric generation (OPG). In the latter process, an input pump photon of a higher frequency \( \omega_p \) and higher intensity is split into two photons of lower frequencies through a down-conversion process in a suitable nonlinear crystal, generating two new beams of different frequencies; one is called the signal \( (\omega_s) \) and the other is called the idler \( (\omega_i) \), as shown in Figure II.11. In the interaction both energy and momentum need to be conserved. The energy conservation condition can be represented as:

\[
\omega_p = \omega_s + \omega_i \tag{II.18}
\]

For the interaction to be efficient, the following momentum (or phase matching) condition needs to be satisfied:

\[
\Delta k = k_p - k_s - k_i = 0 \tag{II.19}
\]

where \( k_p, k_s \) and \( k_i \) are the wave vectors of the pump, signal and idler beams. The signal
frequency to be amplified can be varied in principle from $\omega_p/2$ (so called degeneracy condition) to $\omega_p$, and correspondingly that of the idler will vary from $\omega_p/2$ to 0. Thus at degeneracy, both signal and idler will have the same frequency [22]. To be efficient, the OPG process requires very high intensities and is thus suited to femtosecond laser systems which can easily achieve such intensities even with modest energies. An OPG can be used in two ways to achieve frequency stability. If the OPG crystal is enclosed in a suitable optical cavity and parametric gain exceeds losses, the cavity starts oscillating like an ordinary laser and an optical parametric oscillator is obtained (OPO). A completely different approach involves

![Figure II.11. A schematic diagram of the OPG process.](image)

amplifying a suitably generated weak signal beam (the "seed") in one or more OPG crystals, thus giving rise to an optical parametric amplifier (OPA). OPOs can be pumped by a small-scale femtosecond oscillator with high repetition rates (~ 100 MHz). However, output energies are very low (~ few nanojoules) and tunability is limited by bandwidth of mirror
coatings. OPAs require high pump intensities, provided only by an amplified system (in our case the regenerative amplifier) and operate at low repetition rates (1 to 100 kHz). OPAs provide high output energies and broad frequency tunability.

Figure II.12 is a schematic diagram of the OPA used by our group. The seed is a white-light continuum generated by tight focusing of the amplified beam from the amplifier into a sapphire plate. As a result of the interplay between self-focusing and self-phase modulation, a large amount of spectral broadening takes place. The pump for the OPA (here the second harmonic generated by passing the amplified fundamental through a 2 mm LBO-I crystal) and seed pulses are combined in a suitable non linear crystal (3 mm BBO-II), in a first parametric amplification stage (preamplifier). The delay (as shown in Figure II.13) is used for temporal overlap of the two aforesaid pulses. This BBO-II crystal produces a narrow bandwidth output (another advantage of the type II phase matched crystal is the possibility to separate signal and idler pulses because of their orthogonal polarization). The signal beam is now further amplified by passing the output beam from BBO-II through a BBO-I crystal (the “power” amplifier). The narrow bandwidth of the first stage output is preserved in the second stage of amplification. The purpose of using two amplification stages instead of one long crystal is 2-fold: (a) the GVM (group velocity mismatch) between pump and signal pulses in the first stage can be compensated by a delay line and (b) the scheme gives the flexibility of separately adjusting the pump intensity, and thus the parametric gain, in the two stages. After the second stage, signal and idler beams are separated from pump and each other by using a dichroic filter (F) as shown in Figure II.12.

(iv) The Final Stage: Figure II.13 is a schematic diagram of the final stage of our transient absorption apparatus. The amplified 800 nm 1 kHz pulses are focused into a sapphire plate to
generate white light which is split into 2 portions, the probe and the reference beams. The reference is used to provide shot to shot normalization to eliminate any intensity jitters for the probe pulse. The OPA output is used as the pump which is delayed with the help of a motorized translation stage. The probe and pump pulses are made to overlap both spatially and on the spinning sample cell. The transmitted probe and reference beams are passed through a monochromator and then made to overlap both spatially and temporally on the

Figure II.12. Schematic diagram of the OPA assembly based on reference [23].
spinning sample cell. Both the transmitted probe and reference beams are passed through a monochromator and then by two photodiodes, the signals from which are subsequently processed in a boxcar averager. The data are typically represented in terms of change in absorbance $\Delta A$ which is calculated using the difference between the probe/reference ratio when the pump is blocked and unblocked by a chopper (the chopper is operated at a frequency of 500 kHz so that it blocks every other pump pulse).

![Diagram of pump-probe apparatus](image)

**Figure II.13.** Schematic diagram of the final stage of the pump-probe apparatus. L1, L2, L3 and L4 are lenses with L1 and L2 being used for collimation of pump beam; S, sapphire plate; ND, neutral density filter; PD, photodiode.
References:


5. What is TCSPC? Technical Note by Edinburgh Instruments.


8. The generation of ultrashort laser pulses. Power point presentation on the internet.


CHAPTER 3. EFFECT OF pH ON THE FLUORESCENCE AND ABSORPTION SPECTRA OF HYPERICIN IN REVERSE MICELLES

A paper published in Photochemistry and Photobiology

P. K. Chowdhury, K. D. Ashby, A. Datta, and J. W. Petrich*

ABSTRACT

The well characterized, monodispersed nature of reverse micelles formed by AOT/heptane and their usefulness in approximating a membrane-like environment have been exploited to investigate the effect of pH and water pool size on the photophysical properties of hypericin. Our measurements reveal two titratable groups of pKₐ -1.5 and -12.5. These are assigned to the HypH⁺/Hyp equilibrium (the deprotonation of a carbonyl group) and the Hyp⁻/Hyp²⁻ equilibrium (the deprotonation of a peri hydroxyl group). The low-energy absorbance maxima of HypH⁺, of Hyp and Hyp⁻, and of Hyp²⁻ are 583, 594, and 613 nm, respectively. Neither at pH 13 nor at 1 M HCl is the system entirely in the Hyp²⁻ or the HypH⁺ forms. Ours is the first study of hypericin in reverse micelles as well as the first time-resolved study of hypericin as a function of pH.

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INTRODUCTION

Hypericin (Figure III.1) is one of many naturally and commonly occurring perylene quinone pigments that have stimulated great interest because of their broad spectrum of light-induced biological activities (1-5), among which are virucidal activity against several types of viruses, including the human immunodeficiency virus (HIV) (6-8), as well as antiproliferative and cytotoxic effects on tumor cells (9-11). Owing to this important biological activity, over the past few years we have been studying the photophysics of hypericin and its analogs (12-24). By means of H/D substitution, investigation of O-methylated analogs, and complementary studies using both transient absorption and fluorescence upconversion spectroscopies, we have argued that the major primary photophysical process in organic solvents is excited-state intramolecular hydrogen atom transfer. We have suggested that the protons resulting from a subsequent intermolecular proton transfer reaction may be important for understanding the light-induced biological activity of hypericin and its analogs. In the context of this previous body of work, it is a natural and pressing desire to want to investigate the excited-state phenomena as a function of pH. Such a study is, unfortunately, impossible in homogenous aqueous solution owing to the extreme insolubility of hypericin in water at all but the very extremes of pH. It is known, however, that the interaction of hypericin with membrane (or other subcellular components) is essential to incorporate it into a cell (20, 25, 26). Consequently, not only is there a need to study the photophysical properties of hypericin in membranes or in well-defined systems that mimic a membrane; but such an environment also affords the means by which the excited-state photophysics may be studied as a function of pH.
Reverse micelles formed by dissolving AOT (sodium bis(2-ethylhexyl)sulfosuccinate.) (Fig. III.2a) in heptane provide an ideal system because their size and properties may be very well controlled. AOT/H₂O micelles are well characterized regarding their size, shape and the number of AOT molecules per aggregate (i.e., the aggregation number) (27). Addition of water to AOT/alkane solutions produces systems that resemble small water pools in

**Figure III.1.** (a) Two dimensional structure of hypericin. (b) Hypericin as seen in its side-position.
bioaggregates (27). Also the solubilized water is similar to the interfacial water that is present near the biological membranes of protein surfaces (28). Most workers (29-37) refer to water in the AOT/alkane system as a “microemulsion.” Before discussing the photophysics of hypericin in these microemulsions, it is appropriate to present a very brief summary of their characteristics (27, 29-49, 28, 50-52) that bear upon this study. In the reverse micellar system, the sulfosuccinate head group of AOT is a polar entity constituting the core while the hydrocarbon chain protrudes into the surrounding bulk solution of hydrocarbon, in our case, heptane. Several experiments (41, 48, 50-52) have indicated that the physical properties of the water molecules sequestered in the pool differ considerably from those of bulk water (e.g., polarity (48, 50) and dielectric relaxation time (51, 52)). The polarity of the AOT/alkane solutions increases with the fraction of water inside the pool, the concentration ratio: \( w_0 = [\text{H}_2\text{O}]/[\text{AOT}] \). Some of the salient features of these microemulsions have been summarized in Table III.1 (44).

Jardon and coworkers have produced a large body of careful work on the steady state spectra and the triplet photophysics of hypericin in various conditions (53-58). They were the first to study hypericin as a function of pH in Brij-35 micelles (53); and our work presented here has been greatly influenced by that effort. Based on their findings and taking into account the well characterized, monodispersed nature of the reverse micelles formed by AOT in heptane (Table III.1) and their usefulness in approximating a membrane-like environment, we have investigated the effect of pH and water pool size on the photophysical properties of hypericin. (We note that not all reverse micelles are monodispersed: for example, Triton-X reverse micelles are polydispersed.)
MATERIALS AND METHODS

Hypernicin was purchased from Molecular Probes and was used as received. AOT (sodium diisoctyl sulfosuccinate) purchased from Sigma, was purified by dissolving in methanol and stirring it overnight in presence of activated charcoal. Subsequent filtration and

<table>
<thead>
<tr>
<th>$w_0^a$</th>
<th>$n_{avg}^a$</th>
<th>$r_w (\text{Å})^a$</th>
<th>Number of water molecules per pool$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>35</td>
<td>10</td>
<td>140</td>
</tr>
<tr>
<td>8</td>
<td>72</td>
<td>16</td>
<td>578</td>
</tr>
<tr>
<td>12</td>
<td>129</td>
<td>22</td>
<td>1553</td>
</tr>
<tr>
<td>16</td>
<td>215</td>
<td>29</td>
<td>3446</td>
</tr>
<tr>
<td>20</td>
<td>302</td>
<td>35</td>
<td>6061</td>
</tr>
<tr>
<td>30</td>
<td>613</td>
<td>51</td>
<td>18465</td>
</tr>
</tbody>
</table>

$^a$ Aggregation number, the number of AOT molecules per micelle in the system, $n_{avg}$; radius of the water pool, $r_w$; the concentration ratio of water to AOT, $w_0 = C_{H2O}/C_{AOT}$. There is a sharp rise of $n_{avg}$ with increase of $w_0$; and there appears to be a linear relationship between $w_0$ and the radius of the water pool (32, 41, 43, 44). The aggregation number varies little with solvent or AOT concentration. In a 3% (w/v) solution of AOT in heptane, the aggregation number is 23 (27). The aggregation number of AOT is very insensitive to other solvents (27, 30, 39, 42). In this work, we employ a 4% AOT solution in order to ensure that there is an excess of reverse micelles with respect to hypernicin. Because of the reported insensitivity of $n_{avg}$ to AOT concentration, we assume that the aggregation number remains the same. The concentration ratio is the main criterion determining the microemulsion stability. Finally, since isooctane is very similar to heptane, we cite the reported values for $n_{avg}$ and $r_w$ isooctane (44) and assume that for our purposes they are sufficiently close to those for a system constituted from heptane.

$^b$ This calculation is based on 2 mL of AOT in heptane.
removal of methanol by distillation under vacuum, yielded AOT suitable for use. Spectrophotometric grade heptane purchased from Aldrich was refluxed over calcium hydride and collected by distillation before use.

**Preparation of Sample:** The solution of AOT in heptane was prepared in such a way that the concentration was 0.09 M. Hypericin was insoluble in heptane and dissolved very slowly in the AOT/heptane reverse micellar system. On addition of water, however, hypericin dissolves much more readily. The color of the reverse micellar system with hypericin was reddish at low pH and at intermediate pH values, while at a pH of 13, the solution had a very light greenish tinge. The basic solution, however, slowly reverts to the reddish color characteristic of hypericin in

---

**Figure III.2.** a) The structure of aerosol-OT (AOT). The length of the hydrocarbon chain, 9 Å, is denoted. (b) A schematic diagram of the reverse micelle formed by AOT in heptane, where $r_w$ is the radius of the water pool. Adapted from Day et al. (30). Because the hydrocarbon chain is shorter than both dimensions of hypericin (major axis, 10.5 Å; minor axis, 9.6 Å). It is most likely that two peri hydroxyl and the intervening carbonyl groups protrude into the water pool.
AOT/heptane, indicating a gradual shifting of the equilibrium towards the “normal” form (the 7,14 diketo tautomer, displayed in Figure III.1). The concentration of hypericin was kept at \(-10^{-6}\text{ M}\).

**Steady State Measurements:** Steady-state absorption spectra were recorded on a Perkin-Elmer Lambda-18 double-beam UV-visible spectrophotometer with 1-nm resolution. Steady-state fluorescence spectra (both excitation and emission), were obtained on a SPEX Fluoromax with a 4 nm bandpass (a smaller bandpass, as low as 2 nm, was used in some cases to avoid saturating the detector to preserve linearity in response), and corrected for detector response. For both fluorescence and absorption measurements, a 1-cm path-length quartz cuvette was used.

**Time-Resolved Studies:** The apparatus for time-correlated single photon counting is described elsewhere (12-14). Fluorescence decays were collected by exciting at 295 nm and collecting emission > 550 nm for a maximum of 10000 counts in the peak channel. The polarized fluorescence traces used to obtain fluorescence anisotropy decay parameters were collected to a maximum of 16000 – 18000 counts in the peak channel. The pH was maintained by HCl of appropriate molarity and its subsequent neutralization by 0.1 M NaOH solution. The pH of the resulting solution was recorded by a manual Fisher-Accumet (955) Mini pH meter. All measurements were carried out at 298K.

**RESULTS AND DISCUSSION**

As noted above, hypericin exhibits negligible solubility in heptane. Addition of AOT, however, permits gradual incorporation into solution. Figure III.3 presents a comparison of the steady-state absorption spectra and the fluorescence lifetime of hypericin.
in DMSO and in AOT/heptane. AOT/heptane induces only a small blue shift of 4 nm and a very small lengthening of the lifetime (5.9 as opposed to 5.5 ns) with respect to DMSO. That the AOT/heptane system affords no significant changes in the shape of the hypericin spectrum and that its fluorescence lifetime is nearly the same as that in DMSO and single exponential indicates that there is no hypericin aggregation in AOT/heptane, which is to be expected, given the relative concentration of hypericin and AOT. For our experiments, using 0.09 M AOT in heptane and \( \sim 10^{-6} \) M hypericin, one in every 1000 micelles harbors a hypericin molecule: that is, we have ensured the presence of only one hypericin per micelle. Even if the concentration of hypericin is \( \sim 10^{-5} \) M, there still is only one hypericin for every 100 micelles.

Addition of water to AOT/heptane increases the solubility of hypericin and gives rise to pools whose radius and sequestered number of water molecules are very well defined (Table III.1). Figure III.4 presents steady-state absorbance and emission spectra for hypericin in AOT/heptane/\( w_0 = 4 \) as a function of pH. The spectral changes induced from 1-M HCl to pH 13 are dramatic and demonstrate the utility of the reverse micellar system for the investigation of the effect of pH on the spectra of hypericin. Fluorescence quantum yield data are summarized in Table III.2.

Figure III.5 presents the effect of pH on the steady-state absorbance and emission spectra for a system with \( w_0 = 8 \). The salient feature of this Figure (taken in contrast to Figure III.4) is that between pH 2 and 12, the spectral changes are negligible. The pH must be below 2 or above 12 in order to effect a significant spectral change. The fluorescence quantum yield data (Table III.2) indicate the presence of titratable groups of pK_a \( \sim 1.5 \) and \( \sim 12.5 \). The variation of the absorption spectra as a function of \( w_0 \) is illustrated in Figure III.6:
Figure III.3. (A) Normalized absorbance spectra of hypericin in AOT/heptane (solid) and in DMSO (dotted). (B) (a) Fluorescence decay of hypericin in AOT/heptane: $\lambda_{\text{exc}} = 570$ nm; $\lambda_{\text{em}} \geq 610$ nm, $\tau_F = 5.9$ ns, $\chi^2 = 1.23$. (b) Fluorescence decay of hypericin in DMSO: $\lambda_{\text{exc}} = 570$ nm; $\lambda_{\text{em}} \geq 610$ nm, $\tau_F = 5.5$ ns, $\chi^2 = 1.07$. Both fluorescence decays are well described by a single exponential. The top sets of residuals are those for hypericin in AOT/heptane; the bottom set, for DMSO.
in all cases, the pH is fixed at 1.2, near the pKₐ of the protonated carbonyl. The variation in the spectra from w₀ = 2 to 8 suggest that the properties of the sequestered water change, taking on more bulk characteristics as the pool grows larger.

Fluorescence lifetime and anisotropy decays are summarized in Tables III.3 and III.4, respectively, with polarized fluorescence decay shown in Figure III.7. The fluorescence lifetime is well described by a single exponential of ~5.7 ns until the pH is lowered below 2, at which point a second component appears, whose duration is 3.2 ns. At pH > 12, the
Table III.2. Fluorescence Quantum Yield Data:

<table>
<thead>
<tr>
<th>Sample(AOT/Hep/Hyp)</th>
<th>Quantum Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyp/DMSO</td>
<td>0.35</td>
</tr>
<tr>
<td>( w_0 = 0 )</td>
<td>0.37</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 1.2 )</td>
<td>0.25</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 1.2 )</td>
<td>0.22</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 2.0 )</td>
<td>0.33</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 2.0 )</td>
<td>0.30</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 6.0 )</td>
<td>0.33</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 6.0 )</td>
<td>0.31</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 12 )</td>
<td>0.30</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 12.3 )</td>
<td>0.28</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 12.3 )</td>
<td>0.31</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 12.8 )</td>
<td>0.14</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 12.8 )</td>
<td>0.14</td>
</tr>
<tr>
<td>( w_0 = 4 / \text{pH} = 13 )</td>
<td>0.14</td>
</tr>
<tr>
<td>( w_0 = 8 / \text{pH} = 13 )</td>
<td>0.16</td>
</tr>
</tbody>
</table>

*At the extremes of pH, near the pK of a titratable group, an isosbestic point was selected for the excitation wavelength: 545 nm for low pH; 487 nm for high pH. At intermediate pH, the excitation wavelength is arbitrary and was typically taken as 550 nm. The error estimates for all the quantum yield values lie within ± 0.010 to ± 0.020.

Reverse micelles are insufficiently stable to obtain a fluorescence decay. Fluorescence anisotropy decays are measured at pH 6 for a range of water pool sizes. For \( w_0 = 0, 2, \) and 4, the anisotropy decay is single exponential and its time constant, the rotational diffusion time (\( \tau_{\text{rot}} \)), increases with water pool size (Figure III.8a). The fact that the anisotropy decay of hypericin is single exponential indicates that for these values of \( w_0 \) hypericin is rigidly held and reflects only the overall tumbling of the reverse micelle. At \( w_0 = 8 \) and 20 the anisotropy decay must be described by two time constants. The reverse micelle is sufficiently large, and fluid, so that in addition to probing the overall tumbling of the system, hypericin also undergoes more rapid local motion characterized by a 0.5-ns component.
Figure III.5. (A) Absorbance spectra of hypericin (2.43 × 10^{-6} M) in AOT/heptane. (a) \( w_0 = 0 \) (b) \( w_0 = 8, \text{pH}=2 \) (c) \( w_0 = 8, \text{pH}=6 \) (d) \( w_0 = 8, \text{pH}=12 \). (B) Fluorescence spectra with (a), (b), (c), and (d) representing the same systems as in (A). In both (A) and (B) the spectra have been offset. From pH 2 to 12, the spectral features remain the same.

CONCLUSIONS

Jardon and coworkers have measured the pK\(_a\)s of the bay (3 and 4 positions) and the peri hydroxyl groups (1, 6, 8, and 13 positions) of hypericin in Brij-35 micelles and in aqueous liposome media. Their work indicates a pK\(_a\) of 7 for the bay hydroxyl, of 11 for the peri hydroxyl, and of 1 for the protonated groups (53). The spectral changes observed near pH 7 are very small, indicating that the neutral and monoanionic forms of hypericin have very similar spectra (53). This pK\(_a\) value for the bay hydroxyls is consistent with that for 2,2'-dihydroxybiphenyl derivatives (59) and with electrochemical measurements of hypericin (57). The pK\(_a\)s of the titratable groups in hypericin have also been addressed in the work of
Falk and coworkers (60, 61), Yamazaki et al. (62), and Gerson et al. (63). Recently, Jenks and coworkers (64) have summarized and discussed this literature.

Our measurements do not resolve a $pK_a$ at neutral pH. While we obtain a $pK_a$ at $\sim 1.5$, consistent with that assigned by Jardon and coworkers to the HypH$^+$/Hyp equilibrium,

![Absorbance spectra of hypericin in AOT/heptane as a function of water pool size at the fixed pH of 1.2. The hypericin concentration is the same for the four spectra; and the spectra are stacked to reveal the changes in their features more clearly. There are isosbestic points at 587, 545, and 468 nm.](image)

**Figure III.6.** Absorbance spectra of hypericin in AOT/heptane as a function of water pool size at the fixed pH of 1.2. The hypericin concentration is the same for the four spectra; and the spectra are stacked to reveal the changes in their features more clearly. There are isosbestic points at 587, 545, and 468 nm.
Table III.3. Fluorescence lifetime parameters for AOT/Hep/Hyp $^a$.

<table>
<thead>
<tr>
<th>pH</th>
<th>$w_0$</th>
<th>$a_1$</th>
<th>$\tau_1$(ns)</th>
<th>$\tau_2$(ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M HCl</td>
<td>4 $^b$</td>
<td>0.080±0.003</td>
<td>5.91±0.03</td>
<td>3.22±0.05</td>
</tr>
<tr>
<td>1.2</td>
<td>8</td>
<td>0.27±0.03</td>
<td>5.91±0.03</td>
<td>3.22±0.05</td>
</tr>
<tr>
<td>1.4</td>
<td>8</td>
<td>0.76±0.01</td>
<td>5.9±0.5</td>
<td>3.2±0.5</td>
</tr>
<tr>
<td>1.6</td>
<td>8</td>
<td>0.91±0.02</td>
<td>5.91±0.04</td>
<td>3.22±0.04</td>
</tr>
<tr>
<td>1.8</td>
<td>8</td>
<td>0.91±0.03</td>
<td>5.91±0.04</td>
<td>3.22±0.03</td>
</tr>
<tr>
<td>2.0</td>
<td>8</td>
<td>0.94±0.04</td>
<td>5.91±0.04</td>
<td>3.22±0.06</td>
</tr>
<tr>
<td>6.0</td>
<td>8</td>
<td>1.00</td>
<td>5.60±0.024</td>
<td>-----</td>
</tr>
<tr>
<td>12 $^c$</td>
<td>8</td>
<td>1.00</td>
<td>5.75±0.01</td>
<td>-----</td>
</tr>
<tr>
<td>------</td>
<td>0</td>
<td>1.00</td>
<td>5.89±0.09</td>
<td>-----</td>
</tr>
</tbody>
</table>

$^a$ Fluorescence lifetimes were fit to a sum of up to two exponentially decaying components and had the form: $F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. The absence of values implies that the lifetime was adequately described by a single exponential decay. $\chi^2 < 1.3$ for all data presented in the Table.

$^b$ In concentrated acid solution, at higher values of $w_0$ than 4, the micelles are unstable and the solution becomes turbid.

$^c$ Above pH 12, the reverse micelles are not stable on a time scale sufficiently long to perform a reliable fluorescence lifetime measurement.

Table III.4. Fluorescence anisotropy parameters of AOT/Hep/Hyp/pH=6 as a function water pool size$^a$.

<table>
<thead>
<tr>
<th>$w_0$</th>
<th>$r^1(0)$</th>
<th>$\tau^1_r$(ns)</th>
<th>$r^2(0)$</th>
<th>$\tau^2_r$(ns)</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.23</td>
<td>1.68</td>
<td>-----</td>
<td>-----</td>
<td>1.28</td>
</tr>
<tr>
<td>2</td>
<td>0.21</td>
<td>1.80</td>
<td>-----</td>
<td>-----</td>
<td>1.22</td>
</tr>
<tr>
<td>4</td>
<td>0.20</td>
<td>2.62</td>
<td>-----</td>
<td>-----</td>
<td>1.19</td>
</tr>
<tr>
<td>8</td>
<td>0.11</td>
<td>5.90</td>
<td>0.10</td>
<td>0.550</td>
<td>1.11</td>
</tr>
<tr>
<td>20</td>
<td>0.09</td>
<td>12.0</td>
<td>0.11</td>
<td>0.500</td>
<td>1.27</td>
</tr>
</tbody>
</table>

$^a$ Fluorescence anisotropy decays were fit to a sum of up to two exponentially decaying components and had the form: $F(t) = r^1 \exp(-t/\tau^1_r) + r^2 \exp(-t/\tau^2_r)$. The absence of values for $r^2$ and $\tau^2_r$ implies that the lifetime was adequately described by a single exponential decay. $\lambda_{exc} = 295$ nm; $\lambda_{em} > 550$ nm.
Figure III.7. Polarized fluorescence decay of hypericin in AOT/heptane/w0 = 20/pH=6: $\lambda_{exc}$ = 295 nm; $\lambda_{em}$ ≥ 550 nm, $\chi^2 = 1.27$; $r(t) = 0.11 \exp(-t/0.500 \text{ ns}) + 0.09 \exp(-t/12.0 \text{ ns})$.

Our value for the pK$_a$ of the equilibrium they attribute to Hyp$^-/Hyp^{2-}$, ~12.5, is larger than theirs by as much (66) as 1.5 units. This may be a result of the smaller water pools that the peri hydroxyl groups are exposed to in reverse micelles as opposed to the water in the larger Brij-35 systems, where water is the bulk solvent. It is not clear, however, why a commensurate effect is not evident for the lower of our two pK$_a$ values.

In the context of the assignments made by Jardon and coworkers, the steady-state spectra presented in Figure III.4 indicate that the low-energy absorbance maxima of HypH$^+$
Figure III.8. (a) $\tau_{\text{or}}$ (longer of the two components) vs. $w_0$ for the AOT/Hyp/Hep system. (b) $1/\tau_{\text{or}}$ vs $1/V \times 10^{19}$ where $V$ is the volume of the reverse micelle in units of cm$^3$. In this Figure, $V$ rather arbitrarily computed by taking account the size of the (Table III.1) water pool, the dimensions of the AOT (Figure III.2), and an outer “solvation sphere” of heptane. Naively, one expects that a plot of $1/\tau_{\text{or}}$ vs $1/V$ to be linear yielding a slope of $kT/\eta$ (65). For reverse micellar systems, this does not seem to be the case (66). This may be a result, for example, of incorrectly assuming that the micelle may be approximated as a rigid sphere or that the viscosity, $\eta$, of the solution itself may change with AOT concentration. The work of De Schryver and coworkers, using cresyl violet as a fluorescent probe, presents results similar to ours in their Figure III.7.
(which we attribute to a protonated carbonyl group), of Hyp and Hyp', and of Hyp^{2-} are 583, 594, and 613 nm, respectively. The high-energy emission maxima of HypH^+ and of Hyp and Hyp' are 585 and 595 nm. The dianion is very weakly fluorescent on the steady-state time scale. Jardon and coworkers report a quantum yield of 0.023 (53). Figure III.4 indicates that neither at pH 13 nor at 1 M HCl is the system entirely in the Hyp^{2-} or the HypH^+ forms. (In 1 M HCl, the presence of Hyp is indicated in the absorption spectrum by the slight shoulder corresponding to \(-594\) nm. The existence of Hyp at 1 M HCl is much more apparent in the fluorescence spectrum.)

ACKNOWLEDGEMENTS

This work was supported by NSF grant CHE-9613962 to J. W. P.

REFERENCES


CHAPTER IV. A COMPARISON OF THE EXCITED-STATE PROCESSES OF NEARLY SYMMETRICAL PERYLENE QUINONES: HYPOCRELLIN A AND HYPOMYCIN B

A paper published in the *Journal of Photochemistry and Photobiology A*

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**ABSTRACT**

The excited-state photophysics of two naturally occurring nearly symmetrical perylene quinones are discussed: hypocrellin A and hypomycin B. Hypocrellin A has a hydroxyl group *peri* to a carbonyl group on either end of its long molecular axis in addition to a hydroxyl group on its seven-membered ring. On the other hand, hypomycin B is unique among this class of known naturally occurring perylene quinones in that it possesses only one hydroxyl group, which is *peri* to a carbonyl group. These quinones are investigated in different nonionic micellar environments. For hypocrellin A and hypomycin B, a micelle concentration 10 times in excess of that used for hypericin in a previous study, i.e., 100 times the critical micelle concentration, must be employed to obviate aggregation. Under such

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conditions, the pKₐ of the peri hydroxyl groups of hypocrellin A have been determined to be 8.9. The pKₐ of the protonated carbonyl groups could not be measured. A comparable value is estimated for hypomycin B. The differing solubilities and behaviors of hypericin and hypocrellin in micellar environments are briefly discussed in the context of their biological activity. The excited-state processes in hypocrellin A and hypomycin B are compared on a time scale of several hundreds of picoseconds. No deuterium isotope is observed for hypomycin B. This result is discussed in the light of the previous assignment of the primary photoprocess in hypocrellin A to hydrogen atom transfer.

**INTRODUCTION**

Hypericin and hypocrellin (Figure IV.1) are naturally occurring perylene quinones that have gained great interest recently owing to their light-induced biological activity (1-5). They display virucidal activity against several types of viruses, including the human immunodeficiency virus (HIV) (6-8), as well as antiproliferative and cytotoxic effects on tumor cells (9-11). Hypericin is also a potent antidepressant (12, 13), exhibits light-dependent inhibition of protein kinase C (PKC) (14), and is reported to possess numerous other types of biological behavior (15-20). Hypericin, like other anticancer drugs, also induces apoptosis (21, 10, 22).

Owing to this important biological activity, over the past few years we have been studying the photophysics of hypericin and hypocrellin (23-34). By means of H/D substitution, investigation of O-methylated analogs, and complementary studies using both transient absorption and fluorescence upconversion spectroscopies, we have argued that the
major primary photophysical process in hypericin and hypocrellin A in organic solvents is excited-state hydrogen atom transfer. Considerable effort was required to demonstrate this fact owing to the unusual mirror image symmetry between absorption and emission spectra, the lack of an H/D

![Figure IV.1. Structures of (a) hypocrellin A, (b) hypomycin B, and (c) hypericin.]

isotope effect on the proton transfer reaction in hypericin, and the occasional consideration of this ultrafast reaction in terms of equilibrium Förster-cycle type calculations (35).

We have suggested that the labile protons resulting from the intramolecular proton transfer reaction may be important for understanding the light-induced biological activity of
hypericin and hypocrellin A. Notably, hypericin and hypocrellin A acidify their surroundings upon light absorption (36-38). The role of photogenerated protons takes on significance in the context of the growing body of literature implicating changes in pH with inhibition of virus replication (39), antitumor activity (40, 41), and apoptosis (programmed cell death) (42-44). For example, comparative studies for nine perylenequinones, including hypericin, provide evidence that the quantum yield of singlet oxygen formation is not sufficient to explain the reported antiviral activities of these molecules and that other structural features of perylenequinones are involved (45). In fact, the quantum yield of singlet oxygen from hypericin is much less than had initially been presumed. Recently, Jardon and coworkers have revised their earlier estimation of a singlet oxygen quantum yield of 0.73 (46, 47), essentially equal to the triplet yield, to 0.35 in ethanol and less than 0.02 in water (48). Based on this result, mechanisms involving only oxygen clearly cannot explain all the activity of hypericin. For example, the ability of photogenerated protons to enhance the activity of activated oxygen species may be of importance.

In this article, we shall be concerned with two topics. First, in the context of the previous body of work referred to above, it is a natural and pressing desire to want to investigate the excited-state phenomena as a function of pH. Such a study is, unfortunately, impossible in homogenous aqueous solution owing to the extreme insolubility of these perylene quinones in water at all but the very extremes of pH. Jardon and his coworkers, studying the steady-state spectra and the triplet dynamics of hypericin (49, 50, 47, 51-53), were the first to address this problem in a detailed and systematic matter by incorporating hypericin in Brij micelles. Recently we have extended this idea by investigating hypericin in reverse micelles formed by dissolving AOT (sodium bis(2-ethylhexyl)sulfosuccinate.) in
heptane. Such microemulsions provide an ideal system because their size and properties may be very well controlled. Addition of water to AOT/alkane solutions produces systems that resemble small water pools in bioaggregates (54). Also the solubilized water is similar to the interfacial water that is present near the biological membranes of protein surfaces (55). Here we report the behavior of hypocrellin A and hypomycin B (56) in micellar environments, in particular Brij-35. The behavior of hypocrellin A is significantly different than that of hypericin. Recently, Dumas (57) has performed similar investigations.

Second, given the structure of the perylene quinones of interest here (Figure IV.1), with either one (hypocrellin A) or two (hypericin), hydroxyl groups peri to a carbonyl on either end of the molecule, one is naturally inclined to inquire how many hydrogen atoms are transferred in the excited state. And, if more than one is transferred, one must necessarily inquire whether the process is stepwise or concerted. (It is useful to note the difference between a concerted and a synchronous reaction. A concerted reaction takes place in a single kinetic step, with no reaction intermediate, where some of the changes in bonding take place to different extents in different parts of the reaction. A synchronous reaction is one where all the bond-making and bond-breaking processes take place at the same time and proceed at the same extent during the reaction (58, 59). It is a common error to assume that concertedness implies synchrony.) The availability of hypomycin B (56), where there is only one peri hydroxyl group, and only one intramolecular hydrogen bond, provides an excellent means to investigate these questions.

MATERIALS AND METHODS
Hypocrellin A was purchased from Molecular Probes (Eugene, OR) and was used as received. Hypomycin B was provided by the Shandong researchers and was extracted and prepared as described elsewhere (60). AOT (sodium diisoctyl sulfosuccinate) purchased from Sigma Chemical Co. (St. Louis, MO), was purified by dissolving in methanol and stirring it overnight in presence of activated charcoal. Subsequent filtration and removal of methanol by distillation under vacuum, yielded AOT suitable for use. Brij 35 was obtained from Aldrich (Milwaukee, WI) and was recrystallized twice from ethanol before use. Spectrophotometric grade heptane purchased from Aldrich was refluxed over calcium hydride and collected by distillation before use.

Preparation of Sample: An attempt was made to study hypocrellin A in reverse micelles. The solution of AOT in heptane was prepared in such a way that its concentration was 0.9 M. (The reason for choosing this high reverse micellar concentration is that hypocrellin A has marked solubility in heptane, and hence it was hoped that the higher number of reverse micelles would increase the partitioning of hypocrellin-A at the micelle-water interface). Nevertheless, hypocrellin A was soluble in heptane. Consequently, recourse to Brij micelles was made. An alkaline solution of $2 \times 10^{-2}$ M, 100 times the critical micellar concentration (CMC), of Brij 35 was prepared by dissolving 480 mg Brij 35 in 20 mL of an aqueous solution of NaOH of pH 13. Solid hypocrellin A was added to a portion of this solution and sonicated for five minutes to obtain a clear solution. In order to obtain solutions of different pH, appropriate volumes of 12 M HCl were added with a microliter syringe. The pH of the resulting solutions was recorded by a manual Fisher-Accumet (915) pH meter. For hypomycin B sample preparation, Brij 35 was dissolved in buffers of different pH (7-13). Solid hypomycin B was added to the solutions in a very small amount followed by sonication
so as to ensure complete homogenization of the solution. For hypomycin B, the concentration of Brij 35 solution was also maintained at 100 CMC to avoid complications due to aggregation as much as possible. The concentration of both hypocrellin A and hypomycin B was kept at $\sim 10^{-6}$ M except for the transient absorption measurements of hypomycin B, where the concentration was increased to $10^{-5}$ M to improve the signal to noise ratio.

Steady State Measurements: Steady-state absorption spectra were recorded on a Perkin-Elmer Lambda-18 double-beam UV-visible spectrophotometer with 1-nm resolution. Steady-state fluorescence spectra were obtained on a SPEX Fluoromax with a 4 nm bandpass and corrected for detector response. The samples were excited at 480 nm. For both fluorescence and absorption measurements, a 1-cm path-length quartz cuvette was used.

Time-Resolved Studies: The apparatus for time-correlated single photon counting is described elsewhere (23-25). Fluorescence decays were collected by exciting at 580 nm and collecting emission $> 610$ nm for a maximum of 10,000 counts in the peak channel. The solutions of hypomycin B in Brij at pH values of 12 and above were not stable enough to carry out lifetime measurements.

All measurements were carried out at 298 K.

Time-resolved Pump-Probe Absorption Spectroscopy: The apparatus used for ultrafast kinetic measurements is described in detail elsewhere (61).

RESULTS AND DISCUSSION

Effects of pH on the Absorption and Emission Spectra of Hypocrellin A and Hypomycin B
Whereas hypericin is insoluble in heptane and partitions only at the AOT/water interface (62), hypocrellin A exhibits marked solubility in heptane. This solubility in heptane obviates the performance of unambiguous examination of its photophysics in the microemulsion. Consequently, recourse was made to Brij micelles, as in the work of Jardon and coworkers. It was found, however, that whereas hypericin shows no evidence of aggregation at ten times the CMC (data not shown), i.e., at $2 \times 10^{-3}$ M Brij, hypocrellin A exhibits aggregation at pH 7. A sensitive signature of aggregation is nonexponential fluorescence decay and the quenching of fluorescence (31) (for comparison of aggregation effects, both hypocrellin A and hypericin were maintained at a concentration of $\sim 10^{-6}$ M). A Brij concentration of one hundred times the CMC is required for hypocrellin A to afford an unambiguous single exponential fluorescence lifetime (Figure IV.2). A Brij concentration of $2 \times 10^{-2}$ M was used for all titration studies. At this concentration of Brij, there is 1 probe molecule for every 250 micelles, thus keeping aggregation to the minimum.

Figure IV.3 presents the absorption spectra of hypocrellin A at pH values of 1, 7, and 13. Between pH 13 and 7 there is a distinct change in the spectra, which we attribute to the deprotonation of the peri hydroxyl groups. On the other hand, the spectra at neutral pH and a pH value of 1 are very similar. We anticipated to see a change in this region, attributed to the protonation of the carbonyl groups because Jardon and coworkers have measured a pKₐ of 1 for these groups in hypericin. Consequently, we searched for spectral changes owing to titration of these groups at higher acidity. Figure 4 represents the absorption changes of hypocrellin A from 0.06 to 3.5 M HCl. Although there are well-behaved spectral changes characterized by an isosbestic point at 596 nm, we are hesitant to interpret them in terms of the titration of the carbonyls. In particular, Figure IV.4b gives no evidence of an inflection
point over this range of acid concentration. It is possible that at these concentrations we are disrupting the micelles. We suggest that at low acid concentrations where integrity of micelles is maintained, either protonation of the carbonyls is undetectable spectroscopically or that preferential protonation of the –OCH₃ group occurs.

Figure IV.5 presents the steady-state fluorescence spectra at pH 1, 7, and 13. Again, as in absorption, the spectrum at pH 13 is significantly different from that at 1 and 7. The average fluorescence lifetime of hypocrellin A in Brij micelles is plotted in Figure IV.6. The pKₐ of the

![Fluorescence decay plots](image)

Figure IV.2. Fluorescence decays of hypocrellin A in $2 \times 10^{-3}$ M (10 times CMC), $1 \times 10^{-2}$ M (50 times CMC) and $2 \times 10^{-2}$ M (100 times CMC) aqueous Brij 35 solutions at pH 7. $\lambda_{ex} = 580$ nm, $\lambda_{em} > 610$ nm. The decay for hypocrellin A in 100 CMC of Brij is fit to a single exponential function with a time constant of 1.3 ns. The decay at 50 CMC is biexponential with time constants of 0.18 ns and 1.1 ns, with relative amplitudes of 0.55 and 0.45 respectively. The decay at 10 CMC is fit to a triple exponential function, with time constants of 0.15 ns, 0.75 ns and 2.6 ns, having relative amplitudes of 0.92, 0.05 and 0.03 respectively. The presence of the shorter components in lower concentrations of the micelle indicates the aggregation of hypocrellin A. The residuals shown refer to the fit for the 100 CMC data.
Figure IV.3. Normalized absorption spectra of hypocrellin A in $2 \times 10^{-2}$ M (100 times CMC) Aqueous Brij-35 at pH 1, 7, and 13.

Figure III.4. (a) Absorption spectra of hypocrellin A in $2 \times 10^{-2}$ M (100 times CMC) Brij 35 in (i) 0.06 M, (ii) 0.29 M, (iii) 0.63 M, (iv) 1.5 M, (v) 2 M and (vi) 3.5 M HCl solutions. An isosbestic point is obtained at 596 nm. (b) Plot of absorbance at 360 nm of hypocrellin A in $2 \times 10^{-2}$ M (100 times CMC) Brij 35 in solutions of high acidity, against $-\log [H^+]$ in the rage $0.06 \ M \leq [H^+] \leq 3.5 \ M$, where $[H^+] = \text{concentration of HCl}$.
peri hydroxyl groups is obtained from the inflection point to be 8.9. The use of lifetimes to perform fluorescence titrations provides a distinct advantage over methods using fluorescence quantum yields or optical densities, which require accurate accounting of concentration changes.

Hypomycin B was investigated under the same conditions as hypocrellin A. Figures IV.7 and IV.8 present the steady-state absorption and emission spectra of hypomycin B in 100 CMC

![Normalized fluorescence spectra of hypocrellin A in 2 x 10^2 M (100 times CMC) aqueous Brij 35 at pH 1, 7 and 13. λ_ex = 480 nm.](image)

**Figure III.5.** Normalized fluorescence spectra of hypocrellin A in 2 x 10^2 M (100 times CMC) aqueous Brij 35 at pH 1, 7 and 13. λ_ex = 480 nm.

Brij solutions as a function of pH. Above pH 9, the absorption and emission spectra change significantly, revealing the formation of the anionic form of hypomycin B. Figure IV.9 presents several examples of the fluorescence decays. A summary of the lifetime behavior as a function of pH is given in Table 1. The fluorescence is well described by a single exponential time constant of ~890 ps below pH values of 8; however, above this pH, we see
the appearance of a second, shorter, component, the amplitude of which increases as shown in Table IV.1 with increasing pH values. Hence the average lifetime becomes shorter leading to faster decays as shown in Figure IV.9. The hypomycin B data reveal a titratable group with a pKₐ of approximately 9.7, which is slightly higher than that of hypocrellin A. (We did not have enough material available to construct a detailed titration curve.)

The most significant experiments involving hypomycin B presented here are the picosecond transient absorption measurements in MeOH and MeOD (Figure IV.10). Within

Table IV.1. Fluorescence lifetime parameters of hypomycin B in 100 CMC Brij 35 solutions of different pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>τ₁ (ps)</th>
<th>a₁</th>
<th>τ₂ (ps)</th>
<th>a₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>890</td>
<td>1.00</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>6</td>
<td>890</td>
<td>1.00</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>7</td>
<td>890</td>
<td>1.00</td>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>8</td>
<td>910</td>
<td>0.82</td>
<td>200</td>
<td>0.18</td>
</tr>
<tr>
<td>9</td>
<td>900</td>
<td>0.75</td>
<td>210</td>
<td>0.25</td>
</tr>
<tr>
<td>10</td>
<td>942</td>
<td>0.54</td>
<td>180</td>
<td>0.46</td>
</tr>
<tr>
<td>11</td>
<td>920</td>
<td>0.12</td>
<td>190</td>
<td>0.88</td>
</tr>
</tbody>
</table>

All the samples were excited at 580 nm and emission was collected after 610 nm with a cut-off filter. Fluorescence lifetimes were adequately described by two exponentially decaying components: \( F(t) = a₁\exp(-t/τ₁) + a₂\exp(-t/τ₂) \). The absence of values for \( a₂ \) and \( τ₂ \) indicate that lifetime was adequately described by a single exponential decay. For hypomycin B in Brij micelles in D₂O, the time constant is 900 ps.
experimental error, there is no difference in the decaying transient in the two solvents. This result is discussed below.

**Multiple H-Atom Transfers in the Perylene Quinones?**

Hypomycin B is unique in that it has only one intramolecular hydrogen bond as opposed to two in hypocrellin A and four in hypericin (Figure IV.1). Picosecond transient absorption data for hypomycin B fail to reveal any stimulated emission, let alone rise time in stimulated emission (unlike hypocrellin and hypericin), which we have interpreted in terms of excited-state H-atom transfer. Furthermore, a global analysis of the excited-state kinetics at four different probe wavelengths in MeOD and MeOH yields no significant difference in the excited-state kinetics: the time constants are 82 and 75 ps, respectively. Transient absorption data for hypomycin B in other solvents like DMSO and buffer-DMSO mixtures (data not shown) also failed to reveal any rise time in the stimulated emission. In the context of our previous arguments and criteria for identifying hydrogen atom transfer in hypericin, hypocrellin, and their analogs, one might hastily conclude that hypomycin B does not undergo this process. Compare Figures IV.10 and IV.11. Figure IV.11 clearly reveals the presence of a growth in the kinetics for hypocrellin A, but which is not present for the hypomycin B data in Figure IV.10. If such a conclusion were made, however, prudence demands that it be only tentative.

If subsequent experiments do indeed demonstrate that excited-state hydrogen atom transfer does not occur in hypomycin B, then one may draw the conclusion that multiple transfers (either concerted or stepwise) must occur in these perylene quinones and that by
Figure IV.6. Plot of the average fluorescence lifetime of hypocrellin A in $2 \times 10^{-2}$ M (100 times CMC) aqueous Brij 35 solutions against pH. The data were fit to a sigmoid. The inflection point, obtained from the second derivative of the fitted curve, as shown in the inset, yields a pKa of 8.9. The fluorescence decays were single exponential for all pH values except those in the region of the inflection point (pH ~ 8 to 10). At pH values of less than 8, even up to an acid concentration of 3 M, the lifetime remained constant and single exponential.

frustrating the process in one half of the molecule, the process in the other half is impeded. At this point, such reasoning is speculative and contrary to the growing body of evidence provided by theory and experiment. Quantum mechanical calculations indicate that the double hydrogen atom transfer in hypericin (63) and in the perylene quinone nucleus (64) of hypocrellin is energetically unfavorable compared to the single transfer event. Experiments
Figure IV.7. Absorption spectra of hypomycin B in 100 CMC Brij solutions of pH varying from 5 to 13. All the spectra have been normalized to unity at the peak maximum.

for hypericin in which the one half of the molecule cannot participate in hydrogen atom transfer owing to protonation of the carbonyl group (or even perhaps complexation with a metal ion) (65) also indicate that the transfer process can still occur.

Given the richness of the photophysics of these perylene quinones and their attendant complexity, it is premature to conclude that hypomycin B does not execute an excited-state hydrogen atom transfer. Certainly, much more work is required to reach a proper judgment.
This will include fluorescence upconversion experiments with picosecond resolution in order to interrogate the entire time scale of relevance as well as to monitor only emission, which can simplify the analysis (66). For example, the failure to detect stimulated emission for hypomycin B may simply be the result of the presence of a strongly absorbing species in the same spectral region. Also, experiments must be performed to determine if hypomycin B, like hypericin (36, 37, 67) and hypocrellin A (38), acts an excited-state acid. It is clear, however, that hypomycin B is an important system with which to test and refine our current understanding of these naturally occurring perylene quinones. One of the most important questions that the study of hypomycin B might fruitfully address is the elucidation of the reaction coordinate in the H-atom transfer process in the perylene quinones.

As we have discussed in depth elsewhere, despite the similarities in the structures of hypericin and hypocrellin, which are centered about the perylene quinone nucleus, their excited-state photophysics exhibit rich and varied behavior. The H-atom transfer is characterized by a wide range of time constants, which in certain cases exhibit deuterium isotope effects and solvent dependence. Of particular interest is that the shortest time constant we have observed for the H-atom transfer is ~10 ps. This is exceptionally long for such a process, 100-fs being expected when the solute H atom does not hydrogen bond to the solvent (68). That the transfer time is so long in the perylene quinones has been attributed to the identification of the reaction coordinate with skeletal motions of the molecule (66, 69). The identification of the reaction coordinate and the question of whether one or more H atoms are transferred is related. We have previously observed that when hypericin is bound to human serum albumin, it no longer undergoes an excited-state hydrogen atom transfer. Assuming that the binding occurs through the interaction of one of the two carbonyl groups
Figure IV.8. Emission spectra of hypomycin B in 100 CMC Brij solutions of different pH. $\lambda_{ex} = 480$ nm. All spectra have been normalized to an arbitrary value at the peak maximum. (Beyond pH 9, there is a drastic reduction in fluorescence intensity, indicating that the deprotonated species is far less emissive compared to the normal form of hypomycin B).

of hypericin and the $N_1$-H of the single tryptophan residue (W214), which would necessarily impede H-atom transfer on this half of the hypericin molecule, we suggested that the absence of H-atom transfer in the complex was indicative of concerted, double H-atom transfer in the excited state of hypericin (31). We suggested that H-atom transfer is completely impeded when hypericin binds to HSA because skeletal motion is coupled to the H-atom transfer.
Figure IV.9. Comparison of the fluorescence lifetime decays of hypomycin B in 100 CMC Brij 35 solution of varying pH: (a) pH = 9, (b) pH = 10 and (c) pH = 11. For all the samples $\lambda_{ex} = 580$ nm. The fitting parameters are reported in Table IV.1.

(66, 69, 70). Fluorescence anisotropy measurements of the HSA/hypericin complex indicate that the hypericin is rigidly bound and that there is no rapid restricted motion of hypericin relative to the protein. By analogy, one might argue that if H-atom transfer does not occur in hypomycin B, it is not because the process requires that two H atoms be in flight but because the required skeletal motion is restricted by the presence of the O-C-O bond. Although this response is plausible, it is not easy to reconcile it with the observation that hypericin undergoes H-atom transfer in a glass at low temperatures (the energy of activation is 0.05 kcal/mol) (32), where the amplitude of skeletal motion would seem to be less than that in the HSA matrix. These sorts of problems and questions continue to illustrate the need for further
elucidation of the reaction coordinate for the H-atom transfer in hypericin and its analogs (69).

To conclude this section, we note that our assignment of excited-state H-atom transfer to the primary photoprocess in hypericin, hypocrellin, and their derivatives has occasioned some objections, to which we refer in the introduction and which we address in detail elsewhere, especially in references (66, 69). An additional concern, which has been brought to our attention, that is relevant to address in the light of the previous discussion is the following. As we note above, we have measured the energy of activation for the H-atom transfer in hypericin to be 0.05 kcal/mol (or ~20 cm$^{-1}$). The absence of an isotope effect for the hypericin reaction (and for the ~10-ps reaction in hypocrellin A) indicates that the reaction coordinate is not the hydrogen atom coordinate (which theoretical and experimental results suggest is ~1450 cm$^{-1}$ in the hypericin triplet (70)) and consequently must involve skeletal motions invoked above. It has been suggested by an anonymous colleague that if there is a slow hydrogen atom transfer that is not limited by the hydrogen atom coordinate, "then it must be that vibrational excitation of oxygen or ring modes is what limits the rate. This would correspond to a significant vibrational barrier, and hence large activation energy." We disagree with the last statement. Quantum mechanical calculations (see reference (63) and http://www.msg.ameslab.gov/Group/Supplementary_Material/Hypericin/) indicate that there are four calculated frequencies (unscaled) below 100 cm$^{-1}$: 40 cm$^{-1}$, out-of-plane motion of oxygens and carbons; 48 cm$^{-1}$, oxygen and carbon displacements; 80 cm$^{-1}$, mostly OH oxygen motion; 84 cm$^{-1}$: mostly OH oxygen motion. There is no dearth of low frequency vibrations in large biological molecules as the calculations and many experiments (71-73)
Figure IV.10. Comparison of the kinetic traces of hypomycin B in MeOD (solid lines) and MeOH (dashed lines). The probe wavelengths are given in the right hand top corner of each panel. A global fit was carried out to fit the decays of the several wavelengths. In MeOH, the global time constant was 82 ps while that in MeOD was 75 ps ($\lambda_{ex} = 407$ nm).

suggest; and we believe that such motions may indeed be coupled to the hydrogen atom transfer in these perylene quinone systems.

SUMMARY AND CONCLUSIONS

Jardon and coworkers have measured the pK$_a$s of the bay (3 and 4 positions) and the peri hydroxyl groups (1, 6, 8, and 13 positions) of hypericin in Brij-35 micelles and in
aqueous liposome media. Their work indicates a pKₐ of 7 for the bay hydroxyl, of 11 for the peri hydroxyls, and of 1 for the protonated groups (49). In contrast, hypocrellin A in a similar environment has a pKₐ of 8.9 for the peri hydroxyls; the pKₐ of its protonated carbonyl could not be determined. Recently, using Brij concentrations of $5 \times 10^{-2}$ M (2.5 times the highest concentration we employ, and 250 times the CMC), Dumas (57) has obtained a similar value of 8.2 for hypocrellin A. Another titratable group is resolved with a pKₐ of 11.4. Dumas obtains similar results for hypocrellin B.

Previously we had investigated the light-induced antiviral activity of hypericin and hypocrellin in the presence and absence of oxygen under experimental conditions where the effect of oxygen depletion could be quantified (74). There was a significant reduction of light-induced activity under hypoxic conditions. Interestingly, antiviral activity of hypocrellin was not observed at the low oxygen levels at which hypericin retained measurable virucidal activity. Based on these results, we proposed that additional pathways, such as the generation of protons from excited states of hypericin, may enhance the activity of activated oxygen species. The results presented here suggest a reason why this may be the case. There is much evidence indicating that the photophysical and biological properties of hypericin and its analogs are drastically altered when they exist as aggregates. Aggregates do not exhibit excited-state H-atom transfer, have markedly reduced fluorescence quantum yields, shorter fluorescence lifetimes, and reduced virucidal and cytotoxic activity (31). Under conditions where hypericin exists as the monomer and hypocrellin is still induced to aggregate, such as those modeled in this work, it is not surprising that the biological activity of the latter should be reduced. Similar antiviral and antitumor studies on hypomycin B will provide an interesting comparison with those of hypocrellin A.
Figure IV.11. Kinetic traces of hypocrellin in MeOH and MeOD at four different probe wavelengths. Global fits to the data yield time constants of 67 and 97 ps for MeOH and MeOD, respectively. $\lambda_{ex} = 588$ nm. From reference (27).

Our previous results on hypericin indicate that excited-state H-atom transfer occurs even when one of the carbonyls is prohibited from accepting a hydrogen. The presence of such a transfer is apparent under very acidic conditions in AOT reverse micelles and cannot
be excluded upon chelation of Tb\(^{3+}\) (65). There is, thus, no evidence for a concerted hydrogen-atom transfer mechanism in hypericin. In the present study, contrary to our initial expectations, we are not even able to demonstrate that hypomycin B executes an excited-state H atom transfer; and hence our investigation sheds no light on the general question of how many H atoms are transferred in the perylene quinones and whether the transfer is concerted or stepwise. On the other hand, if further investigation does reveal that H atom transfer does not occur in hypomycin B, the result would have considerable implications for an understanding of the reaction coordinate for the H atom transfer.

ACKNOWLEDGEMENTS

This work was partially supported by NSF grant CHE-9613962 to J. W. P. We thank Professor Pierre Jardon for providing us a copy of the thesis by S. Dumas.

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photosensitized by hypericin. Characterization and study of the mechanism by laser

Phys.* **85**, 971-977.


in the ground state and the first excited singlet state in a nonionic micellar medium. *J. Chim.


oxygen photosensitized by hypericin in the liposomes of dipalmitoylphosphatidylcholine.


CHAPTER V. THE LEGHEMOGLOBIN PROXIMAL HEME POCKET DIRECTS OXYGEN DISSOCIATION AND STABILIZES BOUND HEME

A paper published in *Proteins: Structure, Function and Genetics*.

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**Abstract**

Sperm whale myoglobin (Mb) and soybean leghemoglobin (Lba) are two small, monomeric hemoglobins that share a common globin fold but differ widely in many other aspects. Lba has a much higher affinity for most ligands and the two proteins use different distal and proximal heme pocket regulatory mechanisms to control ligand binding. Removal of the constraint provided by covalent attachment of the proximal histidine to the F-helices of these proteins decreases oxygen affinity in Lba and increases oxygen affinity in Mb, mainly due to changes in oxygen dissociation rate constants. Hence, Mb and Lba use covalent constraints in opposite ways to regulate ligand binding. Swapping the F-helices of the two proteins brings about similar effects, highlighting the importance of this helix in proximal heme pocket regulation of ligand binding.


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The F7 residue in Mb is capable of weaving a hydrogen-bonding network which holds the proximal histidine in a fixed orientation. On the contrary, the F7 residue in Lba lacks this property and allows the proximal histidine to assume a conformation favorable for higher ligand binding affinity. Geminate recombination studies indicate that heme iron reactivity on picosecond time scales is not the dominant cause for the effects observed in each mutation. Results also indicate that in Lba the proximal and distal pocket mutations probably influence ligand binding independently. These results are discussed in the context of current hypotheses for proximal heme pocket structure and function.

Introduction

Myoglobin, the monomeric hemoglobin found in muscle fibers, is widely used as a model system for deciphering the structural and biophysical mechanisms that govern ligand binding in heme proteins. Recombinant myoglobin has provided insight into ligand binding properties, folding and stability of hemoglobins, and is the paradigm for structure and function in this class of proteins. Attempts to extrapolate the understanding of Mb to the more complex structural and ligand binding behaviors seen in human hemoglobin and a host of newly discovered bacterial, protist, and plant hemoglobins and flavohemoglobins have revealed some common features, but many differences from Mb (1,2,3,4). This necessitates the development of parallel model systems which could supplement the existing Mb model system in predicting the diverse functional mechanisms of a variety of hemoglobins.

Leghemoglobins are small, monomeric heme proteins found in root nodules of leguminous plants, which provide an ideal model system for ligand binding since they display an opposite extreme to Mb in many aspects of their function. Leghemoglobins have a higher affinity for oxygen and other ligands generally due to higher association rate
constants and lower dissociation rate constants (5). Both Mb and soybean leghemoglobin (Lba) share a common globin fold, with a His residue at the seventh position on the E helix (the "distal" His$_E^d$) that is located directly above the distal ligand binding site (Figure V.1). In Mb, His$_E^d$ is able to form a strong hydrogen bond with ligands thus influencing ligand binding (6,7). The structure of oxy lupine leghemoglobin indicates a hydrogen bond between the His$_E^d$ side chain and bound oxygen (8). Thus, hydrogen bonding to the bound ligand is thought to occur in leghemoglobins, especially at low pH (9,10). It was originally hypothesized that His$_E^d$ in Lba stabilized bound oxygen and was responsible for high oxygen affinity in this protein. However, replacement of His$_E^d$ with Leu in Lba results in a negligible reduction in oxygen affinity (11). These data suggest that His$_E^d$ is not responsible for the high oxygen affinity in Lba and indicate that Lba and Mb use different mechanisms to regulate ligand binding.

Another factor regulating ligand binding in hemoglobins is the proximal heme pocket environment. The proximal His (His$_F^p$), which forms the only covalent bond between the protein and heme (Figure V.1), is known to be an important factor in regulating ligand binding in hemoglobins (12,13). The best characterized example of proximal regulation of ligand binding affinity in hemoglobins occurs in the α and β subunits of human hemoglobin. The shift from R to T state hemoglobin causes His$_F^p$ to pull the iron atom slightly out of the plane of the heme resulting in a ~800 fold reduction in oxygen affinity (14,15). The degree to which the proximal His$_F^p$ affects reactivity and protein stability in Mb and human hemoglobin has been a recent focus of research (16,17,18).

Barrick and Boxer (19, 20) have developed an ingenious technique to study proximal effects in Mb and hemoglobin. They prepared a mutant of Mb where covalent constraint on
**Figure V.1.** A structural depiction of the similar globin folds of Mb (A, left) and Lba, (C, left) and a view along the bond between the proximal His and the oxy heme iron (to the right of each). The dotted line is parallel to the plane of the His ring, showing eclipsed (Mb) and staggered (Lba) conformations. B: Stereo image of the E (green) and F (yellow) helices of Mb and (D) Lba housing the heme. The H-bonding (pink lines) network involving Ser^F7 in Mb is shown in (B). The F7 residue in Lba is valine and consequently it lacks this H-bonding network. The Mb and Lba structures are from Brucker et al. (55) (PDB accession code 2MBW), and Hargrove et al. (11) (PDB accession code 1BIN), respectively.
His\textsuperscript{F8} from the F-helix was removed. This was accomplished by replacing His\textsuperscript{F8} with a glycine residue and binding an exogenous imidazole molecule in place of the His. The imidazole mimics the His side chain but, since it is not attached to the F-helix, it is not subject to covalent constraints from the rest of the protein. The crystal structure of H(F8)G:imidazole Mb shows a conformation in which the proximal imidazole is staggered with respect to the pyrrole nitrogens of the heme (19). In contrast, His\textsuperscript{F8} in wild type Mb eclipses the pyrrole nitrogens (21,22). It has been hypothesized that the staggered conformation, which allows the heme iron to move more into the plane of the porphyrin ring, is associated with increased ligand affinity. In lupine leghemoglobin, His\textsuperscript{F8} is staggered with respect to the heme pyrrole nitrogens and it has been suggested that this may contribute to the high ligand affinity of leghemoglobins (8,23).

The structure of the Mb proximal heme pocket suggests some mechanisms for orienting the proximal His, thereby regulating ligand binding affinity. A hydrogen-bonding network exists among several proximal pocket amino acids and the heme-7-propionate, which is responsible for limiting movement of His\textsuperscript{F8} (24). This network involves Ser\textsuperscript{F7}, which forms a hydrogen bond directly with the His\textsuperscript{F8} side chain (Figure V.1B). Ser\textsuperscript{F7} is conserved in myoglobins, with the exception of a few examples of Thr. Replacement of Ser\textsuperscript{F7} with amino acid side chains incapable of hydrogen-bonding results in proximal pocket solvation, hemin dissociation, and lower protein stability (24,25,26). When Ser\textsuperscript{F7} is replaced by larger aliphatic side chains (Leu, Val), oxygen affinity is increased. The role of the Ser\textsuperscript{F7}-His\textsuperscript{F8} H-bond in Mb and human hemoglobin has also recently been investigated using trans substitution of His\textsuperscript{F8} with imidazoles (16,27).
While distal pocket regulatory mechanisms can influence the environment of the bound ligand, proximal regulation is expected to directly affect heme iron reactivity (24,28). Determination of heme iron reactivity is complex due to multiple barriers to ligand binding, but measurements of ligand geminate recombination in addition to bimolecular association and dissociation can yield meaningful values (29,30). One method for evaluating heme iron reactivity is measurement of nitric oxide rebinding following an ultrashort photo dissociating laser pulse (31,32,33,34). Since nitric oxide has a very small electronic barrier for heme rebounding relative to oxygen or carbon monoxide, ultrafast measurements of its geminate recombination provide a useful gauge of heme iron reactivity (35,36). While these reactions have been used to determine the effects of distal heme pocket mutations in Mb, they have not yet been applied to evaluating the effects of changes in the Mb proximal heme pocket and there has been no functional evidence for the role of the proximal heme pocket on heme iron reactivity in Lba (33,37).

The present investigation concerns ligand binding to Lba and Mb mutant hemoglobins where the proximal heme pockets have been altered in ways designed to identify factors that dictate differences in ligand binding to these proteins. We have prepared a His$^{F8}$ deletion mutant (H92G) in Lba to evaluate the role of covalent restraint on ligand binding. In addition, S(F7)V and V(F7)S mutations in sperm whale Mb and Lba, respectively, are used to determine the importance of this amino acid in the plant hemoglobin. "Helix swap" mutants of Mb and Lba were also prepared in which an eight amino acid section of each F-helix was replaced with that of the other. Finally, the consequence of the distal pocket H(E7)L substitution has been evaluated in the helix swap proteins in an effort to determine whether proximal and distal regulatory mechanisms are
linked. Bimolecular association and dissociation rate constants for oxygen and carbon monoxide were measured for each protein along with geminate nitric oxide recombination on the picosecond to nanosecond time scales. The results indicate that Mb and Lba use different proximal mechanisms for regulating ligand binding.

Materials and Methods

Mutagenesis of Lba and Mb cDNAs

The Lba cDNA used for mutagenesis has been described previously (11). The recombinant Mb gene was provided by John Olson (38,39). All mutants, with the exception of LbaMbF and MbLbaF, were produced using Stratagene’s Quick-Change Site Directed Mutagenesis kit. Mutagenesis primers were made either by the Iowa State University DNA Facility or One Trick Pony Oligos Division of Ransom Hill Bioscience (Ramona, CA). The LbaMbF mutant was obtained by cassette mutagenesis. “Silent” mutations were introduced into the Lba cDNA to create BamHI and KpnI sites into the middle of the gene which allows the replacement of 18 amino acid residues centered around the proximal His and containing the F-helix of Lba. Using this cassette, the Lba sequence of eight amino acids (LGSVHAQK) centered on His\(^{F8}\) (in bold) were replaced with the sequence LAQSHATK from swMb. The synthetic swMb gene produced by Springer and Sligar (39) contains unique restriction enzyme sites that were used to make the complementary mutation in which the resulting Mb has the Lba F-helix sequence. Plasmids were isolated either by chloroform extraction and isopropanol precipitation or using the QIAGEN QIAprep spin Miniprep plasmid preparation kit. All sequencing was performed by the Iowa State University DNA Sequencing Facility.
Expression and purification of proteins

Expression vectors pET 28a and pET 29a (Novagen) were used to express leghemoglobins and myoglobins, respectively, as described previously for Lba (11). A sample of H93G Mb was provided by Doug Barrick. *Escherichia coli* BL21 (λDE3)-Codon Plus-RP cells (Stratagene, La Jolla, CA) were used as the protein expression host. The cells were grown in 20 L of Terrific Broth medium supplemented with 50μg/ml kanamycin, 50μg/ml chloramphenicol, and 1.0ml antifoam (Sigma) at 37°C. For myoglobin and its mutants, 2ml of 1M iron (III) chloride was added per liter of broth before inoculation. For H92G Lba, imidazole at pH 7.5 was added to the media to a concentration of 10mM. Once the cells attained an optical density that is 1.3 absorbance units higher than the initial, they were induced with isopropyl-D-thiogalactopyranoside (Lab Scientific Inc.) to a final concentration of 0.5mM and incubated for an additional five hours. 4mg/L hemin chloride (SIGMA) (dissolved in a small amount of 0.1M NaOH) was added to the broth during induction.

Cell pellets were thoroughly resuspended in 3ml of lysis buffer (50mM Tris, 100mM NaCl, pH 8.0) per g of pellet. For cell lysis, 300 μg of lysozyme (Research Organics Inc.) per g of cell pellet was added to the resuspension and incubated for 30 minutes with agitation at room temperature. This was followed by addition of 4 mg of deoxycholic acid (SIGMA) per g of cell pellet and incubation at 37°C for 15 minutes. Finally, 30 μg of DNase (SIGMA) per g of cell pellet and appropriate amount of 3000X DNase buffer (1M CaCl₂, 1M MgCl₂, 1M MnCl₂) were added, followed by incubation at room temperature for 15 minutes. The cell pellets, thus treated, were frozen overnight. The cells were then thawed and centrifuged.
for 25 minutes at 25000xg to remove cell debris, leaving either the apoprotein or holoprotein in the supernatant. If the supernatant contained apoprotein (as evidenced by the lack of bright red color and SDS-PAGE), hemin was added to reconstitute the holoprotein. In the case of H92G Lba, 1-10mM imidazole was added in all lysis buffers.

For Lba and its mutant proteins, ammonium sulfate was added to 55% saturation and centrifuged at 25000xg for 25 minutes. The protein in the supernatant was then precipitated by adding ammonium sulfate to 90% saturation, followed by centrifugation at 25000xg for 25 minutes. The pellet was resuspended in 1.9M ammonium sulfate in 20mM Tris-HCl, pH 8.5, loaded onto a Phenyl Sepharose (SIGMA) column and eluted with 0.9M ammonium sulfate. The proteins were then dialyzed into 20mM Tris-HCl, pH 8.5, loaded onto a DEAE (Pharmacia) column, and eluted with 50mM NaCl in 20mM Tris-HCl, pH 8.5.

For Mb and its mutants, ammonium sulfate was added to 60% saturation and centrifuged as described above. The supernatant was then adjusted to an ammonium sulfate concentration of 1.9M and loaded onto a Phenyl Sepharose column. The protein was eluted with 0.9M ammonium sulfate and dialyzed into 20mM Tris-HCl, pH 8.5. The proteins were then passed through a DEAE column twice in 20mM Tris-HCl, pH 8.5.

Spectroscopic and kinetic characterization of wild type and mutant Lba and Mb

Absorbance spectra were measured using a Hewlett Packard 845X UV-Visible spectrophotometer. HbCO and HbO₂ samples were prepared by reducing the proteins with sodium dithionite and loading them into a Sephadex G-25 size exclusion column equilibrated with 100mM phosphate, pH 7.0 saturated in 1atm CO and air, respectively. The dithionite free, ligand-bound samples were collected in sealed cuvettes. Ferrous NO samples were
made by diluting dithionite free CO samples (made in 10% CO, 90% N₂ mixture) 1:1 with NO saturated anaerobic buffer.

The rate constants for bimolecular O₂ and CO binding were measured by rapid mixing and laser photolysis methods (40,41,42) using a previously described laser flash apparatus (43). All kinetic measurements, except the data for H92G Lba and H93G Mb, were taken at 25°C in 100mM phosphate, pH 7.0. Measurements for H92G Lba and H93G Mb were taken in 100mM phosphate, pH 7.0, with 5mM imidazole. Error estimates for association and dissociation rate constants are ~± 10% of the measured value. The binding of imidazole in the proximal or distal pocket was verified by observing the spectra characteristic of 5- or 6-coordinate complex for the deoxy forms of each protein (Figure V.2).

**Measurement of geminate recombination kinetics**

The NO complexed protein samples were prepared in a cuvette (1mm path length) stoppered with a septum in an oxygen free atmosphere by replacing CO with NO as described above. The absorption spectra of the NO forms of the wild type proteins and their mutants used in this study are virtually identical. The protein samples typically had an absorbance of ~0.5 at 407nm. The laser apparatus for pump-probe transient absorption measurements used in the geminate recombination studies has been described in detail elsewhere (44). In brief, a 1kHz home-made, regeneratively amplified Ti:sapphire laser was used to photolyze the sample at 407nm with an energy of 1μJ/pulse. Part of the pulse train was used to generate a white light continuum, which permitted the probing of absorption changes at 438nm signifying the decay of the photolyzed state. To confirm that the observed kinetics were not the outcome of any experimental artifact, the flatness of the translation...
stage was checked by using an MbCO sample on the same time scales. To facilitate
comparison of data for various mutants, the absorbance changes were normalized to one at
the maximum absorbance. Curve fitting of the data obtained on a 400 ps time scale was
performed using the three exponential function: \( \Delta A(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + \text{baseline} \); the parameters from such fits are reported in Table III.

**Measurement of hemin dissociation**

Rate constants for hemin dissociation were measured as described previously (45). Apomyoglobin was used at a concentration of 50\(\mu\)M while holoproteins were used at a concentration of 3\(\mu\)M. The absorbance changes were measured continuously in a Cary 50 Bio Varian UV-visible spectrophotometer equipped with an eighteen-cell changer and a water bath to regulate temperature. The time courses at 403nm and 409nm were used for Lbas and Mbs, respectively, to determine hemin dissociation rate constants. For Lba and its mutants, MES was used as the buffer at pH 5.0 as acetate coordination to Lba markedly inhibits this reaction. For Mb and its mutants, sodium acetate was used at pH 5.0. At pH 7.0 potassium phosphate was used as a buffer. All assays were performed at 20°C.

**Results**

**Removal of covalent constraints on Lba His\(^{F8}\)**

In comparison to Mb, work with the Lba proximal His deletion mutant protein is more complex because the heme pocket of H92G Lba binds imidazole on both the proximal and distal sides with relatively high affinity. In order to develop a model of Lba where the covalent constraints of proximal His binding to the heme iron are removed, kinetic measurements of ligand binding to the constraint-free mutants (H(F8)G) should be taken
Figure V.2. Absorbance spectra of deoxy wild type (---) and H92G Lba (—--) in 100mM phosphate, pH 7.0 and (A) 5mM imidazole (B) 1M imidazole.

when an imidazole is bound only in the proximal pocket of Lba. If imidazole binds only in the proximal pocket, the spectrum of the resulting 5-coordinate complex should be similar to that of ferrous wtLba, which has two distinct peaks with $\lambda_{\text{max}}$ of 428 and 555nm (11). The spectra of deoxy Lba and H92G Lba in 5 mM and 1M imidazole are shown in Figure V.2. At 5mM imidazole, both wild type and H92G Lba have absorbance spectra characteristic of penta-coordinate complexes (Figure V.2A). At very high concentrations of imidazole (e.g. 1M), binding occurs in the distal pocket as well, and a characteristic 6-coordinate spectrum with $\lambda_{\text{max}}$ at 423, 528 and 557nm (Figure V.2B) is observed. Therefore, measurements of
ligand binding to H92G Lba were performed in 5mM imidazole so that imidazole was present as the proximal heme ligand but not bound on the distal side.

Table V.I presents a comparison of the kinetic and equilibrium constants for ligand binding to Lba, Mb, and their respective H(F8)G:imidazole mutant proteins. Replacement of His\textsuperscript{\textit{F8}} with exogenous imidazole in Lba causes a ~3-fold decrease in O\textsubscript{2} and CO affinity. In contrast, the corresponding replacement in Mb results in a ~5-fold and 4-fold increase in O\textsubscript{2} and CO affinity, respectively. Removal of covalent constraints on the proximal His causes opposite effects in Lba and Mb with the major influence being on the rate constants for ligand dissociation.

\textit{Effects of F-helix “swapping” in Lba and Mb on O\textsubscript{2} and CO binding}

The effects brought about by removal of covalent constraints on His\textsuperscript{\textit{F8}} in Lba could possibly be due to helix-imposed proximal environments. This was investigated by exchanging the F-helix environments in Lba and Mb. The O\textsubscript{2} and CO binding kinetics for Lba, Mb, and the corresponding "helix swap" mutant proteins are shown in Table V.I.

Table V.I. Ligand binding to the imidazole complexes of H(F8)G mutants of soybean leghemoglobin and sperm whale myoglobin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(k'_\text{O}_2) (\text{(\mu\text{M}^{-1}\text{s}^{-1})})</th>
<th>(k_{\text{O}_2}) (\text{s}^{-1})</th>
<th>(K_{\text{O}_2}) (\text{(\mu\text{M}^{-1})})</th>
<th>(k'_\text{CO}) (\text{(\mu\text{M}^{-1}\text{s}^{-1})})</th>
<th>(k_{\text{CO}}) (\text{s}^{-1})</th>
<th>(K_{\text{CO}}) (\text{(\mu\text{M}^{-1})})</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Lba</td>
<td>130</td>
<td>5.6</td>
<td>23</td>
<td>15</td>
<td>0.0084</td>
<td>1800</td>
</tr>
<tr>
<td>H(F8)G Lba</td>
<td>120</td>
<td>15</td>
<td>8</td>
<td>11</td>
<td>0.019</td>
<td>580</td>
</tr>
<tr>
<td>wt Mb</td>
<td>17</td>
<td>15</td>
<td>1.1</td>
<td>0.51</td>
<td>0.019</td>
<td>27</td>
</tr>
<tr>
<td>H(F8)G Mb</td>
<td>20</td>
<td>3.9</td>
<td>5.1</td>
<td>1.2</td>
<td>0.011</td>
<td>110</td>
</tr>
</tbody>
</table>

\(k'_l\), \(k_l\), \(k_{\text{CO}}\) are association rate constants, dissociation rate constants, and affinity constants \((k'_l/k_l)\) where \(L\) is either O\textsubscript{2} or CO. All error values are approximately \(\pm 10\%\) of the measured value.
Table V.2. Kinetic binding constants of the wild type proteins, their helix swap mutants, F7 mutants, E7 mutants and His(E7) substituted helix swap mutants.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k'_O_2$ (μM$^{-1}$s$^{-1}$)</th>
<th>$k_O_2$ (s$^{-1}$)</th>
<th>$K_O_2$ (μM$^{-1}$)</th>
<th>$k'_C_O$ (μM$^{-1}$s$^{-1}$)</th>
<th>$k_C_O$ (s$^{-1}$)</th>
<th>$K_C_O$ (μM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Lba</td>
<td>130</td>
<td>5.6</td>
<td>23</td>
<td>15</td>
<td>0.0084</td>
<td>1800</td>
</tr>
<tr>
<td>Lba$\text{Mb_F}$</td>
<td>140</td>
<td>19</td>
<td>7.4</td>
<td>13</td>
<td>0.021</td>
<td>620</td>
</tr>
<tr>
<td>H61L Lba</td>
<td>400</td>
<td>24</td>
<td>17</td>
<td>170</td>
<td>0.0024</td>
<td>71000</td>
</tr>
<tr>
<td>H61L:Lba$\text{Mb_F}$</td>
<td>460</td>
<td>120</td>
<td>3.7</td>
<td>140</td>
<td>0.016</td>
<td>9000</td>
</tr>
<tr>
<td>V91S Lba</td>
<td>130</td>
<td>20</td>
<td>6.5</td>
<td>12</td>
<td>0.019</td>
<td>630</td>
</tr>
<tr>
<td>wt Mb</td>
<td>17</td>
<td>15</td>
<td>1.1</td>
<td>0.51</td>
<td>0.019</td>
<td>27</td>
</tr>
<tr>
<td>Mb$\text{L_ba}_F$</td>
<td>20</td>
<td>3.8</td>
<td>5.2</td>
<td>1.4</td>
<td>0.012</td>
<td>120</td>
</tr>
<tr>
<td>H64L Mb</td>
<td>98</td>
<td>4100</td>
<td>0.02</td>
<td>26</td>
<td>0.024</td>
<td>1100</td>
</tr>
<tr>
<td>H64L:Mb$\text{L_ba}_F$</td>
<td>440</td>
<td>300</td>
<td>1.5</td>
<td>64</td>
<td>0.019</td>
<td>3400</td>
</tr>
<tr>
<td>S92V Mb</td>
<td>21</td>
<td>4.9</td>
<td>4.3</td>
<td>1.3</td>
<td>0.012</td>
<td>110</td>
</tr>
</tbody>
</table>

Data for H64L Mb are from Springer et al. (7). All error values are approximately ± 10% of the measured value.

Replacement of the F-helix of Lba with that of Mb (Lba$\text{Mb_F}$) results in a ~3-fold decrease in O$_2$ affinity. The association rate constant ($k'_O_2$) of Lba$\text{Mb_F}$ is almost the same as wtLba, and the difference in affinity arises due to an effect on the dissociation rate constant ($k_O_2$). The complementary mutant, Mb$\text{L_ba}_F$ (Mb with the F-helix of Lba), shows a ~5-fold increase in O$_2$ affinity compared to wtMb. As with the other helix swap mutant, $k'_O_2$ of Mb$\text{L_ba}_F$ and wtMb are similar and the increase in O$_2$ affinity is mainly due to a decrease in $k_O_2$. CO binding in the helix swap mutants follows a similar trend as the overall affinity decreases ~3-fold in Lba and increases ~4-fold in Mb (Table II).

Effects of F-helix "swapping" on distal pocket mechanisms for ligand stabilization
In Mb it has been conclusively shown that bound O$_2$ is stabilized in the distal pocket through hydrogen bonding with His$^{E7}$ (7). In contrast, it has been demonstrated that hydrogen bonding between bound O$_2$ and His$^{E7}$ in Lba is not nearly as important for stabilizing the bound ligand (11). The effect of F-helix swapping on such interactions in the distal pocket is summarized in Table V.2 in comparison to the single H(E7)L mutations in Mb and Lba which have been described previously (6,11). Insertion of the Mb F-helix in H61L Lba causes an additional increase in the rate constant for O$_2$ dissociation. In comparison, the Lba F-helix stabilizes bound O$_2$ in the background of the Mb H64L protein, as evidenced by the much higher O$_2$ affinity of H64L:Mb$_{LbaF}$ compared to H64L Mb. Similar, albeit smaller effects are observed with CO. Surprisingly, the H64L:Mb$_{LbaF}$ mutant protein has significantly larger O$_2$ and CO association rate constants than H64L Mb. A comparable effect is not observed in the homologous Lba mutant protein.

*The role of residue F7 in proximal regulation*

Ser$^{F7}$ in swMb is part of a network of H-bonding involving the proximal His and heme-7-propionate (Figure V.1B). In pig Mb this residue was shown to affect ligand binding and heme stability (24). The corresponding residue in Lba is Val, the side chain of which is incapable of forming such H-bonds with the adjacent proximal His or heme propionate (Figure V.1C). Substitution of a Ser residue in place of Val in the F-helix of Lba results in a protein with a ~3.5-fold lower O$_2$ affinity compared to wtLba (Table V.2). V91S Lba has an O$_2$ association rate constant similar to the wild type protein and the increase in the O$_2$ dissociation rate constant is responsible for its lowered affinity.
An opposite effect is observed in S92V Mb, which is nearly identical to the result of a similar mutation in pig Mb (24). In S92V swMb, the O₂ affinity increases by ~4-fold compared to wtMb due to a ~3-fold decrease in the O₂ dissociation rate constant while the O₂ association rate constant increases by only ~1.2-fold. The O₂ affinities of the Lba and Mb F7 mutant proteins are similar and they behave as the helix swap mutants in this respect. The same trend is observed for CO binding, where the CO affinities decrease in V91S Lba and increase in S92V Mb.

**Relationship of the proximal heme pocket to heme iron reactivity**

In an effort to explain the results presented in Table V.2, ultrashort laser flash experiments were performed to test the effects of each mutation on picosecond geminate recombination of NO. Time courses following photolysis are shown in Figure V.3. The panels on the left (Figures V.3A, B and C) compare the NO rebinding kinetics of wtMb, MbLbaF and S92V Mb on three different time scales. Figures V.3B and C also show control reactions in which MbCO samples were used. The flatness of these control time courses demonstrates that the subsequent time courses are associated with NO rebinding. It is evident that the traces are nearly identical and overlapping for wtMb and its mutants. The panels on the right (Figures V.3D, E and F) illustrate the decay curves following photo dissociation of the NO complexes of Lba, Lba_{MbF}, and V91S Lba. As in Mb, there are no major differences in their geminate recombination kinetics on the 40-ps time scale. However, on the longer time scales, NO rebinding is slightly faster in the mutant proteins. For purposes of comparison only, the data on the 400-ps time scale were fit to a sum of three exponentials, and the parameters reported in Table III. Rigorous interpretation of these
parameters will await a further investigation when we have obtained data on a longer time scale and have also fit the rebinding kinetics to distributions of rate constants (33,34). However, it is evident from Table V.3 that changes are relatively small compared to the effects of the same mutations on bimolecular ligand binding. Hence, the proximal mutations do not affect NO binding kinetics significantly on fast geminate time scales.

Effects of proximal heme pocket mutations on hemin dissociation

Mb has a higher affinity for heme than Lba (11). Since heme affinity is affected by the environment of the proximal heme pocket (18), the effects of the above mutations on rate constants for hemin dissociation were assessed (Table V.4). Both \( \text{Lba}_{\text{MbF}} \) and V91S Lba lose heme rapidly compared to \( \text{wtLba} \) at pH 7.0 and pH 5.0. The hemin dissociation rate constants for these mutant proteins are \( \sim \)12-fold higher than those of \( \text{wtLba} \). The rate constants are pH dependent and higher at lower pH. \( \text{H61L:Lba}_{\text{MbF}} \) loses heme even faster, but in a pH independent manner. Mb, \( \text{Mb}_{\text{LbaF}} \), and S92V Mb lose heme very slowly at pH 7.0 but each proximal mutation increases the rate of hemin dissociation. The rate constants for hemin dissociation from \( \text{Mb}_{\text{LbaF}} \) and S92VMb are \( \sim \)4-5 fold higher than \( \text{wtMb} \) at pH 5.0. These results are similar to those previously reported for the F7 mutants in pig myoglobin (24). For \( \text{H64L:Mb}_{\text{LbaF}} \) the rate is \( \sim \)200-fold higher at pH 5.0, and \( \sim \)5000-fold higher at neutral pH (Table IV).

Discussion

The role of the proximal heme pocket H-bonding network involving His\(^{93(F8)}\), Ser\(^{92(F7)}\), His\(^{97(FG3)}\), Leu\(^{89(F4)}\), and the heme-7-propionate has been investigated in myoglobins
Figure V.3. An overlay of the room temperature decay curves (NO recombination) of the wild-type proteins and their helix swap and F7 mutants on geminate recombination timescales of 40 ps in (A) and (D), 400 ps in (B) and (E), and 1 ns in (C) and (F), after photodissociation of NO bound hemoglobins. The NO complexed protein samples in 100 mM phosphate buffer, pH 7.0, were pumped at 407 nm and probed at 438 nm. The parameters from fits to these traces (400-ps timescale) are listed in Table V.3. The MbCO traces on the 400-ps and 1-ns timescales are included in (B) and (C) to show the flatness of the translation stage.
from four different species. Smerdon *et al.* (24) first demonstrated the kinetic effects of disrupting the H-bonding network in pig Mb by replacing Ser$^{92}$ with Leu, a residue incapable of H-bonding. This substitution caused His$^{93}$ to rotate about its bond with the heme iron, decreased the oxygen dissociation rate constant, increased solvent access to the proximal heme pocket, and increased the rate constant for hemin dissociation. Ser$^{92}$ substitutions in human, sperm whale and horse Mb have also been investigated (25,46,47). Others have explored the effects of proximal heme pocket changes on heme dissociation, stability, and ligand binding (15,18). The H-bond between Ser$^{92}$ and the proximal heme ligand has also recently been investigated using trans substitutions of free proximal imidazoles (16,27).

**Differences between proximal regulation in Lba and Mb**

Mb uses a combination of covalent attachment and proximal pocket architecture to force the His$^{93(F8)}$ side chain into an eclipsed conformation, which destabilizes distal ligand binding (16,47,48). When covalent attachment is removed, oxygen affinity increases mainly due to a decrease in the dissociation rate constant, and the free proximal imidazole assumes a staggered conformation with respect to the heme pyrrole nitrogens. One structural hypothesis for this effect is that the staggered conformation is preferred in the absence of steric forces that prevent the proximal base from assuming this orientation (16), resulting in higher affinity distal ligand binding. This is also evident from the fact that the distance of the iron from the heme plane in wtMb is 0.23 Å (22), whereas in H93G Mb the iron is nearly co-planar with the porphyrin (0.03Å) (20).

It has been suggested that a similar mechanism might serve to increase oxygen affinity in Lba by allowing the proximal His side chain to attain a staggered conformation
### Table V.3. Kinetics of room temperature geminate rebinding of NO

<table>
<thead>
<tr>
<th>Protein</th>
<th>$A_1$</th>
<th>$\tau_1$(ps)</th>
<th>$A_2$</th>
<th>$\tau_2$(ps)</th>
<th>$A_3^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt LbaNO</td>
<td>0.36</td>
<td>11</td>
<td>0.55</td>
<td>116</td>
<td>0.09</td>
</tr>
<tr>
<td>LbaMbFNO</td>
<td>0.40</td>
<td>16</td>
<td>0.54</td>
<td>92</td>
<td>0.06</td>
</tr>
<tr>
<td>V91SLbaNO</td>
<td>0.35</td>
<td>18</td>
<td>0.54</td>
<td>90</td>
<td>0.11</td>
</tr>
<tr>
<td>wt MbNO</td>
<td>0.47</td>
<td>25</td>
<td>0.42</td>
<td>137</td>
<td>0.11</td>
</tr>
<tr>
<td>MbLbaFNO</td>
<td>0.42</td>
<td>18</td>
<td>0.45</td>
<td>124</td>
<td>0.13</td>
</tr>
<tr>
<td>S92VMbNO</td>
<td>0.49</td>
<td>20</td>
<td>0.42</td>
<td>138</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$^a$The parameters shown here are obtained from fits to the traces collected on 400ps time scale. On this time scale the third time constant is too long to be measured and hence not shown.

### Table V.4. Comparison of the rate constants for hemin dissociation ($k_{H}$) from soybean Lba, sperm whale Mb and their mutant proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{H}$ (h$^{-1}$) at 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
</tr>
<tr>
<td>wt Lba</td>
<td>0.6</td>
</tr>
<tr>
<td>LbaMbF</td>
<td>8.2</td>
</tr>
<tr>
<td>V91S Lba</td>
<td>7.2</td>
</tr>
<tr>
<td>H61L Lba</td>
<td>47</td>
</tr>
<tr>
<td>H61L:LbaMbF</td>
<td>57</td>
</tr>
<tr>
<td>wt Mb</td>
<td>0.005$^b$</td>
</tr>
<tr>
<td>MbLbaF</td>
<td>0.032</td>
</tr>
<tr>
<td>S92V Mb</td>
<td>0.048</td>
</tr>
<tr>
<td>H64L Mb</td>
<td>0.094$^b$</td>
</tr>
<tr>
<td>H64L:MbLbaF</td>
<td>23</td>
</tr>
</tbody>
</table>

$^a$The rates of hemin loss were measured at 20°C to allow comparison with mutant Lbas, which lose heme too rapidly to conveniently work with at 37°C. $^b$These rate constants for hemin loss are taken from Hargrove et al. (56).
with respect to the heme pyrrole nitrogens (8). Under these circumstances, removal of the covalent constraint would lead to even less steric hindrance and result in higher affinity ligand binding. However, our results with H92G Lba show that the covalently unhindered conformation in this mutant protein is not associated with higher affinity distal ligand binding. This suggests that either the staggered conformation is not always associated with highest affinity distal ligand binding, or the free imidazole proximal ligand in H92G Lba does not assume this conformation in the absence of covalent attachment to the F-helix. It is possible that imidazole cannot bond with! heme in a staggered conformation in the crowded Lba proximal heme pocket unless covalent attachment holds the base in a specific position and orientation.

Another structural explanation for the differences between Lba and Mb stems from the different sizes and orientations of the E- and F-helices and the EF loop in the two proteins (Figure V.1A). In the Perutz model for ligand binding in hemoglobin, distal ligand binding reorients the heme iron and requires movement of the proximal His and F-helix since they are all linked through covalent bonding (12,49). The smaller sizes of the E-helix and EF loop coupled with the larger F-helix confer less flexibility on Mb, which resists structural rearrangement of the F-helix upon ligand binding and lowers affinity (Figure V.1B). In Lba, a smaller F-helix, and larger E-helix and EF loop (Figure V.1C) endow the protein with greater flexibility in movement of the F-helix. The importance of the EF loop, which can act as a hinge, has been suggested by Appleby et al. (50). The higher flexibility of Lba helps it to absorb the reorientation motions following distal ligand binding. The opposing motions of the F-helix and surrounding polypeptide are thereby minimized in Lba, resulting in higher ligand binding affinity. Regardless of which structural hypothesis best describes these
proteins, our results indicate that the native Mb proximal heme pocket is designed to
decrease ligand affinity while that of Lba is designed to increase affinity. In the case of Mb,
H-bonding and other steric interactions pull the His$^{93(F8)}$ side chain into a low affinity, more
T-state hemoglobin-like conformation. In Lba, steric factors force His$^{92(F8)}$ into the heme
iron, mimicking R-state hemoglobin.

It has been suggested that the above results stem from increased reactivity of the
heme iron. Measurement of nitric oxide geminate recombination is a good evaluation of
heme reactivity since there is essentially no barrier to ligand binding (30). The most rapid
reactions occur when nitric oxide rebinds from contact pairs or "B states" resulting from
photolysis with an ultrashort laser pulse. Our results indicate that proximal control of ligand
binding is not apparent on these time scales. Therefore, it is likely that the changes in
oxygen affinity resulting from these proximal mutations are due to changes in rate constants
for disruption of the bond between the heme iron and the ligand. A discussion of these
"thermal" dissociation rate constants and geminate binding kinetic parameters comparing
native Lba and Mb can be found elsewhere (51,52).

**The interaction of proximal and distal regulation**

Hemoglobins use distal and proximal mechanisms to regulate ligand binding. Examples of these mechanisms have been demonstrated for many heme proteins, including Mb and human hemoglobin (15,30). The extent to which distal or proximal mechanisms are at work is difficult to determine in monomeric hemoglobins because there are no native T- or R- states to highlight differences in reactivity. It has previously been demonstrated that the distal pocket electrostatic mechanism for regulating ligand binding in Mb is not valid in Lba
The results presented here enhance our understanding of Lba by showing that proximal mechanisms in these two proteins have opposite effects on ligand binding.

It is also important to understand whether proximal and distal regulatory factors act independently, or if they are linked. If mutational effects are independent, the product of the individual effects on a parameter should be observed in a double mutant protein containing both mutagenic changes. If the mechanisms affected by mutation are linked, a double mutant protein would behave differently than expected from the combination of effects associated with individual change. Equation V.1 represents this relationship and is true if the proximal ($k_P$) and distal ($k_D$) effects are independent.

\[
\frac{k_P}{k_{ut}} \times \frac{k_D}{k_{ut}} = \frac{k_{P+D}}{k_{ut}} \tag{V.1}
\]

In Equation V.1, $k_{wt}$, $k_P$, $k_D$, and $k_{P+D}$ are parameters ($k_{O2}$ for example) for the wild-type, proximal, distal and double mutant proteins.

Table V.5 compares the effects of distal and proximal mutations on $O_2$ binding in Lba and Mb. For both proteins, proximal effects result from F-helix swapping (or mutation of F7 residue) and distal effects from the H(E7)L mutation. In Lba, the product of the individual effects is nearly identical to the observed effect of the double mutation for each of $k'O_2$, $k_{O2}$ or $K_{O2}$, whereas in Mb the effects are different. These results for Mb are not surprising because Barrick (27) has shown that distal ligand binding weakens proximal pocket hydrogen bonding, showing a strong coupling between distal and proximal effects.
However, the effects of distal and proximal mutation in Lba are independent, suggesting that proximal and distal regulatory mechanisms may not be strongly coupled in this protein.

Table V.5 Analysis of the interactions of proximal and distal effects on the rate constants for oxygen binding

<table>
<thead>
<tr>
<th>Protein</th>
<th>((k_p / k_w) \times (k_d / k_w))</th>
<th>(k_{p+d} / k_w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lba</td>
<td>(3.3 \times 0.2) 3.5 (21)</td>
<td>0.2 1.4</td>
</tr>
<tr>
<td>Mb</td>
<td>(6.8 \times 0.1) 26</td>
<td>20</td>
</tr>
</tbody>
</table>

\(k_p\) denotes rate constants (\(k'_02\), \(k_{02}\) or \(K_{02}\)) for proximal mutants (helix swap or F7), \(k_d\) denotes rate constant for distal mutant (His(E7)L), \(k_w\) the rate constant for wild type protein and \(k_{p+d}\) denotes rate constant for the double mutants.

**Role of the F-helix in protein stability**

Many reports have shown that mutations at helix position F7 result in proteins which are unstable due to low hemin affinity and increased proximal heme pocket solvation (24,25,26). In Mb, disruption of the H-bonding matrix around His\(^{93(F8)}\) causes bond rotation, pocket solvation, and facilitates hemin dissociation. In Lba, which lacks the same H-bonding matrix, the rate constant for hemin dissociation is much greater than Mb.

If the H-bond between Ser\(^{92(F7)}\) and His\(^{93(F8)}\) is a general factor in reducing hemin dissociation, opposite effects of the helix swap and F7 mutations would be expected in Lba and Mb. As shown in Table IV, this is not the case. Perturbation of either heme pocket results in a marked increase in the rate constant for hemin dissociation. This is not altogether surprising considering the fact that the \(\alpha\) and \(\beta\) subunits of human hemoglobin have very different rate constants for hemin dissociation though both contain Leu at position F7.
Therefore, it seems that heme binding has evolved independently of proximal pocket ligand binding regulatory mechanisms in these proteins.

**Conclusions**

Lba and Mb use different mechanisms to regulate ligand binding. The distal His in Mb imposes a favorable electrostatic environment for stabilizing bound oxygen; in Lba, the distal His plays a meager role. The Mb proximal heme pocket uses hydrogen bonding and steric forces to drive the proximal His side chain into a conformation which destabilizes distal ligand binding; Lba apparently lacks this hydrogen bonding network but uses a compact set of steric interactions to force the proximal His into a conformation which stabilizes distal ligand binding. The details of these interactions and the role of the Lba distal heme pocket in regulating ligand binding are unclear, and high resolution protein structures will be required to understand them.

With respect to the mechanisms that regulate ligand binding, Mb is similar to the $\alpha$ subunits of human hemoglobin. The distal pocket is less reactive and uses a hydrogen bond from His$^{E7}$ to stabilize bound oxygen, association rate constants are generally lower, and heme affinity is high. With respect to the same criteria, Lba is similar to $\beta$ subunits of human hemoglobin. Association rate constants are generally higher, dissociation rate constants are smaller and much less affected by His$^{E7}$ mutation, and hemin affinity is lower than in Mb and $\alpha$ human hemoglobin subunits (11,54). Therefore, comparison of Mb and Lba demonstrate that the mechanisms which govern ligand binding to the subunits of human hemoglobin can occur naturally in distantly related monomeric heme proteins.
Acknowledgements: We would like to thank Doug Barrick for H93G sperm whale myoglobin, John Olson for the synthetic Mb gene, and acknowledge the National Science Foundation (Award No. MCB-0077890) for support of this work.

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CHAPTER VI: EFFECTS OF DISTAL POCKET MUTATIONS ON THE GEMINATE RECOMBINATION OF NO WITH LEGHEMOGLOBIN ON THE PICOSECOND TIME SCALE

A paper published in the Journal of Physical Chemistry B

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ABSTRACT

The picosecond NO geminate rebinding kinetics of wild type leghemoglobin, a monomeric plant hemoglobin with structural similarity to myoglobin, and six mutant proteins at the distal histidine (H61G, H61A, H61V, H61L, H61R, H61F) are investigated. All of the mutant proteins yield rebinding kinetics that are initially more rapid than that of the wild type protein. At long times, the rebinding of H61F becomes slower than that of wild type leghemoglobin. The H61V, H61L, and H61G mutant proteins give extraordinarily rapid and complete geminate rebinding. On a 40-ps time scale, distal effects are overwhelmingly evident for all of the mutants considered. That binding is both rapid and, in several cases, essentially single-exponential is suggestive of the nature of the barrier induced by the distal modification: it must be such that the ligand is prohibited from reorienting with respect to, and diffusing sufficiently far from, the heme iron so that a distribution of return paths is not


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offered to it. Over the past twenty years, the relative importance attributed to the proximal and the distal sides in modulating geminate ligand binding has varied considerably. Our results with leghemoglobin are discussed in terms of the relative contributions of proximal and distal effects to geminate rebinding kinetics.

INTRODUCTION

Site-directed mutagenesis and recombinant protein technology coupled with ever increasing improvements in computational, structural, and kinetic studies has provided a considerable increase in our understanding of ligand binding in myoglobin (Mb) itself. A study of Mb alone, however, cannot provide a completely satisfying paradigm for understanding more complicated hemeproteins, such as newly discovered bacterial, protist, and plant hemoglobins and flavohemoglobins, or, for that matter, even human hemoglobin 1-4. The study of leghemoglobins ideally complements that of myoglobin because, in spite of a structure (Figure VI.1) similar to that of Mb, they display many opposite extremes of reactivity 5-7. When ligands bound to a hemoglobin are photolyzed, the resulting time courses for rebinding are often complex and depend on a number of factors including: (1) reactivity of the heme iron with the ligand; (2) reactivity of the ligand; and (3) the protein matrix surrounding the ligand binding site. A relatively long laser flash (microsecond duration) is used to study the overall bimolecular association reaction, which is influenced by each of the factors listed above. Shorter pulse durations (femtoseconds to nanoseconds) are used to probe geminate recombination that occurs when the dissociated ligand rebinds without exiting the protein matrix. Depending on the combination of the hemoglobin and the
ligand, geminate recombination can occur on ultrafast (< 10-ps) and a number of longer (10-ps to 10-μs) time scales.

Figure VI.1. (a) Structure of the globin fold of leghemoglobin. (b) A closer look at the distal pocket of leghemoglobin and the histidine(E7) residue, which has been replaced by several other amino acids to study the effect of distal pocket mutations.

Traditionally, the longer time-scale experiments were thought to probe the distal heme pocket environment surrounding the ligand binding site, while the femtosecond to picosecond time scale components measured more directly the reactivity of the heme iron as monitored by the rate of bond formation and heme-His(F8) relaxations. However, because ~10-ps geminate recombination has been observed with very few hemoglobins, this assumption has never been thoroughly tested.

**Arguments for proximal effects in geminate recombination**

Early investigations on fast geminate recombination, notably with NO, focused largely on proximal effects \(^{8,9}\). These studies were approached from the point-of-view of
understanding *hemoglobin* (Hb), not myoglobin, and were motivated by the work of Perutz 10 and Gelin and Karplus 11 pointing to an allosteric core consisting of the heme, the proximal histidine, the F helix, and the FG corner, which is in contact with the α1β2 interface. The response of this proximal heme environment to ligand dissociation and rebinding is expected to be significantly different in Mb and Hb.

The behavior of the proximal side can be monitored by means of the stretching frequency of the Fe-His mode in photodissociated MbCO and HbCO. The frequency of this mode is the same at 30 ps in the MbCO photoproducts as in equilibrium unligated Mb 12. In the HbCO photoproduct, however, the frequency of the mode does not begin to change for several nanoseconds 13,14, indicating an interaction of the proximal side with the interface between subunits. Friedman et al. 15 correlated the frequency of the Fe-His mode with the yield of O₂ geminate recombination in Hb and suggested that the degree of relaxation of the proximal side controls the reactivity of the protein. More recently, Friedman and coworkers have considered proximal effects in terms of both the Fe-His mode and the band III absorption (a charge transfer band at ~760 nm peculiar to 5-coordinate ferrous hemes) 16.

The importance of the proximal Fe-His mode motivated our earlier consideration of the ultrafast rebinding in terms of a transient barrier depending on the fluctuating out-of-heme plane displacement of the Fe following ligand dissociation 9. This study examined the geminate rebinding kinetics in terms of various models: single exponential, sum of two exponentials, power law, and time-dependent barrier corresponding to the evolution of the heme iron. Although different functional forms could not unambiguously describe the ligand binding kinetics, the time scale of ligand rebinding was consistent with molecular dynamics
simulations of the Fe-heme motion. This study therefore concluded that an out-of-plane configuration of the Fe with respect to the heme macrocycle reduces the rate of geminate binding, and that modulation of the Fe-heme distance on the time scale of geminate rebinding induces nonexponential rebinding kinetics 9.

**Arguments for distal effects in geminate recombination**

Subsequent work has indicated that this view of the role of the proximal environment, *at least in monomeric hemeproteins*, requires reassessment. Our ultrafast study of the NO geminate rebinding of a wide spectrum of distal mutants of human myoglobin demonstrated that distal effects can profoundly influence the kinetics 17. For example, not only do the distal mutant proteins V68N, V68A, and H64Q exhibit rapid rebinding compared to wild type Mb, but so does the surface mutant protein, K45R. (K45R is at the interface of the heme pocket and solvent and interacts with a propionate. It could affect the pathway for ligand entry or exit.) On the other hand, the mutants K45A and K45Q yield kinetics almost identical to that of the wild type. Gibson, Olson, and coworkers 18,19 have obtained results consistent with these.

Work by Hochstrasser and coworkers 20 has also confirmed the importance of distal effects in sperm whale myoglobin. V68F and V68I have significantly smaller and larger time constants, respectively, for geminate rebinding with NO. They have also shown that Co substitution produces much less pronounced effects on NO geminate recombination with Mb than do distal pocket mutations 20. The Co-substituted myoglobins, nevertheless, do provide slightly faster rebinding kinetics than do their Fe-bearing counterparts, which is indeed consistent (although possibly fortuitously) with a smaller out-of-plane displacement for Co
than for Fe, and with the role of the displacement proposed by Petrich et al. 9 (discussed above).

Négrerie et al. 21 have studied NO geminate recombination in Mb in which the proximal histidine is mutated to glycine, thus furnishing a protein without covalent attachment to the heme iron. At sufficiently high imidazole concentration, the H93G mutant coordinates an imidazole at the proximal position of the heme, which is denoted as H93G(Im). This system consequently provides a means of assessing the contribution of proximal effects to geminate ligand rebinding. The mutation induces a relatively minor perturbation with respect to wild type Mb (Table II). The H93G(Im) kinetics are very similar to that of wild type Mb, with the amount of rapid rebinding component being 42 and 45%, respectively. Surprisingly, the fraction of NO rebound after ~100 ps is larger in the wild type than in the mutant protein, as is indicated by the relative weights of the "baseline" components, a3. The amount of rapid rebinding in H93G, 63%, is more pronounced than in wild type Mb.

Franzen et al. 22,23 demonstrated that the intensity of the Fe-His vibrational mode characteristic of heme doming was fully developed 1 ps after photodissociation of CO, within their experimental resolution, for both Mb and H93G(Im). (The intensity of this mode in Hb was also fully developed, but its frequency was upshifted with respect to its equilibrium value. Those of Mb and H93G(Im) were found to be at their equilibrium value 1 ps after CO photolysis.) Subsequent work by Mizutani and Kitigawa 24 with improved signal-to-noise is consistent with these observations, but indicates that Mb and a model heme compound lacking the protein matrix show ~90% of the intensity of the Fe-His mode within their
instrumental response time of ~2 ps, while the remainder develops with a time constant of a few picoseconds. These workers also detect a small evolution (~2 cm⁻¹) of the frequency of this mode occurring on a 100 ps time scale. These results indicate that the proximal protein environment, at least in monomeric hemoglobins, seems to have a relatively minor effect on the Fe-His mode. In this context, then, it is not surprising that proximal effects on geminate binding are generally small relative to distal effects.

Négrerie et al. 21 have recently studied soluble guanylate cyclase (sGC), in which the binding of NO to the heme iron induces a cleavage of the bond between the proximal histidine and the iron. sGC-NO is an example of a hemeprotein where there is no covalent attachment between the Fe and the proximal side of the protein and its kinetics may thus be compared against other systems in evaluating distal and proximal effects on geminate rebinding. sGC exhibits very rapid NO geminate binding kinetics: 97% of the recombination occurring with a time constant of 7.5 ps. Given that NO binds to protoheme with a 7-ps time constant (Table I) and that H93G Mb is only moderately different with respect to wild type Mb, Négrerie et al. concluded that their sGC result implies that rapid, single exponential rebinding behavior is not a consequence of proximal behavior, but of the distal environment.

Magde and coworkers investigated NO geminate binding kinetics in horse heart and sperm whale myoglobin at pH 4 25. Under these conditions, the proximal histidine-iron bond is broken, providing a local heme environment similar to that of sGC-NO. These workers observed single-exponential NO rebinding kinetics with time constants of 12.5 and 9.5-ps, respectively (Table I). Although it is tempting to interpret these results as supporting the hypothesis 9 concerning the importance of the proximal effects exerted by the Fe-heme
displacement, the protein may be sufficiently altered at pH 4 26-28 so that these results more readily confirm the conclusions of Négrerie et al. based on their sGC-NO data.

**Distal and proximal effects in leghemoglobins**

The first ultrafast NO geminate rebinding study directly addressing proximal effects in leghemoglobin (Lba) and myoglobin mutants ⁶ indicated that these effects are negligible in Mb up to ~900 ps after photodissociation (in S92V and a Lba F-helix substitution). In Lba systems (V91S and a Mb F-helix substitution), proximal effects are negligible on a 40-ps time scale. On the other hand, they are “small” but clearly evident on a 1-ns time scale. This and the present study provide the first systematic investigations of the role of proximal and distal effects on the “ultrafast” geminate recombination of NO with Lba. The results presented here point to considerable distal effects that manifest themselves in the first tens of picoseconds following photodissociation and are consistent with distal heme pocket regulation of geminate recombination.

**MATERIALS AND METHODS**

**Preparation of NO bound Lba samples for geminate recombination measurements**

The mutagenesis, expression, and purification of the Lba proteins employed is reported in detail elsewhere ⁵. The myoglobin mutants discussed are from sperm whale. The NO complexed protein samples were prepared in a cuvette (1-mm path length) stoppered with a septum in an oxygen free atmosphere. LbCO samples were prepared by reducing the proteins with sodium dithionite and loading them into a Sephadex G-25 size exclusion column equilibrated with 100 mM phosphate, pH 7.0 saturated in CO (10% CO, 90% N₂ mixture). The dithionite free, ligand-bound samples were collected in gas tight syringes.
Ferrous NO samples were made by diluting these dithionite free CO samples 1:1 with NO saturated anaerobic buffer. The absorption spectra of the NO forms of the wild type proteins and their mutants used in this study are nearly identical. The protein samples typically had an absorbance of ~0.5 at 407 nm.

**Measurement and analysis of geminate recombination kinetics**

The laser apparatus used for pump-probe transient absorption measurements used in the geminate recombination studies has been described in detail elsewhere. In brief, a 1-kHz homemade, regeneratively amplified Ti:sapphire laser was used to photolyze the sample at 407 nm with an energy of 1 μJ per pulse. A white light continuum was then used to probe the absorption changes at 438 nm. To facilitate the comparison of data for various mutants, the absorbance changes were normalized to one at the maximum change in absorbance. Sample integrity was monitored by measuring the absorption spectra before the start of and after the completion of each experiment. The kinetics on the 40 ps timescale were fit globally according to the following function: $\Delta A(t) = a_1\exp(-t/\tau_1) + a_2\exp(-t/\tau_2)$. That is, $\tau_2$, effectively infinite on this timescale, was held fixed while $\tau_1$ and the amplitudes, $a_1$ and $a_2$, were allowed to vary until the best fits were obtained. On longer time scales, a simple sum of exponentials was used. An alternative, model-independent means of analyzing the data is to fit the kinetic traces to a distribution of rebinding rates. Such an analysis has been applied on several occasions to geminate rebinding of NO. This method obtains the rate distributions from the kinetic curves by using the maximum entropy method (MEM). In this approach, an entropy function is maximized and a distribution of probabilities of the underlying rate components is obtained. The resulting distribution is the most probable, but
not unique rate distribution describing the observed kinetics 31-33. Self consistency checks for the use of the maximum entropy method are 1) that the fit to the MbCO rebinding kinetics yields only time constants that are smaller than the time scale of the experiment (not shown); 2) that the ultrafast NO rebinding of, for example, the H61L Lb mutant yields a single narrow peak in the rate distribution; and 3) that superimposable (within the experimental noise) kinetic traces of different mutants yield similar distributions of recombination rates. The maxima of the MEM distributions were normalized to unity to facilitate comparison among the mutant proteins.

**Bimolecular recombination kinetics**

The association rate constants for bimolecular NO binding were measured by laser photolysis methods 34,35 using a previously described laser flash apparatus 36. All kinetic measurements were taken at 25°C in 100-mM phosphate, pH 7.0. The NO rebinding after photolysis was followed at 428 nm (the deligated peak for Lba). The kinetic traces are single exponential and a suitable curve fitting yields the rate constants. The dissociation rate constants were measured with a Cary 50-Bio spectrophotometer by displacing bound NO with CO in the presence of dithionite. Error estimates for the rate constants are ~ ± 10% of the measured value.

**RESULTS AND DISCUSSION**

Figures VI.2 and VI.3 present geminate recombination kinetics on time scales of 40- and 400-ps, respectively, for the six distal mutant proteins and wild-type Lba. It is remarkable that for H61G, H61V, and H61L the rebinding is more than 80% complete within 80 ps after NO photolysis. Such efficient geminate recombination is comparable to that of
protoheme-NO in ethylene glycol water, myoglobin at pH 4, and soluble guanylate cyclase, which afford the most rapid and complete rebinding of which we are aware. Tables VI.1-3 summarize these geminate and bimolecular rebinding parameters.

As noted earlier, the first ultrafast NO geminate rebinding study directly addressing proximal effects in leghemoglobin and myoglobin mutants indicated that they are negligible in Mb up to ~900 ps after photodissociation (in S92V and a Lba F-helix substitution, Figure VI.3A). In Lba systems (V91S and a Mb F-helix substitution), proximal effects are negligible on a 40-ps time scale. On the other hand, they are “small” but clearly evident on a 1-ns time scale (Figure VI.3A). The present work points to considerable distal effects that manifest themselves in the first tens of picoseconds following photodissociation. To a first approximation, we might conclude that proximal or distal effects on ligand binding are a direct consequence of structural factors on the respective sides of the heme—although, the situation may be more complicated, as we suggest below.

The geminate rebinding of the distal mutants of leghemoglobin considered in the present work can be fruitfully considered in the light of an analysis of rate distributions. The mutant proteins fall into two classes. The kinetic data obtained on a 400-ps time scale indicate bimodal distributions for wild-type leghemoglobin and for its mutants, notably H61A and H61F (Figure VI.3B). While the two lobes for the wild-type distribution are well separated and of comparable amplitude, those for the H61A and the H61F mutants are closer, with the lower amplitude lobes appearing as shoulders in the distributions. It is interesting that the shoulder for H61A occurs at larger rate constants compared to that for H61F, which occurs at smaller rate constants. In marked contrast, the distributions for the H61V and the H61L mutant proteins display one band, which is narrow and sharply peaked at large rate
Figure VI.2. An overlay of the room temperature decay curves (NO geminate recombination) of the wild type protein (wtLb) and its corresponding distal mutant proteins, where wt stands for wtLb and the letters F, A, R, V, L and G stand for the mutant proteins H61F, H61A, H61R, H61V, H61L, and H61G, respectively. Decays collected on a 40-ps timescale are displayed in this figure. The samples were photodissociated at 407 nm and probed at 438 nm.

Figure VI.3A. (a) Kinetic traces (1-ns full timescale) for wtLba and its proximal mutants; (b) kinetic traces (1-ns full timescale) for wtMb and its proximal mutants. The mutant proteins S92VMb and V91SLba were selected, as discussed in detail elsewhere, because they are complimentary substitutions resulting in an Lba-like proximal pocket in Mb (S92V) and a Mb-like proximal pocket in Lba (V91S).
Table VI.1. NO Geminate Rebinding Kinetic Parameters of the Fe(II) forms of Soybean Lba, Its Distal Mutant Proteins, and Others.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(a_1)</th>
<th>(\tau_1) (ps)</th>
<th>(a_2)</th>
<th>(\tau_2) (ps)</th>
<th>(a_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type Lba</td>
<td>0.23</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61A</td>
<td>0.49</td>
<td>19.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61R</td>
<td>0.59</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61G</td>
<td>0.80</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61L</td>
<td>0.90</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61F</td>
<td>0.38</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lba H61V</td>
<td>0.67</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH-NO</td>
<td>0.92</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanylate cyclase</td>
<td>0.97</td>
<td>7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm whale Mb pH 4</td>
<td>1.00</td>
<td>9.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse heart Mb pH 4</td>
<td>1.00</td>
<td>12.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Unless otherwise indicated, fitting results for geminate rebinding kinetics obtained on a full scale of 40 ps. Kinetics are globally fit, as discussed in the text, to the form \(\Delta A(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)\), where \(\tau_2\) is too long to be fit accurately on this time scale and is effectively infinite. \(\tau_2\) is fixed at 2 ns for the global fits. The prefactors are normalized such that \(a_1 + a_2 = 1\).

* PTH (protoheme) in ethylene glycol/water (95/5). The kinetics are cited from reference 9. \(\lambda_{pump} = 580\) nm; \(\lambda_{probe} = 425\) nm.

* Soluble guanylate cyclase 21

* Reference 25

Table VI.2. Fit Parameters for Picosecond NO Geminate Rebinding Data Obtained on Longer Time Scales: Distal and Proximal Effects

<table>
<thead>
<tr>
<th>Protein</th>
<th>(a_1)</th>
<th>(\tau_1) (ps)</th>
<th>(a_2)</th>
<th>(\tau_2) (ps)</th>
<th>(a_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Mb</td>
<td>0.19</td>
<td>30</td>
<td>0.57</td>
<td>150</td>
<td>0.24</td>
</tr>
<tr>
<td>wt Lba</td>
<td>0.12</td>
<td>20</td>
<td>0.62</td>
<td>190</td>
<td>0.26</td>
</tr>
<tr>
<td>Lba H61A</td>
<td>0.19</td>
<td>11</td>
<td>0.80</td>
<td>114</td>
<td>0.01</td>
</tr>
<tr>
<td>Lba H61R</td>
<td>0.60</td>
<td>25</td>
<td>0.35</td>
<td>150</td>
<td>0.05</td>
</tr>
<tr>
<td>Lba H61G</td>
<td>0.75</td>
<td>15</td>
<td>0.25</td>
<td>105</td>
<td>------</td>
</tr>
<tr>
<td>Lba H61L</td>
<td>0.48</td>
<td>5</td>
<td>0.52</td>
<td>30</td>
<td>------</td>
</tr>
<tr>
<td>Lba H61F</td>
<td>0.22</td>
<td>12</td>
<td>0.48</td>
<td>170</td>
<td>0.30</td>
</tr>
<tr>
<td>wt Mb (^b)</td>
<td>0.45</td>
<td>11</td>
<td>0.50</td>
<td>92</td>
<td>0.05</td>
</tr>
<tr>
<td>Mb H93G(Im) (^b)</td>
<td>0.42</td>
<td>14.7</td>
<td>0.39</td>
<td>82</td>
<td>0.19</td>
</tr>
<tr>
<td>Mb H93G (^b)</td>
<td>0.63</td>
<td>14.6</td>
<td>0.25</td>
<td>95</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Unless otherwise indicated, the fits reported are for data obtained on a full scale of 400-ps.

* Sperm whale myoglobin and its proximal histidine mutant. See discussion in text. The data were obtained on a full-scale of 100 ps 21.
constants, consistent with the rapid, nearly single exponential rebinding kinetics (Figure VI.3B).

The first bimolecular recombination kinetic data of leghemoglobin and its mutants are presented in Table VI.3 and in Figure VI.4 for purposes of comparison. The rebinding kinetics are well described by a single exponential as is indicated by the representative kinetic traces and MEM fits and distributions. The availability of the bimolecular data provides a useful perspective from which to evaluate the geminate data. For example, among the proteins studied, the H61F mutant protein has the largest bimolecular rebinding rate constant and about the smallest geminate rebinding rate constant. These two observations can be reconciled if it is postulated that the mutation introduces an opening in the heme pocket, which, while permitting the ligand to stray further from the Fe (thus providing slow geminate rebinding), also permits easy reentry for ligands that have already exited (fast bimolecular rebinding). Such an opening also explains the ~5-fold higher dissociation rate constant in H61F, as opposed to the other mutant proteins (Table VI.3). On the other hand, the lower NO dissociation rate constant in H61R might be a result of strong hydrogen bonds with the bound ligand.

Similarly, if the rate of geminate ligand rebinding is enhanced, it must be because the barrier to ligand diffusion away from the heme has been increased, and vice versa. In addition, slow geminate rebinding may be the result of stabilization of the ligand by interactions with amino acids in the pocket—i.e., a so-called “docking site.” “Docking” for CO in Mb has been indicated in spectroscopic and structural studies. For example, it has long been considered that the rapid geminate rebinding of nitric oxide with hemeproteins is a consequence of a very small electronic barrier for heme rebinding relative to oxygen or
carbon monoxide. The origin of this rapid rebinding has been called into question by Anfinrud and coworkers, who have attributed it to distal effects. They compared...
the rebinding of CO with Mb and with microperoxidase. Microperoxidase is an enzymatically digested cytochrome c oxidase consisting of a heme with a "proximal" histidine, which is part of an 11-peptide fragment. This peptide is not long enough to wrap around the heme to form an organized distal environment. CO recombination with microperoxidase is very rapid, with an initial component of 110 ps and with more than 80% of the ligands being rebound after 1 ns. One nanosecond after photodissociation, essentially no CO has recombined with Mb. In this context, the rapid NO recombination observed in the H61V, H61L, and H61G leghemoglobin mutants could be explained in terms of a reduction of attractive forces between the distal "docking site" residues and the NO.

Finally, it may be overly simplistic to distinguish between proximal effects independently of distal effects. It is possible that certain proximal mutations may influence the distal barrier. It is also possible, as suggested by the simulations of Lambry, that the trajectory of the dissociated ligand causes it to collide with the distal pocket in such a way that fluctuations in the heme pocket are very rapidly set into motion, subsequently influencing the rebinding kinetics.

CONCLUSIONS

We have presented the first ultrafast spectroscopic study addressing the distal effects on the geminate recombination of NO with leghemoglobin and its mutants. The H61V, H61L, and H61G mutant proteins show extraordinarily rapid and complete geminate rebinding, approaching that exhibited by PTH-NO, myoglobin at pH 4, and guanylate cyclase (Table VI.I). The picosecond NO geminate rebinding kinetics of wild type leghemoglobin and six of its mutant proteins at the distal histidine-61 (H61G, H61A, H61V, H61L, H61R,
H61F) have been investigated. All of the proteins yield rebinding kinetics that are initially more rapid than that of the wild type. At long times, the rebinding of H61F becomes slower than that of wild type. The H61V, H61L, and H61G mutants give extraordinarily rapid and complete geminate rebinding. No distal or proximal mutations decrease the initial rate of
geminate rebinding in leghemoglobin, and no mutations in myoglobin decrease the initial rate without at the same time permitting the ligand to escape to the solvent 18.

Table VI.3. Bimolecular Rate Constants for Wild Type Lba and Its Mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k'_{NO}$ ($\mu$M$^{-1}$s$^{-1}$)</th>
<th>$k_{NO}$ (s$^{-1}$)</th>
<th>$K_{NO}$ (µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Lb</td>
<td>190</td>
<td>0.00002</td>
<td>9,000,000</td>
</tr>
<tr>
<td>H61G</td>
<td>330</td>
<td>0.00002</td>
<td>17,000,000</td>
</tr>
<tr>
<td>H61A</td>
<td>300</td>
<td>0.00002</td>
<td>15,000,000</td>
</tr>
<tr>
<td>H61V</td>
<td>230</td>
<td>0.00002</td>
<td>12,000,000</td>
</tr>
<tr>
<td>H61L</td>
<td>320</td>
<td>0.00002</td>
<td>16,000,000</td>
</tr>
<tr>
<td>H61F</td>
<td>360</td>
<td>0.00001</td>
<td>3,600,000</td>
</tr>
<tr>
<td>H61R</td>
<td>310</td>
<td>0.00001</td>
<td>31,000,000</td>
</tr>
</tbody>
</table>

$k'_{NO}$ are association rate constants; $k_{NO}$, dissociation rate constants; and $K_{NO}$, affinity constants ($k'_{NO}/k_{NO}$). Errors in the rate constants are approximately $\pm$ 10% of the measured value.

A growing body of data supports the importance of distal effects for inducing rapid, essentially single-exponential geminate rebinding in monomeric heme proteins. That suggests that the distal barrier prohibits the ligand from reorienting or diffusing very far from the heme iron, so that a distribution of return paths is not offered to it.

While proximal effects can be induced (ref 6 and Table VI.2), they are not as dramatic as distal modifications. Also, the data indicate that in monomeric proteins, the role of the displacement of the Fe out of the heme plane is much less important than previously believed. A complete assessment, however, of proximal effects, must await a prudent consideration of multimeric proteins exhibiting cooperativity before general conclusions are drawn concerning their importance. Finally, it may be overly simplistic to distinguish between proximal effects independently of distal effects. It is possible that certain proximal
mutations may influence the distal barrier or that the distal barrier is influenced by the impact of the dissociated ligand.

ACKNOWLEDGEMENTS

This work was supported by NSF grant MCB-0077890 to MSH and JWP. We thank Michel Négrerie and Jean-Christophe Lambry for very helpful discussions and Andrew Shreve and Stefan Franzen for providing us the maximum entropy method software.

REFERENCES


CHAPTER VII: DYNAMIC SOLVATION IN ROOM TEMPERATURE IONIC LIQUIDS

A paper submitted to the Journal of Physical Chemistry B


Abstract

The dynamic solvation of the fluorescent probe, coumarin 153, is measured in five room temperature ionic liquids using different experimental techniques and methods of data analysis. With time-resolved stimulated emission and time-correlated single-photon counting techniques, it is found that the solvation is comprised of an initial rapid component of ~55 ps. In all the solvents, half or more of the solvation is completed within 100 ps. The remainder of the solvation occurs on a much longer time scale. The emission spectra of coumarin 153 are nearly superimposable at all temperatures in a given solvent unless they are obtained using the supercooled liquid, suggesting that the solvents have an essentially glassy nature. The physical origin of the two components is discussed in terms of the polarizability of the organic cation for the faster one and the relative diffusional motion of the cations and the anions for the slower one. A comparison of the solvation response functions obtained from single-wavelength and from spectral-reconstruction measurements is provided. Preliminary
fluorescence upconversion measurements are presented against which the appropriateness of the single-wavelength method for constructing solvation correlation functions and the use of stimulated emission measurements is considered. These measurements are consistent with the trends mentioned above, but a comparison indicates that the presence of one or more excited states distorts the stimulated emission kinetics such that they do not perfectly reproduce the spontaneous emission data. Fluorescence upconversion results indicate an initial solvation component on the order of ~7 ps.

Introduction

Room-temperature ionic liquids (RTILs) are becoming an increasingly rich area of study\(^1,2\). They have been used as novel solvent systems for organic synthesis\(^3-16\), for liquid-liquid extraction\(^17,18\), in electrochemical studies\(^19\), and as ultralow volatility liquid matrices for matrix-assisted laser desorption/ionization (MALDI) mass spectrometry\(^20\). RTILs have other properties that make their application in chemical systems attractive. Some are immiscible with water and nonpolar organic solvents. They are stable to temperatures in excess of 300°C, but they have negligible vapor pressures, thus making them "green" solvents by reducing environmental levels of volatile organic carbons (VOCs). Their viscosities can easily be varied by changing their cationic or anionic constituents.

Most ionic liquids are said to have similar polarities, close to those of short-chain alcohols\(^21-24\). The solvatochromic effect of Reichardt’s dye\(^21\) and Nile Red\(^22\) as well as of fluorescent probes\(^23,24\) and the Rohrschneider-McReynolds gas-liquid chromatography (GLC) method\(^25\) have been used to characterize ionic liquids by obtaining a general, one-dimensional polarity-based parameter. This approach has not been successful for RTILs
because they all fall within the same narrow range of values. Yet, two different ionic liquids that have essentially identical “polarity,” as measured by such methods, can produce very different results when used as solvents for organic reactions, gas-liquid chromatography, or extractions. Most recently, we have used the solvation parameter model developed by Abraham, which has been developed to characterize either liquid- or gas-phase interactions between solute molecules and liquid phases. This model is based on a linear free energy relationship. The solute retention factor is determined chromatographically for a set of probe solutes, and a multiple linear regression analysis relates it to a set of five parameters that characterize the solvent.

Solvent properties can dramatically influence the rate of chemical reactions. Assuming equilibrium solvation along the reaction coordinate, many of these effects can indeed be explained by classical thermodynamics, as the construction of the above linear free energy relationships attempts. A thermodynamic approach is valid, however, only if the motion of the solvent molecules is very fast compared with the motion along the reaction coordinate, so that the solvent is always in equilibrium with the solute. In particular, the solvent relaxation timescales influence the dynamics of electron transfer, proton transfer, and other charge transfer reactions by exerting a time-dependent dielectric friction. In these cases, reaction rates may be limited by the rate of solvent relaxation. A well established method to measure time-dependent solvation is by means of time-resolved fluorescence spectroscopy. After short-pulse excitation, the fluorescence spectrum of a probe solute red shifts in time as the surrounding solvent reequilibrates to the new, excited-state charge distribution. This time-dependent fluorescence shift provides a direct measure of the kinetics of solvation occurring at the microscopic level relevant to chemical reactions.
Before the availability of room temperature ionic liquids, transient solvation by ions was studied by Huppert and coworkers using molten salts \(^{31,32}\) and by Maroncelli and coworkers using nonaqueous solutions of dissolved ions \(^{33}\). The dynamic aspects of solvation by room temperature ionic liquids are becoming an object of experimental and theoretical studies. The dynamic solvation and the reorientational behavior of several probe molecules in different ionic liquids have recently been investigated\(^{34-39}\). These workers have shown that the solvation dynamics is biphasic. A molecular dynamics simulation by Shim et al. \(^{40}\) has been interpreted in terms of the faster component corresponding to diffusional motion of the anion and the slower component corresponding to collective motion of the anion and the cation. In addition, Hyun et al. \(^{41}\) and Giraud et al. \(^{42}\) have used the optical Kerr effect to study the low-frequency vibrational motions (< 200 cm\(^{-1}\)) of these solvents.

In this article, we address the dynamic aspects of solvation by RTILs using Stokes shift data of the fluorescent probe, coumarin 153 (Figure VII.1), from the subpicosecond to the nanosecond time regimes. We investigate four RTILs based on the 1-butyl-3-methylimidazolium cation, BMIM\(^{+}\), and either the Cl\(^{-}\), BF\(_4\)^{−}, PF\(_6\)^{−}, or the (CF\(_3\)SO\(_2\))\(_2\)N\(^{-}\) anions (Figure VII.1). They are typically referred to as [BMIM\(^{+}\)][Cl\(^{-}\)], [BMIM\(^{+}\)][BF\(_4\)^{−}], [BMIM\(^{+}\)][PF\(_6\)^{−}], and [BMIM\(^{+}\)][NTf\(_2\)^{−}], respectively. We compare these RTILs with the organic subunits, butylimidazole and methylimidazole. A fifth ionic liquid studied is [NHE\(_3\)^{+}][TFA\(^{-}\)], triethylammonium trifluoroacetate. Major conclusions are that the rapid initial phase of solvation arises from the cation and that the simulations of Shim et al. \(^{40}\) may not provide an appropriate comparison for large organic fluorescent solvation probes, such as coumarin 153. In the course of this work, comparisons were made between different methods of constructing the solvation correlation function and of obtaining the solvation data.
itself. Consequently, this work addresses aspects of methodology nearly as much as the problem of solvation by RTILs.

The plan of the article is as follows. After a description of the various experimental and data-treatment methods, we present the results. It became apparent during the course of the investigation that they depend sometimes rather significantly on the type of experiment or data treatment used. The discussion section is separated into three parts. The first is a short consideration of the steady-state spectra of the RTILs. The second deals with the early stages of this work, where the long-time behavior (up to 4 ns) of RTILs was studied by time-correlated single-photon counting and the early events by transient absorption (stimulated emission), the latter of which employed a particular method of constructing the solvation correlation function using a single probe wavelength. Although these methods produced results obeying reasonable trends, it became apparent that neither measurement of stimulated emission nor single-wavelength analysis was adequate. The third part of the discussion provides a comparison with results from upconversion measurements that offer a more traditional and direct measure of spontaneous emission. Salient points are summarized in the conclusions section.

Materials and Methods

Room Temperature Ionic Liquids. 1-butyl-3-methylimidazolium chloride [BMIM][Cl] is produced by refluxing equimolar amounts of 1-methylimidazole with 1-chlorobutane at 70 degrees for 72 hours. The resulting [BMIM][Cl] is washed with ethyl acetate and dried under vacuum. 1-Butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4] is produced by mixing 10 grams [BMIM][Cl] (0.057 mol) in ~100 mL acetone. 6.29 grams (0.057 mol)
of sodium tetrafluoroborate is added and stirred for 24 hours. The resulting sodium chloride is filtered off and the acetone removed under vacuum. 1-Butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF₆] is produced by dissolving 164.49 grams (0.94 mol) [BMIM][Cl] in ~250 mL water and reacting an equimolar amount (0.94 mol) of hexafluorophosphoric acid (HPF₆) and stirring for 24 hours. The resulting [BMIM][PF₆] is washed with water until the washings are no longer acidic. The IL is then dried under vacuum. Hexafluorophosphoric acid is corrosive, toxic and should be handled with care. 1-Butyl-3-methylimidazolium bis[(trifluoromethyl)-sulfonyl]imide, [BMIM][NTf₂], is produced by dissolving 34.0 grams (0.118 mol) N-lithiotrifluoromethanesulfonimide in ~150 mL water and mixing with 20.67 grams (0.118 mol) [BMIM][Cl] also dissolved in ~150 mL water. The mixture is stirred for 12 hours. The aqueous portion is removed and the resulting ionic liquid washed with water and then dried under vacuum. All RTILs produced using the [BMIM][Cl] salt are subjected to the silver nitrate test to ensure no chloride impurities remain in the samples. [NHE₃][TFA] is prepared by adding a slight molar excess of trifluoroacetic acid dropwise to triethylamine in a 50 mL round bottom flask. The mixture is then heated at 40 °C and stirred for 2 hours. Excess trifluoroacetic acid is removed under vacuum and the remaining ionic liquid dried through a silica gel column and stored under P₂O₅.

Spectroscopic Measurements and Data Analysis. Butylimidazole (>98% purity) and methylimidazole (>99% purity) were obtained from Aldrich (St. Louis, MO) and dried over molecular sieves (type 4A) before use. During spectroscopic measurements, the quartz cuvettes were kept tightly sealed so as to prevent moisture from being absorbed by the ionic liquids. The temperature dependent measurements were carried out in a thermoelectric
Figure VII.1. The solvation probe, coumarin 153; 4 RTILs ([BMIM⁺][Cl⁻], [BMIM⁺][BF₄⁻], [BMIM⁺][PF₆⁻], and [BMIM⁺][NTf₂⁻]) formed from the 1-butyl-3-methylimidazolium cation and the four anions indicated ([NTf₂⁻] is (CF₃SO₂)₂N⁻); one RTIL formed from the triethylammonium cation and trifluoroacetate ([NHEt₃⁺][TFA⁻]); and butylimidazole and methylimidazole.

temperature controlled cuvette holder (Quantum Northwest, WA), permitting regulation over the range -40-100 °C. All ionic liquids were carefully dried before optical measurements were performed. Silica gel of 60-200 mesh is activated at 150 °C. A small silica gel column
is prepared using a Pasteur pipette and the pure ionic liquid is added to the column. Depending on the viscosity of the ionic liquid, a bulb is used to force the ionic liquid through the silica gel column. Once the ionic liquid emerges from the column, it is stored in a dessicator under P₂O₅ until further use. The RTILs were subsequently dried over molecular sieves: Type 4A, GRADE 514, 8-12 Mesh and with effective pore size of 4 angstroms.

Steady-state Measurements. Steady-state excitation and emission spectra were recorded with a SPEX Fluoromax with a 4-nm bandpass and were corrected for detector response. A 1-cm pathlength quartz cuvette was used for the measurements. The steady-state spectra can be used to compute the reorganization energy, \( \lambda \), by means of:

\[
\lambda = \hbar \frac{\int_{0}^{\infty} d\nu (\sigma_a(\nu) - \sigma_f(\nu))\nu}{\int_{0}^{\infty} d\nu (\sigma_a(\nu) + \sigma_f(\nu))}
\]

The \( \sigma_a, \sigma_f \) are the absorption (or excitation) and emission spectra, respectively, on a wavenumber scale. The reorganization energy is widely used as a measure of the strength of interactions between a chromophore and its surrounding dielectric media in solvation dynamics studies. It is usually taken as half of the Stokes shift. This estimation is accurate if the excitation and emission spectra are Gaussian, but it becomes unreliable if they are not. The actual computation of \( \lambda \) is accomplished by first manipulating the emission and excitation spectra to permit their addition and subtraction. This requires normalized spectra consisting of equally spaced points. We interpolate and renormalize them so as to obtain spectra having 20-cm\(^{-1}\) spacing between each point and then shift the crossing point of the two curves so that it lies at 0 cm\(^{-1}\). The spectral baselines are then corrected by subtracting the lowest intensity and renormalizing. This manipulation is motivated by the low intensity
emission near 800 nm and questions concerning the utility of the correction factors of our fluorimeter in this region. In any case, baseline subtraction is minor and changes the final result by approximately 1%. An appropriate number of zeros is added to the high energy end of the emission spectrum and to the low energy end of the excitation spectrum so that the curves can be added and subtracted along their entire breadths. \( \lambda \) may now be calculated according to eqn. 1. In practice, however, the integration is more conveniently performed from negative infinity to zero instead of from zero to positive infinity to avoid interference from transitions to higher-lying excited states. Taking these limits of integration is permitted as long as there is mirror image symmetry between the emission and excitation spectra. Time-resolved spectra are treated similarly, but the emission data first must be continued down to zero intensity, since it is impractical to obtain time- and spectrally-resolved data out into the wings of the emission spectrum. This continuation is done simply by connecting the last two points of the available data with straight lines that extend to zero. These new curves consisting of the data points and the straight lines are interpolated in the same way as the steady state excitation curve. The value of \( \lambda \) is sensitive to the way in which the spectrum is continued. We have used both straight-line and log-normal continuations, but have opted for the simplest. The errors introduced in the continuation are effectively cancelled when time-dependent \( \lambda \)'s are compared with each other (as opposed to the steady-state spectrum) as they are in Table VII.1, which furnishes \( \lambda \) at 100 and 4000 ps. The rest of the process proceeds as above. All data manipulations were performed with Microcal Origin 7.0.

*Time-resolved Measurements.* The laser source for the time-correlated single-photon counting measurements was a home-made mode-locked Ti-sapphire laser, tunable from 780 to 900 nm with a repetition rate of 82 MHz. The fundamental from the Ti-sapphire oscillator
was modulated by a Pockels cell (Model 350-160, Conoptics Inc) to reduce the repetition rate
to about 8.8 MHz and was subsequently frequency doubled by focusing tightly into a 0.4-mm
BBO crystal. The resulting blue light, which had a central wavelength of 425 nm, provided
the excitation source. The fluorescence decays were collected at the magic angle
(polarization of 54.7° with respect to the vertical). Emission was collected through a single
monochromator (ISA H10) fitted with a slit having an 8-nm band pass. A half-wave plate
before a vertical polarizer ensured the polarization of the excitation light. The instrument
response function of the apparatus had a full-width-at-half-maximum (FWHM) of 80 ps. A
cuvette of 1-cm pathlength was used for the time-resolved measurements of C153 in the
different solvents. To construct the time resolved spectra, a series of decays (~3000 counts
in the peak channel) were collected over as much of the fluorescence spectrum as possible
and were fit to a maximum of three exponentials, yielding fits with chi-squared values ~1.
Transient spectra were reconstructed from these fits by normalizing to the steady state
spectra of the samples according to the equation 29:

\[
S(\lambda,t) = D(\lambda,t) \frac{S_0(\lambda)}{\int_0^\infty D(\lambda,t) \, d\lambda}
\]

(2)

D(\lambda,t) is the wavelength-resolved fluorescence decay; S_0(\lambda), the steady-state emission
intensity at a given wavelength. We have employed the traditional approach of fitting the
time-resolved spectra to a log-normal line-shape function, from which we extract the peak
frequency, \nu(t), as a function of time. The solvation dynamics were described by the
normalized correlation function as follows 29:

\[
C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)}
\]

(3)
v(0) is the frequency at zero time, that is, immediately after excitation. v(∞) is the frequency at "infinite time," the maximum of the steady state fluorescence spectrum. The decays used to construct the time resolved emission spectra were typically collected over a range of wavelengths from 470 nm to 610 nm at intervals of 10 nm: unless otherwise indicated, a total of fifteen transients were used to construct the time-resolved emission spectra, from which the C(t) were obtained.

*Transient Absorption (Stimulated Emission) Measurements.* The instrument function of our time-correlated single-photon counting system has a FWHM of 80 ps. In order to investigate more rapid phenomena, better time resolution was required. This was provided by a home­made regeneratively amplified Ti:sapphire system $^{44,45}$, providing 130-fs pulses, which we used to perform pump-probe transient stimulated emission measurements. Samples were excited at 407 nm. ([BMIM]$^+${[C1]}) was not investigated with this system because our rotating sample holder is not temperature controlled and because the solvent slowly solidifies at room temperature.

The use of stimulated emission to measure solvation requires special conditions—notably that excited-state (and ground-state) absorption does not contaminate the signal. Such a situation should not be assumed to be the norm. Ernsting and coworkers $^{46}$ have studied the time-dependent spectra of coumarin 153. Their results suggested that the spectral regions near 480 and 560 nm are free of absorbing species. This was fortuitous because Gardecki and Maroncelli $^{47}$ indicated that 480 and 560 nm could be used for coumarin 153 to construct single-wavelength solvation correlation functions. Such a situation seemed to be optimal experimentally since laboratories are more typically equipped to perform transient absorption than fluorescence upconversion experiments and because a single-wavelength
analysis is obviously not as time-consuming as one requiring data collection over a range of wavelengths. Several research groups have discussed and employed the single-wavelength solvent correlation function, \( C_{sw}(t) \) \(^{47-49}\). As a result, we measured stimulated emission kinetics at 480 nm and 560 nm, and typically collected over a full scale of 100 ps.

To have a picture of the solvation over the entire time scale, starting from "time zero" (as defined by the 130-fs pulses of the transient absorption apparatus) to about 4 ns, the longest time scale we investigated with our photon-counting system, we spliced the data from the two experiments together and constructed the single-wavelength solvation response function at the probe wavelength of 480 nm. The decay curves from the stimulated emission and the photon-counting measurements were first fit to multiexponential functions. The data points for the first 100 ps were taken from the fitted stimulated emission kinetics; and from 100 ps to 4 ns, from the fitted photon-counting experiments. The spliced curves were normalized according to the following equation \(^{47}:\)

\[
F'(v,t) = F(v,t) \exp\left\{+K_{sw}^{\infty}t\right\}
\]

where \( F(v,t) \) is the spliced emission curve and \( \exp\left\{+K_{sw}^{\infty}t\right\} \) is the population factor. For our normalization, we used the inverse of the average fluorescence lifetime (1/\( \langle \tau_F \rangle \)), obtained from measuring the fluorescence decay while collecting light from most of the emission band, to approximate \( K_{sw}^{\infty} \). An average lifetime is employed since the fluorescence decay is not well described by a single exponential. Once the population factor was taken into account, the normalized single wavelength solvation response curve, \( C_{sw}(t) \), was constructed:
\[ C_{SW}(t) = \frac{F'(\nu_{SW}, t) - F'(\nu_{SW}, \infty)}{F'(\nu_{SW}, 0) - F'(\nu_{SW}, \infty)} \]  

*Fluorescence Upconversion Measurements.* The system used for these measurement is that of the amplified Ti:sapphire referred to above for the stimulated emission experiments. The fundamental output from the amplifier (815 nm) is doubled by a type-I LBO crystal (2 mm). The frequency doubled blue pulses (407 nm) are separated from the fundamental by a dielectric mirror coated for 400 nm and are focused onto a rotating cell containing the sample using a 5-cm convex lens. The remaining fundamental was used as the gate to upconvert emission. Fluorescence was collected by an LMH-10x microscopic objective (OFR Precision Optical Products) coated for near UV transmission. The gate and the emission are focused by a quartz lens (12 cm) onto a type-I 0.4-mm BBO crystal (MgF\(_2\) coated, cut at 31° and mounted by Quantum Technology, Inc). The polarization of both the gate and excitation source was controlled with a set of zero-order half-wave plates for 800 nm and 400 nm, respectively. The upconverted signal is then directed into an H10 (8 nm/mm) monochromator (Jobin Yvon/ Spex Instruments S. A. Group) with a 5-cm convex lens coupled to a Hamamatsu R 980 PMT equipped with a UG11 UV-pass filter and operated at maximum sensitivity. The PMT output was amplified in two stages (total by a factor of 25, 5 for each stage) by a Stanford research Systems SR-445 DC-300 MHz amplifier with input terminated at 500 Ω and was carefully calibrated after a long (1-2 hours) warm-up. Photon arrival events were registered with SR-400 gated photon counter operated in CW mode with a threshold level of -100 mV. This signal was fed into boxcar averager. A part of blue pulse train was used to normalize pump beam fluctuations. A translation stage (Compumotor) with
a resolution of 0.06 mm/step used to delay the exciting pulses and a computer with Keithley
Metrabyte (DAS 800) interfacing card for driving the motor. The instrument response
function was obtained by collecting the cross-correlation function of the blue and red pulses;
the resulting third harmonic intensity was plotted against delay time. The cross-correlation
functions typically have a FWHM of ~1 ps. This instrument response is a little over three
times as broad as that obtained with our unamplified system. We attribute this to the
absence of compensating prisms after frequency doubling, the presence of the rotating
sample cell, and perhaps a nonideal optical geometry, which nevertheless permits the facile
interchange between pump-probe transient absorption and fluorescence upconversion
measurements. All curves were fit and deconvolved from the instrument function using an
iterative convolute and compare least-squares algorithm.

RESULTS

Table VII.1 summarizes most of the results obtained in this study. (Upconversion
results will be discussed separately.) Figure VII.2 presents excitation and emission spectra of
coumarin 153 in the three ionic liquids for which we were able to obtain spectra above and
below their melting points (while the ionic liquids still remained liquid). The significant
feature is that the emission spectra of coumarin 153 are superimposable at all temperatures in
a given solvent unless the spectra are obtained using the supercooled liquid.

Figure VII.3 presents two typical wavelength-resolved decay profiles of coumarin
153 in [BMIM'][NTf₂]. Figure VII.4 gives the solvation correlation functions, C_{SR}(t),
constructed from the time-correlated single-photon counting data using the spectral
reconstruction method.
Figure VII.2. Excitation and emission spectra of coumarin 153 in [BMIM][PF₆] at 90°C (solid) $\lambda = 2662$ cm$^{-1}$, 25°C (dotted) $\lambda = 2592$ cm$^{-1}$, and -10°C, supercooled (dashed) $\lambda = 2272$ cm$^{-1}$; [BMIM][Cl] at 70°C (solid) $\lambda = 2582$ cm$^{-1}$, and 30°C, supercooled (dashed) $\lambda = 2294$ cm$^{-1}$; [BMIM][NTf₂] at 90°C (solid) $\lambda = 2649$ cm$^{-1}$, 25°C (dotted) $\lambda = 2607$ cm$^{-1}$, and -10°C, supercooled (dashed) $\lambda = 2466$ cm$^{-1}$. All samples were excited at 420 nm. For excitation spectra, the emission monochromator was at 600 nm.
Figure VII.3. Representative wavelength-resolved decays for C153 [BMIM$^+$][NTf$_2^-$] at 490 and 600 nm. A typical instrument function profile is included. The trace at 600 nm reveals the growth of a solvent relaxed state (which is absent in the profile at 490 nm). The decays used to construct the time resolved emission spectra were typically collected over a range of wavelengths from 470 nm to 610 nm at intervals of 10 nm: a total of fifteen time-resolved fluorescence traces were used to construct the time-resolved emission spectra, from which the C(t) were obtained.

The single-wavelength solvent response functions, C$_{SW}$(t), obtained at 480 nm from combining the stimulated-emission and the photon-counting results are shown in Figure VII.5. These results are direct measurements of the early rapid solvation process in these ionic liquids, at least 50% of which is completed in the first 100 ps. In general, this method reports a fast solvation component of 40-70 ps, which is considerably longer than the < 5 ps suggested by Maroncelli and coworkers$^{37,39}$ based on their estimation of the resolution of their time-correlated single-photon counting apparatus. The significance and accuracy of our result will be discussed in more detail at the end of this section.
Figure VII.4. $C_{SR}(t)$ curves and corresponding fits for C153 in (A) [BMIM$^+$][NTf$_2^-$] (hollow circles) $[C(t) = 0.72 \exp(-t/350 \text{ ps}) + 0.28 \exp(-t/1670 \text{ ps})]$, [BMIM$^+$][PF$_6^-$] (solid circles) $[C(t) = 0.36 \exp(-t/360 \text{ ps}) + 0.64 \exp(-t/1360 \text{ ps})]$, [BMIM$^+$][BF$_4^-$] (solid triangles) $[C(t) = 0.62 \exp(-t/60 \text{ ps}) + 0.38 \exp(-t/1100 \text{ ps})]$, and [NHEt$_3^+$][TFA$^-$] (hollow squares) $[C(t) = 0.58 \exp(-t/120 \text{ ps}) + 0.37 \exp(-t/940 \text{ ps})]$ and (B) [BMIM$^+$][Cl$^-$] at 30°C (solid squares) $[C(t) = 0.24 \exp(-t/270 \text{ ps}) + 0.76 \exp(-t/2530 \text{ ps})]$, [BMIM$^+$][Cl$^-$] at 70°C (solid circles) $[C(t) = 0.63 \exp(-t/120 \text{ ps}) + 0.37 \exp(-t/1290 \text{ ps})]$, [BMIM$^+$][NTf$_2^-$] (hollow circles), and butylimidazole (solid triangles) $[C(t) = \exp(-t/70 \text{ ps})]$. 
Figure VII.5. $C_{SW}(t)$ for Cl53 obtained at 480 nm at room temperature. The plots are stimulated emission kinetics obtained over 100 ps with 130-fs pulses and, where necessary, time-correlated single-photon counting data obtained over 4 ns with an 80-ps instrument function. The two traces were spliced together at 100 ps in order to present a continuous trace of the solvation dynamics. Global fits to the $C_{SW}(t)$ are summarized in Table VII.2. The $C_{SW}(t)$ for methanol is presented as a “control” experiment, yielding an average solvation time of 3.5 ps. That the fast solvation components of the BMIM$^+$ liquids resemble that of butylimidazole and that the fast component is seen to become more rapid as the solvent is changed to methylimidazole or to methanol suggests that the ~55-ps event in the ionic liquids is a plausible result. See, however, the correlation functions obtained from fluorescence upconversion data: Figures VII.7,8, and 10.

A comparison of the solvation correlation functions obtained from the single-wavelength and the spectral reconstruction methods is given in Figure VII.6 and in Table VII.1 in order to provide a check on the appropriateness of using the stimulated emission data to measure the initial rapid phase of solvation. The agreement between the two methods is often quite good, as in the cases of [BMIM$^+$][Cl$^-$] at 70°C, [BMIM$^+$][BF$_4^-$], and butylimidazole. It is rather poor in the cases of [BMIM$^+$][PF$_6^-$] and [BMIM$^+$][NTf$_2^-$] (not
shown). The agreement in general appears poorer upon comparison of the average solvation times in Table VII.1 than when visually inspecting the form of the correlation functions. This is because the curves fail to superimpose in the long-time region, where even low-amplitude slow dynamics can contribute considerably to the average. Even in this long-time region, however, the form of the two correlation functions may remain similar, as can be seen by comparing the data for [BMIM][Cl] at 70°C and [BMIM][BF4].

Fluorescence upconversion measurements provide an independent gauge of the time scale for this initial rapid process. Whereas stimulated emission (i.e., transient absorption) measurements are sensitive to both emitting and absorbing species, fluorescence measurements report only on spontaneous emission and thus provide a more straightforward means of probing the solvation behavior of interest. Upconversion data are provided in Figures VII.7-9 for [BMIM][PF6], butylimidazole, and methanol. For each of the solvents, the transients obtained using the two methods are compared at 480 and 560 nm, the wavelengths suggested for use in single-wavelength analysis for coumarin 153. For each solvent, there are deviations arising from excited-state absorbance at these wavelengths, which conspire to distort the time scale for solvation.

DISCUSSION

Steady-State Spectra

Steady-state spectra taken as a function of temperature are presented in Figure VII.2 for three RTILs. Spectra obtained at temperatures above the melting point are nearly superimposable. These three solvents can be supercooled easily. Once the temperature is lowered below the melting point, crystallization occurs slowly. In the case of [BMIM][Cl],
Figure VII.6. A comparison of the solvation correlation functions obtained from the single-wavelength (solid lines), $C_{SW}(t)$, and the spectral reconstruction (dashed lines), $C_{SR}(t)$, methods. All data were obtained from time-correlated single-photon counting experiments. A complete comparison is given in Tables VII.1 and 2. The agreement ranges from very good for $[\text{BMIM}^+] [\text{Cl}^-]$ at 70°C and $[\text{BMIM}^+] [\text{BF}_4^-]$ to poor for $[\text{BMIM}^+] [\text{PF}_6^-]$.

At a temperature lower than the melting point, crystallization occurs slowly. In the case of $[\text{BMIM}^+] [\text{Cl}^-]$, it can occur over a period of several days. Upon supercooling, a significant change in the spectra is observed, which is manifest essentially in their position. There is less solvent relaxation, which we have quantified by means of the reorganization energy, $\lambda$ (Table VII.1 and Figure VII.2). In the case of $[\text{BMIM}^+] [\text{Cl}^-]$, $\lambda$ decreases from 2582 cm$^{-1}$ at
Figure VII.7. Normalized upconversion traces, I(t), for C153 in [BMIM+]\([PF_6^-]\) at different wavelengths. \(\Delta A(t)\) is the kinetic trace for the pump-probe stimulated experiment at the corresponding wavelength. All upconversion traces are fitted globally with time constants of 5, 210, and 5100 ps.

70°C to 2294 cm\(^{-1}\) for the supercooled liquid at 30°C. This shift suggests that the solvent is essentially "glassy" in the supercooled state. For [BMIM+]\([Cl^-]\), supercooling increases the average solvation time by about a factor of four. An interesting aspect of the spectra is that
Figure VII.8. Normalized upconversion traces, I(t), for C153 in butylimidazole at different wavelengths. ΔA(t) is the kinetic trace for the pump-probe stimulated experiment at the corresponding wavelength. All upconversion traces are fitted globally with the following time constants: 28, 102, and 4700 ps.
Figure VII.9. Normalized upconversion traces, $I(t)$, for C153 in MeOH at the 480 and 560 nm along with the stimulated emission traces, $\Delta A(t)$, obtained at the same wavelengths.

Figure VII.10. Solvation correlation functions, $C_{SR}(t)$, for [BMIM$^+$][PF$_6^-$] (solid line) and butyl imidazole (dashed line). The correlations functions are well described by the following forms:

[BMIM$^+$][PF$_6^-$]: $0.14 \exp(-t/7 \text{ ps}) + 0.86 \exp(-t/160 \text{ ps})$;
butylimidazole: $0.09 \exp(-t/7 \text{ ps}) + 0.91 \exp(-t/72 \text{ ps})$.

It is likely that the relative amplitude of the short component in these correlation functions is reduced with respect to those obtained from stimulated emission measurements owing to the poorer time resolution of $\sim 1 \text{ ps}$.
the change in reorganization energy between the normal (90°C) and the supercooled liquids (-10°C) is much smaller for [BMIM⁺][NTf₂⁻] than for [BMIM⁺][Cl⁻] and [BMIM⁺][PF₆⁻]: Δλ = 183, 288, and 390 cm⁻¹, respectively (see the caption to Figure VII.2). That the change is the smallest for [BMIM⁺][NTf₂⁻] may be attributed to it having the lowest viscosity of the three solvents: upon supercooling, it is immobilized to a lesser degree than [BMIM⁺][Cl⁻] or [BMIM⁺][PF₆⁻]. We were unable to perform the same temperature dependent analysis with the BF₄⁻ solvent because it melts at -82°C and our apparatus did not permit us to obtain such a temperature. Maroncelli and coworkers 37,39 have recently shown that ionic liquids conform to standard glass behavior by fitting their kinetic data to stretched exponentials and by showing that it varies linearly with viscosity.


Inspection of Table VII.1 indicates that there is a considerable disparity in the values of the average solvation times obtained from different laboratories using the spectral reconstruction method. Reasonable agreement is obtained between our result for [BMIM⁺][PF₆⁻] and those of Maroncelli and coworkers using 4-AP and coumarin 102 37. There is also reasonable agreement between our result for [BMIM⁺][NTf₂⁻] and those of Karmakar and Samanta using coumarin 153 and Prodan 36. Some of the disparity may be attributed to the number of counts collected in the fluorescence decay, which affects the signal-to-noise ratio, and to the full-scale time window used, which determines how accurately the long-time component is determined. In our case, we use ~4 ns, which is smaller than that used by other groups. A more significant origin of the discrepancies may arise from the preparation and purity of the ionic liquids themselves. Trace amounts of water
and chloride impurities are known to have large effects on the viscosities and densities of RTILs. Notably, Bright and coworkers report that the average solvation time of Prodan in [BMIM\(^+\)][PF\(_6^-\)] decreases from 6.5 to 3.9 ns as the water content is increased from <50 ppm to 1.8 wt %. Since the focus of this article is not to obtain a quantitative comparison of the long-time behavior of the RTILs, these differences do not cause excessive concern. They should, however, give an indication to future workers of the spectrum of results that have been obtained with an eye to assuring reproducibility when examining the glassiness of the RTILs.

Of more immediate interest in this work is the initial rapid phase of solvation in the ionic liquids. Several previous studies have suggested, and our results indicate (Figures VII.4 and 5), that more than 50% of solvation relaxation is very rapid. The origin of these fast solvent fluctuations is not clear. We have hypothesized that the fast relaxations result from the organic cations. In order to test this idea, we attempted to compare the solvation dynamics of butylimidazole with those of the four ionic liquids bearing the BMIM cation. A comparison of [NHEt\(_3^+\)][TFA\(^-\)] with triethylamine was, unfortunately, not possible because triethylamine quenches excited-state coumarin by electron transfer. We attempted other comparisons with RTILs formed from butylpyridinium and pyridinium organic cations with BF\(_4^-\) and NTf\(_2^-\). These also presented complications in the excited-state kinetics that prevented a further comparison of solvation dynamics of the RTILs with their organic counterparts.

The organic solvent, butylimidazole, has a solvation time of between 40-70 ps (Figures VII.4 and 5, Tables VII.1 and 2). Butylimidazole has a very low viscosity, and amplitudes of the fast components of the solvation times for the ionic liquid counterparts...
seem to scale with viscosity, as indicated by Figure VII.5 and Table VII.2. The latter presents the results of a global fitting analysis to the $C_{SW}(t)$ functions (except where indicated). In this procedure, the $C_{SW}(t)$ were fit to two decaying exponential components where the smallest time constant was fixed to agree roughly with that of butylimidazole, 55 ps. The amplitudes of the two components and the time constant of the longer were permitted to vary. A 55-ps component was present in all the fits. Such a value seems reasonable given that the time constant and the average solvation time for butanol, obtained by fluorescence measurements, are 47 and 63 ps, respectively. Another point of comparison is provided by the $C_{SW}(t)$ of methylimidazole (Figure VII.5 and Table VII.2), which is dominated by a rapid component of 8.5 ps. The time constant and average solvation time for methanol, as measured by fluorescence techniques, are 2.3 and 5.0 ps, respectively. Our $C_{SW}(t)$ for coumarin 153 in methanol (Figure VII.5) obtained from stimulated emission kinetics is described by an average solvation time of 3.5 ps. This suggests that ionic liquids based on the methylimidazolium cation will have much faster initial solvation components owing to the shortening of the aliphatic chain from butyl to methyl, just as the solvation by the alcohols is faster for methanol than for butanol. A more direct test of this hypothesis will require measurements of the solvation times of different RTILs at the same viscosity as well as a comparison with ionic liquids based on other cations.

On the other hand, another interpretation of the origin of the initial fast component has resulted from a recent computer simulation. Shim et al. place a dipolar excitation on a model diatomic solute in a RTIL. They observe fast initial relaxation apparently arising from translations of the anions. Given the small size of the model solute and the large changes in net charge, the motions of the resulting concentrated anionic charges may dominate the
relaxation. In the experimental solvation dynamics studies of organic dye molecules such as coumarin 153 in RTILs, the charge distribution change is scattered across the whole probe molecule, which is most likely well solvated by the cations given the hydrophobic nature of C153 in the ground state. We suggest, therefore, that the most possible scenario is that the cation motions dominate the contribution of the initial fast relaxation upon excitation, which is consistent with our experimental observations. In order to elucidate the nature of the fast relaxations, a realistic model of C153 in RTILs will be needed.

Analysis Based Upon Fluorescence Upconversion Results

Finally, we wish to comment on the relative merits of using stimulated emission measurements, single wavelength construction of \( C(t) \), and direct fluorescence measurements. The comparison provided here, in particular the data presented in Figures VII.6-9, indicate that while the stimulated emission measurements provide a good qualitative picture of the dynamics, they deviate considerably from the fluorescence upconversion measurements. We have already noted the discrepancies in the subnanosecond and nanosecond time regimes using single wavelength and "complete" spectral data from photon-counting measurements (Figure VII.6). The fluorescence upconversion measurements permit us to make this comparison in the picosecond time regime. Examination of the traces at 480 and 560 nm indicates that there is at least one absorbing species that contributes to the stimulated emission signal. In the absence of absorption, the two techniques should give identical results. But in fact, for [BMIM\(^+\)][PF\(_6^-\)] and butylimidazole, excited-state absorption causes the stimulated emission kinetics at 480 and 560 nm to decay and rise, respectively, slower than those of their spontaneous emission counterparts. This translates into an overestimation of the time scale for solvation by a factor of \( \sim 8 \). Deviations are also apparent
for methanol. In particular, while the fluorescence upconversion trace in methanol decays essentially to zero, the stimulated emission trace levels off at about 40 ps to a steady-state value of about 20% of the initial signal intensity, again indicating the presence of one or more absorbing states.

Figure VII.10 presents the $C_S(t)$ for [BMIM$^+$][PF$_6^-$] and buytlimidazole obtained from the data in Figures VII.7 and 8, respectively. These solvation correlation functions can both be fit to the same initial rapid component of 7 ps; and, as such, the role of the organic cation suggested by the stimulated emission studies is corroborated. A more comprehensive comparison is, however, required and is currently being undertaken.

CONCLUSIONS

Our results are generally consistent with those of previous workers $^{34-39}$, but there are significant discrepancies in the average solvation times reported for some ionic liquids, most likely arising from the determination of the longer-lived dynamics. Solvation times obtained from the spectral resolution and the single wavelength methods are compared. Our stimulated emission experiments with subpicosecond time-resolution are the first to probe directly the initial rapid solvation component that had been suggested in the earlier studies. They yield a time constant in the range of 40-70 ps (Figure VII.5, Table VII.2). Nevertheless, fluorescence upconversion measurements indicate that the presence of excited-state absorption increases this time by approximately a factor of 8 from its value as obtained by monitoring spontaneous emission directly. Consequently, while stimulated emission measurements can be useful in indicating general trends, direct measurements of spontaneous
emission and use of spectral reconstruction methods are required for quantitative work. These results lead one to inquire into the nature and the number of the excited states contributing to this absorption since their presence could profoundly affect the interpretation of solvation dynamics data, although studies to date have argued that this is not the case. Finally, a comparison of the solvation times in the 1-butyl-3-methylimidazolium ionic liquids with that of butylimidazole itself (and methylimidazole) leads us to consider the role of the polarizability of the cationic partner in giving rise to the initial rapid solvation component.

ACKNOWLEDGEMENTS

DWA was supported by NIH grant RO1 GM53825-08. We thank Dr. Ranjan Das for technical assistance in collecting the fluorescence upconversion data. Mark Maroncelli provided stimulating comments, and we thank him for sharing his Kerr-gated fluorescence data with us before publication.
Table VII.1
Spectral characteristics of coumarin 153 in some RTILs

<table>
<thead>
<tr>
<th>Ionic Liquid a</th>
<th>m.p. (°C) b</th>
<th>η (cP)</th>
<th>&lt;v&gt;\text{abs} (cm^{-1})^d</th>
<th>&lt;v&gt;\text{em} (cm^{-1})^d</th>
<th>λ (cm^{-1})^e</th>
<th>λ_{100 ps} (cm^{-1}) f</th>
<th>λ_{4000 ps} (cm^{-1}) f</th>
<th>&lt;τ&gt;\text{SG} (ns) b</th>
<th>&lt;τ&gt;\text{SW} (ns) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>[BMIM][Cl] (30°C)</td>
<td>65</td>
<td>11000</td>
<td>23490</td>
<td>18570</td>
<td>2294</td>
<td>1940</td>
<td>2112</td>
<td>2.0</td>
<td>1.7</td>
</tr>
<tr>
<td>[BMIM][Cl] (70°C)</td>
<td>65</td>
<td>334</td>
<td>23540</td>
<td>18090</td>
<td>2582</td>
<td>2285</td>
<td>2627</td>
<td>0.53</td>
<td>0.33</td>
</tr>
<tr>
<td>[BMIM][PF_6] (20°C)</td>
<td>-8</td>
<td>371</td>
<td>24080</td>
<td>18420</td>
<td>2592</td>
<td>2015</td>
<td>2313</td>
<td>1.0</td>
<td>0.38</td>
</tr>
<tr>
<td>[BMIM][BF_4] (20°C)</td>
<td>-82</td>
<td>154</td>
<td>23670</td>
<td>18350</td>
<td>2481</td>
<td>1968</td>
<td>2203</td>
<td>0.46</td>
<td>0.31</td>
</tr>
<tr>
<td>[BMIM][NTf_2] (20°C)</td>
<td>-4</td>
<td>52</td>
<td>24110</td>
<td>18530</td>
<td>2607</td>
<td>1965</td>
<td>2180</td>
<td>0.72</td>
<td>0.17</td>
</tr>
<tr>
<td>butylimidazole (20°C)</td>
<td>&lt;50</td>
<td>24040</td>
<td>18700</td>
<td>2622</td>
<td>2263</td>
<td>2344</td>
<td>0.070</td>
<td>0.041</td>
<td></td>
</tr>
<tr>
<td>methylimidazole (20°C)</td>
<td>&lt;50</td>
<td>24009</td>
<td>18330</td>
<td>2672</td>
<td>2484</td>
<td>2480</td>
<td>0.050</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>[NHEt_3][TFA] (20°C)</td>
<td>26100</td>
<td>19450</td>
<td>2836</td>
<td>2451</td>
<td>2764</td>
<td>0.42</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a For abbreviations, see the caption to Figure VII.1. Although the melting point of [BMIM][Cl] is ~65°C, it usually takes several days at room temperature before it solidifies.

b From reference 57.

c From reference 52.

d \( \langle v \rangle \sim \frac{\int v f(v) dv}{\int f(v) dv} \), computed using 70% of the emission and excitation spectra in order to exclude the contributions from absorption to states higher than \( S_1 \) in energy.

e Computed from equation 1. See text and reference 43.
The reorganization energies at 100 and 4000 ps are computed as discussed in the experimental section. Note that because of the limited data set used to obtain the time resolved spectra, the λ at 4000 ps deviate from the steady-state values, for which in most cases we would expect them to be approximately the same.

The fractional amount of solvation at 100 ps, for example, can be determined by \( f_{100 \text{ ps}} = \frac{\lambda_{400 \text{ ps}} - \lambda_i}{\lambda_{4000 \text{ ps}} - \lambda_i} \), where \( \lambda_i \) is the intramolecular contribution to the reorganization energy. Maroncelli and coworkers have addressed the estimation of \( \lambda_i \) by proposing methods to compute the “zero-time” spectrum arising only from solvation.

Average solvation times obtained from the spectral reconstruction data, computed from the fit parameters given in the caption to Figure VII.4. The comparison of the average solvation times is made using only data accumulated from time-correlated single-photon counting data. Errors in the solvation times are \( \sim \pm 15\% \).

Average solvation times obtained from the single-wavelength data, computed from the fit parameters given in the caption to Figure VII.5. The average time is computed from the spliced picosecond and nanosecond experiments.

25 °C using 4-aminophthalimide (4-AP).

25 °C using coumarin 102.

25 °C using coumarin 153.

20 °C using 6-propionyl-2-dimethylaminonaphthalene (Prodan).

At room temperature using coumarin 153.

25 °C using coumarin 153. The average solvation time is computed from parameters obtained from a stretched exponential fit.

20 °C using coumarin 153.

20 °C using Prodan.

20 °C using 4-AP.

298 K using Prodan.
Table VII.2

Global fitting of the initial rapid phase of $C_{SW}(t)$ of some RTILs $^a$

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>$\eta$ (cP) $^b$</th>
<th>$a_1$</th>
<th>$\tau_1$ (ps)</th>
<th>$\tau_2$ (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{BMIM}^+][\text{BF}_4^-]$ (20°C)</td>
<td>154 $^{52}$ 233 $^{57}$</td>
<td>0.39</td>
<td>55</td>
<td>1000</td>
</tr>
<tr>
<td>$[\text{NHEt}_3^+][\text{TFA}^-]$ (20°C)</td>
<td>0.61</td>
<td>55</td>
<td>650</td>
<td></td>
</tr>
<tr>
<td>$[\text{BMIM}^+][\text{PF}_6^-]$ (20°C)</td>
<td>371 $^{52}$ 312 $^{57}$</td>
<td>0.62</td>
<td>55</td>
<td>2250</td>
</tr>
<tr>
<td>$[\text{BMIM}^+][\text{NTf}_2^-]$ (20°C)</td>
<td>52 $^{57}$</td>
<td>0.73</td>
<td>55</td>
<td>1500</td>
</tr>
<tr>
<td>butylimidazole (20°C)</td>
<td>&lt;50</td>
<td>0.98</td>
<td>55</td>
<td>120</td>
</tr>
<tr>
<td>methylimidazole (20°C)</td>
<td>&lt;50</td>
<td>0.80</td>
<td>8.5</td>
<td>190</td>
</tr>
<tr>
<td>methanol (20°C)</td>
<td>0.59</td>
<td>0.35</td>
<td>0.32</td>
<td>5.2</td>
</tr>
</tbody>
</table>

$^a$ $C_{SW}(t)$ obtained from 480-nm stimulated emission data as described in the text are fit to a sum of exponentials, $C_{SW}(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$, where $a_1 + a_2 = 1$. In all cases (except the last two solvents) the rapid solvation component is fixed at 55 ps. Given the 100-ps full scale used in most cases (for $[\text{NHEt}_3^+][\text{TFA}^-]$, a 400-ps scale was used), the longer-lived components should not be regarded as accurate estimations of the slower solvation phase. The $C_{SW}(t)$ are displayed in Figure VII.5.

$^b$ We have no viscosity information for $[\text{NHEt}_3^+][\text{TFA}^-]$ and have not been able to obtain any for butylimidazole or methylimidazole from either the literature or the supplier.
REFERENCES


CHAPTER VIII: THE COMPLEX OF APOMYOGLOBIN WITH THE FLUORESCENT DYE, COUMARIN 153

A paper accepted in the journal of Photochemistry and Photobiology

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Abstract

Understanding a protein’s dielectric response requires both a theoretical model and a well-defined experimental system. The former has already been proposed by Song (*J. Chem. Phys.* 116, 9359 (2002)). We suggest that the latter is provided by the complex of coumarin 153 (C153) with apomyoglobin. C153 has been exhaustively studied and has proven to be an excellent probe of the solvation dynamics of polar solvents. Myoglobin is one of the most thoroughly studied proteins. Myoglobins from a wide range of species have been subject to X-ray structural analysis and site-directed mutagenesis. Here we demonstrate the existence of a robust C153-apomyoglobin system by means of molecular dynamics simulations, equilibrium binding studies using a Job’s plot and capillary electrophoresis, circular dichroism, and time-resolved fluorescence.

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The reorganization energy of C153 bound to apomyoglobin is compared with that of C153 in bulk solvent using the method of Jordanides et al (J. Phys. Chem. B 103, 7995 (1999)).

Introduction

In the past decade, it has been well established by numerous experimental and theoretical studies that solvation dynamics in polar solvents can be described by linear response theory [1-10]. In general, the full frequency dependent dielectric function of the polar solvent gives a good description of the solvation dynamics from the ultrafast regime to that of diffusive relaxation. Some direct comparisons between theory and experiments have been established [11-14]. The success is largely attributable to the dielectric fluctuations of polar solvents being described accurately by simple linear response models, such as the dielectric continuum model [14-16]. On the other hand, the dielectric response in proteins is more complicated. There exist many length scales due to the structural constraints created by the carbon back bone. Some studies indicate that a linear response model may be valid from atomistic simulations [17, 18]. A simple dielectric continuum description of the protein is clearly insufficient, even though such a description has been widely used to correlate experimental data [19-22]. A full atomistic description is very time consuming for large proteins and may also hinder the distillation of transparent physical pictures [23, 24].

We have previously proposed to seek a physically well-defined middle ground between the continuum and a full atomistic description of the protein’s dielectric response in order to develop a new model that recognizes the highly inhomogeneous nature of a protein and at the same time avoids the enormous computational cost of atomistic model simulations. To this end, we proposed a collection of structurally constrained polarizable dipoles embedded in a dielectric continuum solvent to describe the dielectric response of a protein.
Other models of solvents can also be used provided that the response function of the solvent can be obtained from other sources. The main assumption of our model is the existence of a set of frequency-dependent polarizabilities for each residue, portable to all proteins in nature. The polarizabilities can be obtained by performing detailed molecular dynamics simulations for small proteins or by solvation dynamics measurements with various mutations. The dielectric response of large proteins can be computed from these intrinsic parameters. Each dipole is located on the center of mass of a residue, and for each such a residue there is a frequency-dependent polarizability $\alpha(\omega)$ (in general a complex quantity such as the dielectric constant function). The total number of polarizable dipoles is thus equal to the total number of residues and their positions are completely specified by the native structure of the protein.

Studies of the solvation dynamics of proteins offer a powerful means to test the validity of models of their dielectric response. In spite of considerable efforts towards the understanding of the dielectric relaxation processes in proteins [25-28], up to now a reliable estimate for the dielectric response function of proteins is still lacking. Early studies indicated that a slow relaxation (~1 ns) indeed exists in myoglobin [29, 30] in contrast to the polar solvents. This is not unexpected due to the structural constraints. But the role of a protein’s interior motions in its dielectric relaxation is presently unclear from various experimental studies of solvation dynamics in protein environments. Homoelle et al. have suggested that the dynamical fluctuations observed in phycobiliproteins involve substantial interior motions of the protein [31]. Fraga and Loppnow [32] have shown that the resonance Raman spectra are affected by the different residue compositions of the blue copper proteins from different species. On the other hand, other experimental and theoretical studies of lysozyme seem to suggest that the major contribution of the observed dynamical fluctuations
comes from the surrounding water solvent and the water molecules attached on the protein surface [33].

Zewail and coworkers used tryptophan fluorescence to study solvation dynamics in proteins. The solvation dynamics were significantly slower for the surface tryptophan residues in Subtilisin *Carlsberg* and in monellin than for that of tryptophan in bulk water. They argued that the slow relaxation is due to the water molecules constrained on the protein surface [27, 34, 35]. Given, however, the ~500 cm⁻¹ difference in the reorganization energies for the surface tryptophans in the two proteins, it seems that there is also a considerable relaxation arising from the different amino acids neighboring the tryptophans.

These differences in the interpretations of various experiments are in no small part due to the lack of a reliable dielectric response function for the studied proteins from either experiments or computer simulations. The knowledge of the protein dielectric response provided by our model could shed light on this since it presents a residue level dielectric response function of proteins. What is also required, however, is a well-characterized experimental system that facilitates the interpretation of the data. To this end, we propose the complex of coumarin 153 and apomyoglobin. There are three main considerations for the choice of this system. First, coumarins in general, and coumarin 153 (C153) in particular, are well characterized and widely used chromophores for solvation dynamics studies [36-46]. Second, we can produce a broad range of mutant proteins [47, 48] in which one or several amino acids are strategically replaced, so as to test our theoretical model. Third, the structures of many myoglobins and their mutants have been determined to high resolution [49, 50]. We consequently propose that a fruitful system for studying the protein dielectric response is myoglobin in which the heme has been replaced by coumarin 153. The
availability of mutant proteins with amino acid replacements near the heme binding site will eventually permit a quantitative evaluation of local contributions of specific residues to the solvation dynamics of proteins.

Previous attempts to exploit the myoglobin system to study the solvation response of proteins have been made [29, 30] using the fluorescent probes, 2,6-ANSDMA and DANCA, respectively (Figure VIII.1). The former probe molecule afforded a single exponential response of 9.1 ns; the latter, a more complicated response with both shorter and longer response times. The discrepancy between the results for these two probe molecules as well as the predominance of the long-lived response time caused us to search for other probes. We thus initially considered the probe molecules 1,8-ANS and biliverdin, both for which there are structures of their complexes with apomyoglobin [51, 52]. Neither of these chromophores is, however, ideal because their absorption spectra are complicated by overlapping electronic states. Even if internal conversion from higher-lying states to the lower fluorescent state is faster than solvation dynamics, as has been suggested to be the case in tryptophan [27, 34, 35, 53], an accurate determination of the reorganization energy based on the steady-state spectra becomes very difficult. We consequently opted for coumarin 153, which not only has been studied in a very wide range of solvents and in the gas phase, but whose excited-state solvation has been demonstrated not to involve any contributions other than those from S₁ [38]. Hochstrasser and coworkers have studied the solvation of coumarin 343 in calmodulin [41]. These workers attached coumarin 343 to the N-terminus of a tridecapeptide, which was designed to bind tightly to calmodulin. In this system, solvation is complete in 100 ps.
Materials and Methods

Coumarin 153 was purchased from Exciton Inc. (Dayton, OH) and used without further purification. Apomyoglobin was prepared using the procedure described elsewhere [54]. Coumarin 153 has low solubility in water. Consequently, addition of apoprotein providing
sufficient time for equilibration (45 minutes) ensured that it was taken up by complex formation. In order to obtain complexes with different C153/apoMb ratios, increasing amounts of C153 were added to a 5 x 10^{-5}-M protein solution. The C153 solution was prepared by adding microliter amounts of a concentrated stock solution of C153 in methanol to water and sonicating for ~1 hour in the dark to ensure complete homogenization of the resulting solution. All the samples were then allowed to equilibrate for 45 minutes prior to the steady-state and time-resolved measurements. Completion of equilibration was checked by recording the steady state spectra at 10-minute intervals after the addition of C153 until no change in Stokes shift was observed.

*Binding constant measurements, fluorescence.* Two stock solutions were prepared of equal concentration, 5x10^{-5} M, of both apoMb and coumarin 153. From these stock solutions, seven 200 µL samples were prepared with an increasing mole fraction, X, of coumarin: 0.1, 0.2, 0.25, 0.5, 0.75, 0.8, and 0.9. After the samples were allowed to stand for approximately 45 minutes to ensure complete equilibration, fluorescence intensities were measured and plotted against mole fraction of coumarin, X, to verify that the maximum intensity occurred at a mole fraction of 0.5, indicating a one-to-one binding stoichiometry. (C153 has very low solubility in water, and the protein ApoMb by itself has no measurable fluorescence in the region where the complex emits. Thus, the fluorescence is directly proportional to the concentration of the protein-coumarin complex.) Using this maximum intensity and the intensity at one other mole fraction, the binding constant, K_B can be determined [55], where

\[
K_B = \frac{(I \times I_0) / (I_0 - I)([apoMb]_T - [C153]_T)}{I_0}
\]

I_0 is the maximum fluorescence intensity at X = 0.5. I is the fluorescence intensity at a given mole fraction, X. [apoMb]_T = total concentration of apoMb at a given X, and the subscript T refers to the total concentration.
\([C153]_T\) is the concentration of coumarin 153 at a given \(X\). The dissociation constant for the complex is \(K_D = 1/K_B\).

**Binding constant measurements, capillary electrophoresis (CE).** Frontal analysis CE experiments were performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system equipped with a 488-nm laser-induced fluorescence (LIF) detector. Untreated fused-silica capillaries with 50 \(\mu\)m ID and 360 \(\mu\)m OD was purchased from Polymicro Technologies, Inc. (Phoenix, AZ). The capillaries were 30 cm in length (20 cm to detection window). When a capillary was first used, it was rinsed for 1 min with water, 5 min with 1 M NaOH, and 1 min with water. Prior to each injection, the capillary was washed for 0.5 min with water, 0.5 min with 1 N NaOH, and 0.5 min with water, followed by 2 min with running buffer. The premixed coumarin-apomyoglobin samples were injected at 0.5 psi for 40 seconds to the capillary. Experiments were performed at 10 kV and a temperature of 25°C. Sample emission was monitored at 520 nm. Data were collected by P/ACE system MDQ software. Sodium phosphate, and phosphoric acid were purchased from Fisher Scientifics (Fair lawn, NJ). 0.2 M Na-phosphate buffer at pH 9.0 was used. The phosphate buffer was filtered through 0.22 mm Non-pyrogenic filter (Costar, Corp.Corning, NY). In the frontal analysis technique, coumarin 153 and apomyoglobin were premixed and injected as a large sample plug onto the capillary column. At pH 9, the apomyoglobin is negatively charged. The electrophoretic mobility of the free coumarin 153 is different from the mobility of the protein and protein-coumarin complex. After injection of large mixed sample plug, the free coumarin will migrate away from the protein. Equilibrium is maintained where the zones temporarily overlap. The equilibrium free coumarin concentration is calculated from the height of the resulting plateau. The height of the free coumarin plateau decreases with the
addition of apomyoglobin because of its binding to coumarin. The plateau height at \([\text{apoMb}] = 0 \ \mu\text{M}\) was used as the coumarin standard. In this assay, a series of sample mixtures with a fixed protein concentration and increasing coumarin concentrations are injected. Performing such experiments at different coumarin concentrations allows the determination of binding constant according to the following equation. \(\frac{r}{1-r} = n K_B [C153]_f\), where \(r\) is the fraction of bound chromophore, \(n\) is the number of binding sites, \([C153]_f\) is the free chromophore concentration in the running buffer, and \(K_B\) is the binding constant between apoMb and C153.

**Steady-state spectroscopies.** Steady-state absorption spectra were recorded on a Perkin-Elmer Lambda-18 double-beam UV-visible spectrophotometer with 1-mm resolution. The concentration of the apoprotein was determined by monitoring the absorbance at 280 nm, where the molar extinction coefficient is 15.2 mM\(^{-1}\)cm\(^{-1}\). Steady-state fluorescence spectra (both excitation and emission), were obtained on a SPEX Fluoromax with a 4-nm bandpass, and corrected for detector response. For both fluorescence and absorption measurements of the complex, a 3-mm pathlength quartz cuvette was used. Steady-state circular dichroism (CD) spectra were performed on a JASCO CD spectrometer (J-710). A 3-mm pathlength cell was also used for these measurements. The concentration of the samples was kept close to micromolar range to avoid saturation of the detector. The data so presented are an average of 3 scans and were collected at an interval of 1 nm.

**Time-resolved spectroscopies.** The laser source for the time-correlated single-photon counting measurements was a home-made mode-locked Ti-sapphire laser, tunable from 780 to 900 nm with a repetition rate of 82 MHz. The fundamental from the Ti-sapphire oscillator was modulated by a Pockels cell (Model 360-80, Conoptics Inc) to reduce the repetition rate
to about 8.8 MHz and was subsequently frequency doubled by focusing tightly into a 0.4-mm BBO crystal. The blue light, which had a central wavelength of 425 nm, provided the excitation source. Emission from the samples of the complex was collected at $\lambda_{em} > 550$ nm with a bandpass filter in order to reduce the contribution of scattered excitation light interfering with the sample fluorescence. The fluorescence lifetime decays were collected at the magic angle (polarization of 54.7° with respect to the vertical). A half-wave plate was used before the excitation polarizer to ensure vertical polarization of the excitation light. To obtain the rotational dynamics, emission from the samples was collected parallel and perpendicular to the direction of polarization of the excitation pulse. The instrument response function of the apparatus had a full-width–at-half-maximum (FWHM) of 80 ps. The fluorescence lifetime decays were typically collected upto a maximum of 10,000 counts in the peak channel of the multi-channel analyzer (MCA) while for the anisotropy decays, the corresponding maximum value was 12,000 counts. Sample integrity was checked by monitoring the excitation and emission spectra before and after the measurements. The coumarin concentration was kept at $\sim 5 \times 10^{-6}$ M throughout. A 3-mm cell pathlength was used for the time-resolved measurements of the complex.

*Molecular dynamics simulations.* The starting configuration of horse heart myoglobin is from the protein DATA BANK (PDB id 1WLA) with TIP3P water models. Standard constant pressure-temperature MD was performed using the ORAC package with the Amber force field [56]. In all simulations, short-range non-bonded interactions were calculated up to a 10 Å cutoff, whereas long-range electrostatic interactions were treated by the SPME method using a very fine grid, 128 point per axis, with periodic boundary conditions, and Ewald convergence parameter of 0.43 Å$^{-1}$. Three different Nosé-Hoover thermostats were
coupled to solute, solvent, and total center of mass. An external pressure of 0.1 MPa was applied all along the trajectory. A five time-step rRESPA [57] algorithm with times of 0.5-1.0-2.0-4.0-12.0 fs was used with bond constraints on hydrogen covalent bonds handled by a Shake-Rattle-like algorithm. The final system was first equilibrated with velocity rescaling for 60 ps at 50 K and 80 ps at 300 K. Following this initial equilibration, we ran the system for one additional nanosecond at constant temperature (T = 300 K) and pressure (P = 0.1 Mpa). To achieve full relaxation, the simulation box was entirely flexible for the first 300 ps, whereas for the remainder of the run, only isotropic changes of the box were allowed [58]. Finally, the system was simulated for an additional 10 ns. Shown in Figure VIII.2 are snapshots at around 3 ns for the horse heart and the sperm whale apomyoglobin complexes. One might object that the way to find the equilibrium configuration is to raise the temperature and then to cool the system. This is not recommended. Raising the temperature will mostly likely denature the protein, and the heme-pocket may adopt some highly unlikely conformations that would not be present in the native structure. Since the insertion of C153 in our experiments is done with the native myoglobin structure, the method we employ is physically reasonable.

Results and Discussion

The binding of coumarin 153 to apomyoglobin has been characterized by molecular dynamics simulations (which predict tight, stable binding in the heme pocket), measurement of its dissociation constant using two different methods, circular dichroism (which demonstrates no changes in secondary structure for the complex with respect to that of the native holo protein), and its reorientation time using measurements of the polarized fluorescence decay.
Figure VIII.2. Snapshot of equilibrated C153-apomyoglobin in water from 3-ns molecular dynamics simulations using the Amber95 force field: (a) sperm whale apomyoglobin; (b) horse heart apomyoglobin. The C153 is shown in the space-filling models. Key histidine residues in the heme pocket are also shown. Residue Val68 (one of the residues in contact with C153 in the hydrophobic heme-pocket with small nuclear polarizability) is used in one of our mutations [47, 48]. (c) The time dependence of the relative orientation of C153 with respect to His 93 (see text) from the MD trajectory of the horse heart apomyoglobin-C153 complex.
A 10-ns MD simulation using the Amber force field indicates that C153 is stable in the heme-pocket and an equilibrium configuration is found (Figures VIII.2a and b). Also shown in Figure VIII.2c is a trajectory of the angle between C153 and His 64, which is defined by the vector from N to ester O of C153 and the vector of two Ns on the His 64 sidechain. This angle is averaged at 98° with a standard deviation of 8°. Similar results are obtained for the angle between C153 and His 93.

Using two different methods, we have obtained dissociation constants of 5.65 ± 0.25 μM (Job’s plot) and 13 ± 2 μM (CE) for coumarin 153 and horse heart apoMb, which are comparable to or smaller than those discussed previously for other fluorescent probes [29, 30] (Figures VIII.3 and VIII.4). The Job’s plot of the complex (Figure VIII.3) clearly reveals that the maximum of the fluorescence intensity occurs at coumarin mole fraction of 0.5, which is a clear indication of 1:1 stoichiometry for the complex in the given concentration range of protein. One explanation for the difference in the two results is that the CE experiments were performed using a higher C153/apoMb ratio because the detector was equipped with a 488-nm light source, which is not optimal for exciting coumarin 153 fluorescence.

Independent confirmation that the coumarin binds in the heme pocket is provided by the similarity of the circular dichroism (CD) spectra of native myoglobin with that of the C153/apoMb complex (Figure VIII.5). A comparison of the fluorescence anisotropy decay of coumarin 153 in solution (DMSO) (Figure VIII.6) and bound to apoMb (Figure VIII.7) also argues for C153/apoMb complex formation. While the free coumarin exhibits a very fast depolarization time of 100 ps in DMSO, this time is lengthened to 9.2 ns upon binding, the latter time consistent with the rotational correlation time that would be expected for the
Figure VIII.3. Job's plot for the complexation of coumarin-153 with apoMb. That the fluorescence intensity peaks at a mole fraction value of 0.5 (dotted lines) for C153 is a clear indication of a 1:1 complex formed between the probe molecule and the protein. The data presented are an average of four measurements. The calculated dissociation constant for the complex is $5.65 \pm 0.25 \mu M$.

~18 kDa protein. More significantly, the anisotropy decay of bound coumarin is single exponential (9.2 ns), within our time resolution. This can be interpreted in terms of a rigidly bound coumarin whose fluorescence is depolarized exclusively by the overall rotational motion of the apomyoglobin itself. A single exponential decay would not be expected for a surface bound chromophore [59, 60]. That the fluorescence decay of C153 is single exponential also suggests that, within the sensitivity of our experimental apparatus, it binds in the heme pocket in only one conformation.

Finally, there is a significant Stokes shift of bound C153 with respect to C153 in methanol that demonstrates the difference between the heme pocket and the bulk solvent.
Figure VIII.4. Electropherogram (top panel) obtained using the frontal analysis capillary electrophoresis method with coumarin-apoMb. Experimental conditions: pressure injection at 0.5 psi for 40 s; separation voltage, 10 kV; LIF detector with excitation at 488 nm and emission at 520 nm. 0.2 M sodium phosphate buffer at pH 9.0. The first peak plateau is due to the coumarin; the second, to the coumarin-apoMb complex. The final result is based on 3 individual experiments: $K_D = 13 \pm 2 \mu M$. The ordinate label RFU stands for "relative fluorescence units." The bottom panel is a plot of $r/(1-r)$ against free coumarin, where $r$ is the fraction of C153 bound per protein. The slope of a linear fit to the experimental data points gives the binding constant ($K_B$) of C153 in the complex. The value of $K_B$ obtained from this figure is $0.086 \mu M^{-1}$, which gives $K_D = 11.6 \mu M$. 
Figure VIII.5. The CD spectra of apoMb (solid), Mb (dashed), apoMb/C153 complex (dotted). The figure clearly shows the retention of the native structure of Mb once C153 binds inside the vacant heme pocket of apoMb. Protein concentrations are approximately 10^{-6} M, and the apoMb/C153 complex has a concentration ratio of 2:1. Similar spectra were obtained with other ratios in a range from 9:1 to 2:1.

Figure VIII.6. Polarized fluorescence decay of C153 in DMSO: $\lambda_{ex} = 420$ nm; $\lambda_{em} > 470$ nm, $\chi^2 = 1.3$; $r(t) = 0.32 \exp(-t/110 \text{ ps})$. The anisotropy measurement was repeated 4 times yielding a reorientation time, $\tau_r = 100 \pm 10$ ps and a limiting anisotropy, $r(0) = 0.32 \pm 0.02$. The fluorescence lifetime was $5300 \pm 50$ ps. The top panel shows the residual from the fits.
in solvating the fluorescent state. The spectra in Figure VIII.8 can be used to compute the reorganization energy, \( \lambda \), by using the method of Jordanides et al. of [33]:

\[
\lambda = \frac{\int d\omega [\sigma_a(\omega) - \sigma_f(\omega)]\sigma}{\int d\omega [\sigma_a(\omega) + \sigma_f(\omega)]}
\]

The \( \sigma_a,\sigma_f \) are the absorption (or excitation) and emission spectra, respectively on a wavenumber scale. The reorganization energy is widely used as a measure of the strength of interactions between a chromophore and its surrounding dielectric media in solvation dynamics studies. It is usually taken as half of the Stokes shift. This estimation is accurate if the excitation and emission spectra are Gaussian, but it becomes unreliable if the spectra are not Gaussian. The reorganization energy computed using the above expression is 2280 cm\(^{-1}\) for C153 complexed with apomyoglobin as opposed to 2800 cm\(^{-1}\) for C153 in methanol, which indicates the difference in the probe’s environment in these two distinctly different systems.

The preponderance of evidence listed above supports C153 binding to apomyoglobin, specifically in the heme pocket, in a manner illustrated in Figure VIII.2. This leaves the dye in a hydrophobic environment consisting of a large number of amino acid side chains that are responsible for stabilizing and isolating the heme prosthetic group to prevent dissociation and oxidation in the native protein. In sperm whale myoglobin, the principal side chains impinging on C153 are two His residues (at positions 64 and 97 in the primary sequence), Phe43, Val68, Leu29, and Leu89.
Figure VIII.7. Polarized fluorescence decay of the coumarin 153-horse heart apomyoglobin complex. The concentration of protein (5.5 x 10^{-5} M) is 10 times that of coumarin to eliminate multiple binding of C153 to the protein. \( \lambda_{\text{ex}} = 425 \text{ nm}; \lambda_{\text{em}} > 550 \text{ nm}, \chi^2 = 1.2; r(t) = 0.29\exp(-t/9300 \text{ ps}). \) The anisotropy measurement was repeated 3 times. The average value of rotational time is 9200 ± 100 ps and the limiting anisotropy is \( r(0) = 0.29 \pm 0.01. \) The fluorescence lifetime of coumarin bound to apomyoglobin was 5050 ± 50 ps (3 measurements). For the lifetime measurements, \( \lambda_{\text{ex}} = 425 \text{ nm}; \lambda_{\text{em}} > 550 \text{ nm} \) and \( \chi^2 = 1.2. \) Fitting the decay to a double exponential gave no improvement over the chi-squared value already obtained from the single exponential fit. The top panel shows the residuals from the fits.

Conclusions

We have furnished an exhaustive characterization of the complex of the fluorescent probe, C153, with apomyoglobin indicating that it binds moderately strongly and very rigidly in the heme pocket. Since protein is clearly a heterogeneous environment with a spatially dependent polarizability, a precise knowledge of where and how the fluorescence probe
binds is crucial to any thorough analysis of the protein dielectric response and our ability to distinguish the protein's response from that of the solvent. Future work will analyze the contribution of the individual amino acid residues in the heme pocket to the dielectric response of the protein.

**Figure VIII.8.** Steady state spectra of the horse heart apomyoglobin/coumarin 153 complex (solid lines) and of coumarin 153 in methanol (dashed lines). According to our method of evaluating the reorganization energy [33] the whole excitation or absorption spectrum is needed. If there are more than one electronic excited states close to the fluorescence state it will be very difficult to get an accurate excitation spectrum of that state, which is the case, for example, for ANS, whose excitation spectrum is congested with at least two overlapping peaks in the blue region. The reorganization energy calculated by our method for C153 in the complex was 2280 cm\(^{-1}\) while that in methanol was 2800 cm\(^{-1}\).

Acknowledgements
This work was partially supported by NSF grant MCB-0077890 to MSH and JWP. XS acknowledges the financial support provided by a grant from the Petroleum Research Fund, administrated by the American Chemical Society. Mr. J. Graboski assisted with the preparation of the apomyoglobin samples. We thank Conoptics, Inc. for their generous loan of the Pockels cell to reduce the repetition rate of our Ti:sapphire system. We also thank Tijana Grove for assisting us with the CD measurements.

References


CHAPTER IX: SUMMARY AND CONCLUSIONS

This thesis has focused on a variety of time-resolved spectroscopic techniques and their applicability in probing the excited state photophysics and photoprocesses of a range of biologically and environmentally relevant systems.

The well characterized AOT reverse micelles have been known to dissolve large amounts of water in their polar cores. These reverse micelles also mimic a biological membrane. This dual nature of AOT has allowed us, as revealed in chapter III, to study the photophysics of hypericin as a function of pH (which is not possible under normal conditions because of the insolubility of hypericin in water). The variations in the steady-state spectra and time-resolved data of hypericin with pH and as a function of water pool size point towards an orientation in which the peri-hydroxyl and carbonyl groups of hypericin protrude into the polar micellar core. Variation of anisotropy as a function of water pool size reveals the extent of rigidity of the hypericin bound to the micelle. With increase in the value of \( w_0 \) (= 8, 12), the hypericin becomes more and more flexible as can be seen by the use of a second rotational time constant to describe the local motion of hypericin inside the micelle. That the \( pK_a \) value of \( \sim 12.5 \) corresponding to the Hyp\(^+\)/Hyp\(^2-\) equilibrium is larger than the previously reported value by as much as 1.5 units is probably a result of the hydroxyl groups being exposed to smaller water pools. The water in the polar core of the reverse micelles has been found to behave differently than bulk water in many ways.

Chapter IV deals with the comparison of the excited state photophysics of two nearly symmetrical perylene quinones, hypocrellin A and hypomycin B. Hypomycin B is unique as it has only one hydroxyl group peri to a carbonyl group as opposed to two in hypocrellin A and four in hypericin. At lower concentrations of Brij micelles (10 times the CMC),
hypocrellin A aggregates. This result is important because it is known that aggregates do not exhibit excited state hydrogen atom transfer, have markedly reduced fluorescence quantum yields, shorter fluorescence lifetimes and reduced virucidal and cytotoxic activity. pH dependent studies of hypomycin B carried out in conditions similar to hypocrellin, reveal a titratable group with a pKa of ~9.7 for the former. Within experimental error, hypomycin B does not exhibit deuterium isotope effect (unlike hypocrellin A). The transient absorption data of hypomycin B do not reveal any stimulated emission at any of the probe wavelengths studied, let alone a rise time in the stimulated emission. This either means that the compound does not undergo an excited-state H-atom transfer or that the transfer is too fast to be resolved by our instrument. Whatever the case maybe, the picosecond data do not shed any light as to how many H-atoms may be transferred in the perylene quinones and whether the transfer is concerted or stepwise.

Chapter V focuses on the different mechanisms that the two heme proteins myoglobin (Mb) and soybean leghemoglobin (Lba) use to regulate ligand binding. In the proximal pocket of Mb, Ser(F7), which is a part of an extended hydrogen bonding network, holds the proximal histidine, His(F8), in place through hydrogen bonding thus leading to destabilization of the bound ligand. In Lba however, Ser(F7) is replaced by Valine (Val) which cannot hydrogen bond with the proximal histidine. As a result Lba binds ligands (like O₂) with higher affinity. The mutants used in this were chosen so as to modulate the proximal pocket environment in the proteins so as to find out what their effects might be on geminate ligand recombination and equilibrium ligand affinities in Mb and Lba. Binding constant values of the helix swap mutants reveal that for MbLbaF there is a 5-fold increase in the equilibrium constant value for oxygen binding to heme when compared to wild type (wt) Mb.
A 3-fold reduction in oxygen affinity is observed for Lba_{MbF} when compared to that of wt Lba. For wt Mb and its mutants, the kinetic traces almost overlap on all the time scales shown, thus revealing that proximal mutations have almost no effect on geminate recombination of NO. For Lba however, the mutants do show faster decays on the 400 ps and 1 ns time scales. Binding constant studies also reveal that for Lba, the distal and proximal regulatory factors act independently while for Mb distal ligand binding weakens proximal hydrogen pocket hydrogen bonding.

Having looked at the proximal contributions to geminate ligand recombination, chapter VI throws light on the distal pocket effects. The distal histidine (His(E7)) of Lba was replaced by six amino acids of varying size and polarity. Transient absorption measurements on the 40 ps timescale show considerable distal pocket effects which are also evident from the difference in the geminate rebinding parameters obtained from fitting the kinetics. The data on the 400 ps timescale have also been analyzed in terms of rate distributions (based on the MEM method), the results of which were consistent with those obtained from fitting the kinetic traces. The H61V, H61L and H61G mutants show extraordinarily rapid and complete geminate rebinding. In comparing the results to those obtained for the proximal mutations in chapter V, it appears that the distal pocket has a much greater effect on geminate recombination.

Chapter VII includes an exhaustive characterization of the dynamics of five room temperature ionic liquids using coumarin 153 as the probe molecule. The results suggest that the initial phase of the rapid solvation is due to the polarizability of the cationic part of the ionic liquids. The amplitude of the rapidly decaying component correlates well with the viscosity of the RTILs. Pump-probe stimulated emission measurements and fluorescence
upconversion measurements on the same system (e.g. [BMIM][PF6]) have been found to give different results. This discrepancy is probably due an interference of excited state absorption on the pump-probe kinetics, since it is absent in the upconversion studies which only measure spontaneous emission.

In chapter VIII, exhaustive characterization of the complex of the fluorescent probe coumarin 153 (C153) with apomyoglobin has been carried out using two types of binding studies (Job’s method based on fluorescence and capillary electrophoresis), circular dichroism (CD) measurements and time-resolved fluorescence anisotropy measurements. The anisotropy measurements reveal that C153 is rigidly bound in the heme pocket as evident from the single exponential rotational time of ~9 ns which correlates with the protein and coumarin moving together as a whole unit. This is the first step towards the use of C153 to probe the time-dependent dielectric response of the protein and also analyze the contribution of the individual amino acid residues in the heme pocket towards the total protein solvation response.