2-1994

Single-Exponential Fluorescence Decay of the Nonnatural Amino Acid 7-Azatryptophan and the Nonexponential Fluorescence Decay of Tryptophan in Water

Y. Chen
Iowa State University

F. Gai
Iowa State University

Jacob W. Petrich
Iowa State University, jwp@iastate.edu

Single-Exponential Fluorescence Decay of the Nonnatural Amino Acid 7-Azatryptophan and the Nonexponential Fluorescence Decay of Tryptophan in Water

Abstract
The fluorescence decay of an optical probe, the nonnatural amino acid 7-azatryptophan, is measured as a function of pH, in varying mixtures of H20 and D2O and in various nonaqueous solvents. The spectroscopic distinguishability of 7-azatryptophan is demonstrated by the comparison of its fluorescence lifetime in mixtures of N-acetyltryptophanamide (NATA) with that of mixtures of 5-hydroxytryptophan in NATA. The observation of single-exponential fluorescence decay for 7-azatryptophan in water is discussed in terms of nonradiative processes that compete effectively with charge transfer from the excited-state 7-azaindole to the side chain groups and in terms of the dependence of the charge-transfer reaction on the excited-state energy of 7-azaindole. We propose that the absence of nonexponential fluorescence decay (owing to the relative insignificance of charge transfer to the side chain as a nonradiative process in 7-azatryptophan in water) arises from an unfavorable free energy of reaction. This free energy is determined largely by the energy of the fluorescent state, which lies 46 nm (9.8 kcal/mol) below that of tryptophan when the solvent is water.

Disciplines
Chemistry

Single-Exponential Fluorescence Decay of the Nonnatural Amino Acid 7-Azatryptophan and the Nonexponential Fluorescence Decay of Tryptophan in Water

Y. Chen, F. Gai, and J. W. Petrich*

Department of Chemistry, Iowa State University, Ames, Iowa 50011

Received: November 9, 1993®

The fluorescence decay of an optical probe, the nonnatural amino acid 7-azatryptophan, is measured as a function of pH, in varying mixtures of H_2O and D_2O and in various nonaqueous solvents. The spectroscopic distinguishability of 7-azatryptophan is demonstrated by the comparison of its fluorescence lifetime in mixtures of *N*-acetyltryptophanamide (NATA) with that of mixtures of 5-hydroxytryptophan in NATA. The observation of single-exponential fluorescence decay for 7-azatryptophan in water is discussed in terms of nonradiative processes that compete effectively with charge transfer from the excited-state 7-azaindole to the side chain groups and in terms of the dependence of the charge-transfer reaction on the excited-state energy of 7-azaindole. We propose that the absence of nonexponential fluorescence decay (owing to the relative insignificance of charge transfer to the side chain as a nonradiative process in 7-azatryptophan in water) arises from an unfavorable free energy of reaction. This free energy is determined largely by the energy of the fluorescent state, which lies 46 nm (9.8 kcal/mol) below that of tryptophan when the solvent is water.

Introduction

In a series of articles,[1-8] we have suggested that the nonnatural amino acid 7-azatryptophan is in many instances preferable to tryptophan as a probe of protein structure and dynamics. These arguments have been based on the amenability of 7-azatryptophan to incorporation in synthetic peptides and bacterial protein and the retention of activity of peptides and proteins containing it. The underlying basis for the preference of 7-azatryptophan to tryptophan lies, however, in the distinguishability of its absorption and fluorescence spectra and in the single-exponential fluorescence decay of 7-azatryptophan over most of the pH scale.

Tryptophan has been the most widely used intrinsic optical probe of protein structure and dynamics. There are, however, two major problems attendant to the use of tryptophan, especially in fluorescence measurements. First, since tryptophan is a naturally-occurring amino acid, there are often several tryptophans in a protein molecule whose emission must be distinguished. Second, the fluorescence decay of tryptophan itself in aqueous solution is nonexponential.[9-13]

The single-exponential 3-ns fluorescence decay of *N*-acetyltryptophanamide (NATA) in water at 20 °C provides a well-known exception to nonexponential fluorescence decay in small tryptophan-containing molecules.[10] It is obvious, however, that NATA cannot serve as a model for tryptophan in proteins, as is sometimes proposed, since tryptophyl peptides such as X-Trp-Y and larger polypeptides containing one tryptophan (glucagon, mellitin, ACTH, and ACTH fragments) exhibit qualitatively similar nonexponential fluorescence decay kinetics: approximately commensurate proportions of a ≤1-ns component and a ~3-ns component.[9-17]

We have consequently investigated alternatives to tryptophan. Our work has led us to the amino acid analog 7-azatryptophan (Figure 1). The greatest value of 7-azatryptophan is to probe the interactions on a smaller peptide or protein containing it with another protein that may contain several tryptophans.[3] Since we have demonstrated that 7-azatryptophan is amenable to peptide synthesis and that it can be incorporated into bacterial protein,[1,3] we can synthesize a model peptide with 7-azatryptophan appropriately located in the sequence or prepare by site-directed mutagenesis small proteins containing 7-azatryptophan. These intrinsically tagged molecules can then be studied individually or in complex with their target. The utility of 7-azatryptophan will greatly be extended by the powerful techniques based on suppressor tRNA that are now emerging.[18]

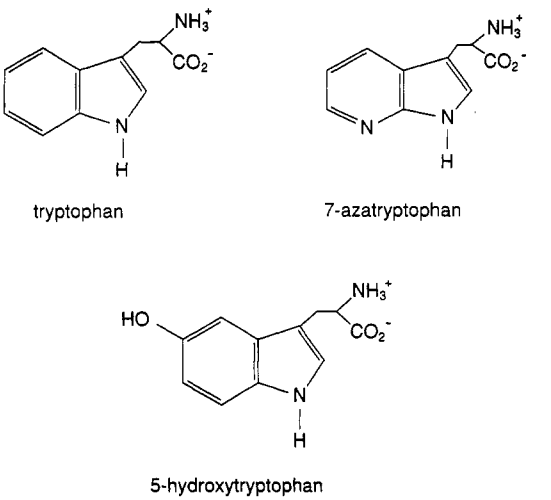

Figure 1. Structures of tryptophan (Trp), 7-azatryptophan (7AT), and 5-hydroxytryptophan (5HT).

It is conceivable that in some cases 7-azatryptophan may have a nonexponential fluorescence decay when it is incorporated into the protein matrix. It is important to appreciate, however, that in using 7-azatryptophan as the optical probe our explanation of its fluorescence decay in the system of interest is not hobbled from the outset by the complicated photophysics of the probe itself or when the probe is incorporated in a tripeptide such as NAc-Pro-7-azatrp-Asn-NH_2.[3] In other words, if a situation should arise in which 7-azatryptophan does exhibit a nonexponential fluorescence decay in a protein, we shall know with certainty that this decay is due to the protein matrix, and we shall not be obliged to take into account the contributions from the adjacent peptide bonds as we are with tryptophan.

Experimental Section

Time-correlated single-photon counting measurements were performed with the apparatus described elsewhere.[5,6] The dryness of the polar aprotic solvents was determined by the absence of

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts*, February 1, 1994.

0022-3654/94/2098-2203$04.50/0

TABLE 1: Proton Inventory for 7-Azatryptophan at 20 °C[a]

n^b	τ_F (ps)	n^b	τ_F (ps)	n^b	τ_F (ps)
0.0	780 ± 10	0.4	1235 ± 15	0.8	2103 ± 10
0.1	882 ± 10	0.5	1407 ± 17	0.9	2385 ± 15
0.2	977 ± 8	0.6	1596 ± 8	1.0	2780 ± 20
0.3	1084 ± 10	0.7	1808 ± 15		

[a] The isotope effect on the fluorescence lifetime is only slightly dependent on the state of protonation of 7-azatryptophan: $\tau_F(pD=7)/\tau_F(pH=7)$ = 2780 ps/780 ps = 3.56; $\tau_F(pD=11)/\tau_F(pH=11)$ = 1970 ps/570 ps = 3.46. [b] Mole fraction D_2O.

TABLE 2: Rate Constants for Proton-Transfer Steps in 7-Azatryptophan[a]

proton-transfer step	rate constant ($s^{-1} \times 10^{-9}$)
k^{HH}	1.28 ± 0.02
k^{DD}	0.36 ± 0.01
$(k^{HH}k^{DD})^{1/2}$	0.68 ± 0.01
k^{HD} [b]	0.60 ± 0.01

[a] Fluorescence lifetime measurements from which the rate constants were obtained were performed at 20 °C. [b] Obtained from eq 22 in ref 7. This method of analysis assumes that $k^{HD} = k^{DH}$. The reported results were determined by assuming that isotopic fractionation factor $\phi^R = 1$ in both the ground and the excited states. If $\phi^R = 1.6$, then k^{HD} = (0.72 ± 0.01) × 10^9 s^{-1}. The methods of analysis of the proton inventory data are discussed in detail elsewhere.[7]

a tautomer band in the fluorescence spectrum of 7-azaindole and 7-azatryptophan. The similarity of the absorption spectra of 7-azaindole and 7-azatryptophan in these solvents indicates that aggregation of 7-azatryptophan does not occur. Other experimental details are provided in refs 7 and 8. The nonexponential fluorescence decay of tryptophan is considered here in terms of a finite number of conformational isomers,[10,12,19-22] but distributions of isomers have been discussed as well.[23]

Results

Spectroscopic Distinguishability of 7-Azatryptophan. An important feature of 7-azatryptophan that renders it preferable to tryptophan as an optical probe is its distinguishability in absorption and fluorescence spectra and its single-exponential fluorescence lifetime.[1,3,5] Recently, there have been reports suggesting that 5-hydroxytryptophan is a useful biological probe as well.[24] While in some cases 5-hydroxytryptophan may prove useful (if relatively long excitation wavelengths, ~320 nm, are employed), Figure 2 demonstrates that because its fluorescence spectrum and lifetime are similar to those of tryptophyl chromophores, it is much more difficult to distinguish from tryptophan than is 7-azatryptophan. This is due in large part to the 3.8-ns lifetime of 5-hydroxytryptophan, which is similar to that of the long component of tryptophan. Figure 2 indicates that in mixtures of 5-hydroxytryptophan and the tryptophyl chromophore, NATA, in a ratio as high as 1/10, the presence of 5-hydroxytryptophan cannot be discriminated from the mixture. This is demonstrated by the identical χ^2 values obtained from fits to single-exponential and double-exponential functions. On the other hand, even when the ratio is as low as 1/40 *in 7-azatryptophan*, the 7-azatryptophan can be detected in the mixture.

pH Dependence of the Fluorescence Lifetime of 7-Azatryptophan. There are several states of ionization available for 7-azatryptophan in water. Like tryptophan, the cationic, zwitterionic, and anionic forms of the side chain may be present. We must, however, also take into account N_7, which we know to be titratable based on previous work.[2,5] For 7-azaindole, the pK_a of N_7 is 4.5. Potentiometric titrations of 7-azatryptophan similar to those for 7-azaindole[2] indicate that its ground-state pK_a values are 2.70, 3.85, and 9.35. The pK_a values correspond respectively to the following groups: CO_2H, N_7, and NH_2. Below pH 2, both the amino group and N_7 (as well as the acid group) are protonated, yielding a dication. When 2 < pH < 4, three species can coexist in equilibrium: the dication mentioned above, the zwitterion protonated at N_7, and the zwitterion deprotonated at N_7 (the conventional zwitterion). From pH 4 to 9, the predominant from is the zwitterion. From pH 10 to 13, the anion predominates.

The dependence of the average fluorescence lifetime of 7-azatryptophan on pH is illustrated in Figure 3. For pH < 4, double-exponential fluorescence decay is observed. Above pH 4, the fluorescence lifetime is single exponential. Between pH 4 and 10, the lifetime is constant at ~780 ps. At pH > 10, the lifetime remains single exponential, but it decreases.

A problem of considerable interest for understanding the excited-state tautomerization reaction of 7-azaindole in alcohols[5] and the nonradiative process for 7-azaindole in water[7] and for interpreting proton inventory data[7] is the determination of the excited-state pK_a values. For pH values between 2 and 4, the species NH_3^+–N_7^+H–CO_2H, NH_3^+–N_7^+H–CO_2^-, and NH_3^+–N_7–CO_2^- are in equilibrium. Although we know with certainty the fluorescence lifetime of the last form, the deprotonated zwitterion, we do not know the lifetimes of the first two species, and thus any calculation of the excited-state pK_a of the first two species becomes intractable. For pH > 9 there is a ground-state equilibrium between the deprotonated zwitterion and the anion, NH_3^+–N_7–CO_2^- and NH_2–N_7–CO_2^-. In the ground state it is known for glycine[25] that the rate for the back-reaction (the protonation of the amino group by water) is only 48 × 10^5 s^{-1}. Even if in the excited state the rate for the back-transfer is several orders of magnitude larger, equilibrium between the conjugate acid and base will not be established owing to the relatively short-lived excited states involved (≤1 ns), and the excited-state pK_a cannot be determined.

Fluorescence Lifetime of 7-Azatryptophan in Mixtures of H_2O and D_2O; The Proton Inventory. Elsewhere[7] we examined the nonradiative processes of 7-azaindole in ROH/ROD solvent mixtures, where R = CH_3, CH_3CH_2, or H. These proton inventory experiments[26] are designed to determine the number of protons involved in the nonradiative process and whether this process is stepwise or concerted. The proton inventory data for 7-azatryptophan are remarkably similar to those for 7-azaindole (Figure 4 and Tables 1 and 2). These data for 7-azatryptophan argue against any significant involvement of the side chain in the proton-transfer step in this pH region and suggest that the nonradiative process involving the N_1 ligand in H_2O/D_2O mixtures is fundamentally different from that in alcohols.

We note that the interpretation of the proton inventory data is subject to several assumptions. Among the most important of these involves the rate of H or D exchange to the solute with the solvent and the value of the fractionation factor. We have discussed this in detail elsewhere.[7]

Dependence of the Fluorescence Lifetime of 7-Azatryptophan on Solvent. As is borne out from the comparison of the mixture of 5-hydroxytryptophan and 7-azatryptophan in Figure 2, the distinguishability of 7-azatryptophan with respect to 5-hydroxytryptophan or NATA (or tryptophan) is obtained not only from its shifts in absorbance and emission spectra but also from a distinctly different fluorescence lifetime: ~0.8 ns as opposed to ≥3 ns. It is important to understand why 7-azatryptophan is characterized by a 780-ps lifetime in water at 20 °C at neutral pH and, most importantly, why its lifetime is single-exponential. To this end, we have performed fluorescence lifetime measurements of 7-azatryptophan in a variety of solvents. The results are summarized in Table 3. The significant result, which shall be discussed in detail below, is that in solvents that shift the fluorescence maximum of 7-azatryptophan to higher energies nonexponential fluorescence decay is observed.

Discussion

Comparison of 7-Azatryptophan and Tryptophan. It has been proposed that the nonexponential fluorescence decay of tryptophan arises from different conformational isomers of the side chains with respect to the indole ring that have different excited-state

TABLE 3: Comparison of Fluorescence Lifetimes[a]

compound	solvent	λ_{max}^{em}/(nm)	A_1[b]	τ_1 (ps)	τ_2 (ps) [ref]
7-azaindole	H_2O	385	1.00	900 ± 15	
7-azatryptophan[c]	H_2O	397	1.00	780 ± 10	
indole	H_2O	341	1.00	4550 ± 20	
tryptophan[c]	H_2O	351	0.22 ± 0.01	670 ± 50	3200 ± 100 [10]
tryptophan ethyl ester, pH 5[d]	H_2O	345	0.59 ± 0.06	260 ± 20	870 ± 50 [10]
tryptophan ethyl ester, pH 9[d]	H_2O	354	0.27 ± 0.05	930 ± 30	2400 ± 200 [10]
5-hydroxytryptophan	H_2O	335	1.00	3800 ± 100	
7-azaindole	MeOH	364	1.00	140 ± 3[e]	
7-azatryptophan	MeOH	382	1.00	140 ± 3[e]	
indole	MeOH	330	1.00	3400 ± 100	
tryptophan	MeOH	340	0.05	200 ± 100	1900 ± 50[f] [33]
7-azaindole	DMSO	361	1.00	9300 ± 200	
7-azatryptophan	DMSO	385	0.10 ± 0.02	1080 ● 50	19200 ± 60
indole	DMSO	330	1.00	4800 ± 200	
tryptophan	DMSO	344	0.12 ● 0.02	1200 ± 200	7200 ± 400
			0.06	2600 ± 400	8000 ± 60 [33]
tryptophan ethyl ester	DMSO	339	0.48 ± 0.02	480 ± 30	1820 ± 80
7-azaindole	CH_3CN	353	1.00	5700 ± 200	
7-azatryptophan	CH_3CN	374	0.43 ± 0.02	1050 ± 100	11000 ± 400

[a] Experiments are performed at 20 °C. [b] Lifetimes are fit to a double exponential of the form $F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where $A_1 + A_2 = 1.00$. [c] Zwitterionic form. [d] $pK_a^1 \sim 7$. Hydrochloride salt. [e] Emission is collected from the "normal" band: 320 nm < λ^{em} < 480 nm. [f] The fluorescence decay for tryptophan in methanol is fit well to a triple exponential. The third component comprises 10% of the emission intensity and has a lifetime of 7 ns. This component is attributed to the anionic form of tryptophan.[33]

charge-transfer rates.[10] It seems likely that 7-azatryptophan would exist in similar conformational isomers. Why, then, is the fluorescence lifetime of 7-azatryptophan in water single exponential (when emission is collected over most of the band[4,5]), and what is the role of the side chains in the photophysics of 7-azatryptophan? There are two possible answers to these questions: In 7-azatryptophan, the charge-transfer process to the side chain is deactivated, or there is a more efficient process that competes with charge transfer in water. We shall consider both possibilities.

Elsewhere we have shown that in water 7-azatryptophan exhibits behavior that is very similar to that of 7-azaindole.[4,5] The majority of solute molecules are solvated in such a fashion that excited-state tautomerization is blocked.[5,27] The fluorescence lifetimes of these blocked states are 910 ps for 7-azaindole and 780 ps for 7-azatryptophan at 20 °C. It is possible that the nonradiative process responsible for the 780-ps lifetime in 7-azatryptophan has a rate greater than, or at least comparable to, the rates of charge transfer from the 7-azaindole ring to side chain groups.

Evidence for the existence of such a process is provided by a proton inventory[7,26] of the excited-state pathways of decay of 7-azatryptophan. The results of these experiments indicate[7] that the majority of the excited-state population of 7-azaindole in water does not decay by a two-proton process similar to that observed in alcohols, as has been proposed. Another mechanism for the nonradiative decay of 7-azaindole is suggested: proton abstraction from the N_1 nitrogen by a coordinated water molecule. The proton inventory data are nearly identical for 7-azatryptophan (Figure 4, Tables 1 and 2), and we propose that here proton abstraction from N_1 is also an efficient nonradiative decay pathway that is responsible for the 780-ps lifetime.[7,28]

While the proton inventory experiments suggest another significant nonradiative process, they do not address the question of whether charge transfer to the side chain is still a viable mode of nonradiative decay. In order to answer this question, experiments were performed in nonaqueous solvents (Table 3). Alcohols that are polar and protic are represented in Table 3 by methanol. Methanol is capable of forming the idealized cyclic intermediate that is proposed to be crucial to the execution of excited-state tautomerization of 7-azaindole. In methanol this process occurs in 140 ps. In most linear alcohols it occurs on an ~200-ps time scale.[2,29] We have already argued that the population of such a cyclic intermediate is negligible in water. It is also unlikely that a stable cyclic intermediate could be formed with polar aprotic solvents like DMSO or CH_3CN. In the polar aprotic solvents 7-azatryptophan, on the other hand, now displays a nonexponential fluorescence decay as does tryptophan.

Because of the evidence for nonradiative decay by charge transfer in tryptophan in both aqueous[10] and nonaqueous solvents and because nonexponential fluorescence decay is induced in 7-azatryptophan in DMSO and CH_3CN, *we propose that charge transfer to the side chain is an effective nonradiative pathway for 7-azatryptophan in these solvents.* That the side chain is responsible for the observed nonexponential decay is demonstrated by the single-exponential fluorescence decay of 7-azaindole in the same solvents (Table 3). If charge transfer to the side chain is a significant pathway of nonradiative decay in these polar aprotic solvents, it is important to understand why. The answer to this question may be found by noting that nonexponential fluorescence decay in 7-azatryptophan is accompanied by a shift in the fluorescence maximum to higher energies (Table 3). On the other hand, 7-azatryptophan in methanol yields the same result as 7-azaindole: a single-exponential lifetime of 140 ps. Apparently, double-proton transfer competes effectively with charge transfer to the side chain in this solvent.

If the blue shift in the emission maximum of 7-azatryptophan observed upon changing solvent from H_2O to DMSO or CH_3CN may be attributed to destabilization of the excited state (which seems likely considering the sensitivity of the indole excited state to solvent perturbation[6,8]), then one may apply an argument based on charge-transfer theory[30] to explain the nonexponential decay of 7-azatryptophan in polar aprotic solvents. The free energy change for the charge-transfer process is related to the excited-state energy of the donor, $E(S_1)$, by the following relations:[31,32]

$$-\Delta G^\circ = E(S_1) - E(D/D^+) + E(A/A^-) + e^2/\epsilon R \quad (1)$$

where $E(D/D^+)$ is the oxidation potential of the donor, $E(A/A^-)$ is the reduction potential of the acceptor, and the last term is a correction for the Coulombic energy of interaction of the charge-separated pair. The Coulombic term is often neglected in order to take into account charge delocalization. If, then, in changing the solvent from water to DMSO or CH_3CN the oxidation and reduction potentials undergo little change, the largest contribution to the free energy for charge transfer must be attributed to the excited-state energy of the donor species. This change in solvent renders the charge-transfer reactions more exothermic (see below and Figure 6).

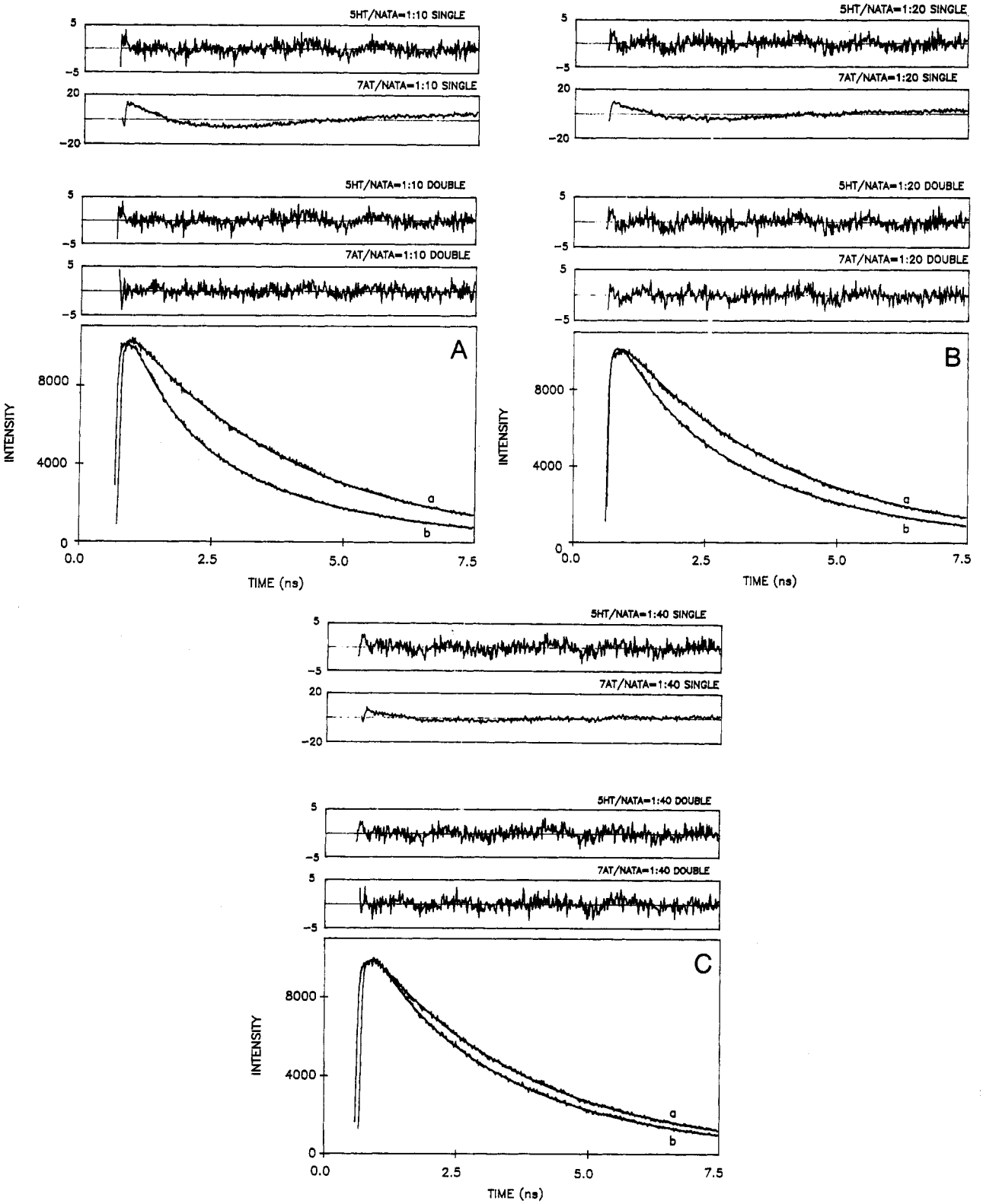

Figure 2. Spectroscopic distinguishability of 7-azatryptophan and 5-hydroxytryptophan with respect to tryptophyl absorption and emission at 20 °C and neutral pH. In all cases, λ_{ex} = 305 nm and $\lambda_{cm} \geq$ 335 nm. In water, the fluorescence maximum of 7-azatryptophan is 395 nm, and that of 5-hydroxytryptophan is 340 nm. For each panel, (a) refers to 5HT/NATA mixtures and (b) to 7AT/NATA mixtures. Panels A, B, and C present the results of fitting the fluorescence decays of mixtures of 7-azatryptophan (7AT) or 5-hydroxytryptophan (5HT) and NATA. (A) [7AT]/[NATA] = [5HT]/[NATA] = 1/10. 5HT/NATA: single-exponential fit, τ = 3240 ps, χ^2 = 1.32; double-exponential fit, $F(t)$ = 0.35 exp($-t$/3220 ps) + 0.65 exp($-t$/3250 ps), χ^2 = 1.32. 7AT/NATA: single-exponential fit, χ^2 = 2390 ps, χ^2 = 20.0; double-exponential fit, $F(t)$ = 0.42 exp($-t$/822 ps) + 0.58 exp($-t$/3000 ps), χ^2 = 1.16. (B) [7AT]/[NATA] = [5HT]/[NATA] = 1/20. 5HT/NATA: single-exponential fit, τ = 3200 ps, χ^2 = 1.29; double-exponential fit, $F(t)$ = 0.33 exp($-t$/3220 ps) + 0.67 exp($-t$/3190 ps), χ^2 = 1.30. 7AT/NATA: single-exponential fit, τ = 2660 ps, χ^2 = 9.3; double-exponential fit, $F(t)$ = 0.24 exp($-t$/736 ps) + 0.76 exp($-t$/2950 ps), χ^2 = 1.19. (C) [7AT]/[NATA] = [5HT]/[NATA] = 1/40. 5HT/NATA: single-exponential fit, τ = 3120 ps, χ^2 = 1.25; double-exponential fit, $F(t)$ = 0.48 exp($-t$/3500 ps) + 0.52 exp($-t$/2773 ps), χ^2 = 1.24. 7AT/NATA: single-exponential fit, τ = 2760 ps, χ^2 = 4.1; double-exponential fit, $F(t)$ = 0.16 exp($-t$/732 ps) + 0.84 exp($-t$/2950 ps), χ^2 = 1.24.

To summarize, if charge transfer to the side chain is possible for 7-azatryptophan in water, it is not observed because of the presence of another nonradiative process with a greater rate. We propose that this process is abstraction of the N_1 proton by a

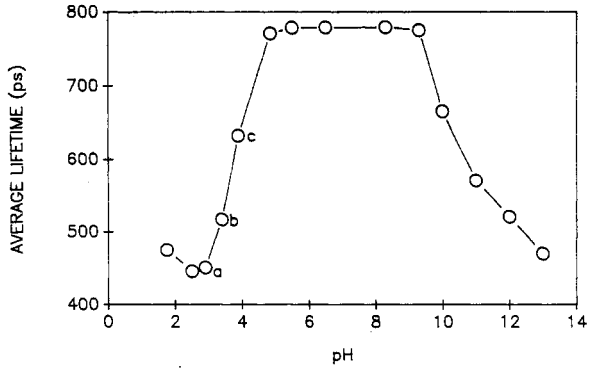

Figure 3. Fluorescence lifetime titration of 7-azatryptophan at 20 °C. The lettered points indicate pH values where the fluorescence lifetime is nonexponential. (a) pH 2.9; $F(t) = 0.95\exp(-t/430\text{ ps}) + 0.05\exp(-t/848\text{ ps})$. (b) pH 3.4, $F(t) = 0.73\exp(-t/421\text{ ps}) + 0.27\exp(-t/778\text{ ps})$. (c) pH 3.9; $F(t) = 0.30\exp(-t/360\text{ ps}) + 0.70\exp(-t/750\text{ ps})$. At all the other pH values investigated the fluorescence lifetime was single-exponential.

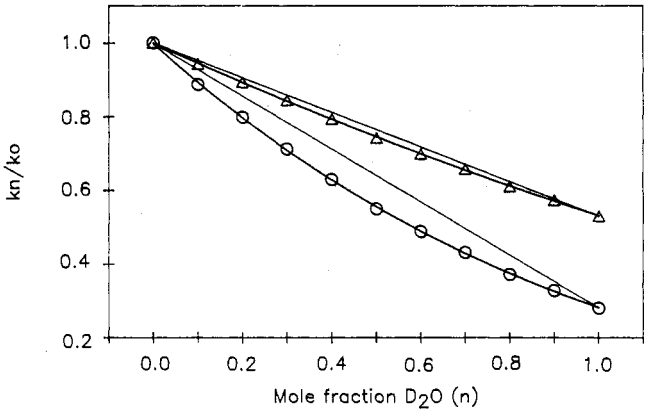

Figure 4. Proton inventory data for 7-azatryptophan in H_2O and D_2O at 20 °C. The open circles represent k_n/k_0 vs n, where n is the mole fraction (atom fraction) of the deuterated solvent. The pH at $n = 0$ is 6. The solid line through the open circles represents the fit to the expression $k_n/k_0 = (1 - n + 0.52n)(1 - n + 0.69n)^2$. The straight line plotted directly above is the result expected for a one-proton process. The open triangles represent $(k_n/k_0)^{1/2}$ vs n. The solid line through the open triangles is only meant to guide the eye. This plot deviates significantly from the straight line just above it. Hence, the proton inventory data in water for 7-azatryptophan are, like those for 7-azaindole,[7] inconsistent with the two-proton process observed in alcohols. See ref 7 for a more detailed discussion of the analysis and the significance of these data.

coordinated water molecule. In polar, aprotic solvents, however, proton abstraction from N_1 is no longer feasible, and nonexponential fluorescence decay can be observed because the donor, the excited-state 7-azaindole moiety, lies higher in energy than it does in water.

The Rate of Charge Transfer to the Side Chain: Comparison of Tryptophan and 7-Azatryptophan. The expression for the rate of nonadiabatic charge (electron) transfer is[30]

$$k = 2\pi/\hbar|H_{DA}|^2\text{FC} \qquad (2)$$

H_{DA} is the matrix element coupling the donor, D, and the acceptor, A, and FC is the nuclear Franck–Condon factor, which may be estimated quantum mechanically, semiclassically, or classically. In the classical limit the Franck–Condon factor is given by

$$\text{FC} = [1/(4\pi\lambda 4RT)]^{1/2}\exp\{-[(\Delta G^\circ + \lambda)^2/(4\lambda RT)]\} \qquad (3)$$

where ΔG° is the standard free energy change for the reaction and λ is the reorganization energy, which contains both solvent and vibrational contributions. From these equations an expression may be obtained that relates the rate of charge transfer to the

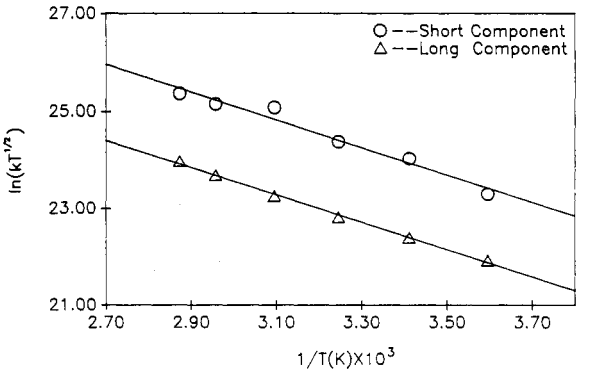

Figure 5. Application of eq 4 to the nonradiative rates obtained from the two lifetime components[10,11] of zwitterionic tryptophan as a function of temperature. Both lifetime components yield the same activation energy of 5.63 kcal/mol. The prefactor, A, for the short-lived component is $3.96 \times 10^{14}\ s^{-1}\ K^{-1/2}$ and for the long-lived component, $8.40 \times 10^{13}\ s^{-1}\ K^{-1/2}$.

absolute temperature:

$$\ln(kT^{1/2}) = \ln A - (\Delta G^\ddagger/RT) \qquad (4)$$

where $\Delta G^\ddagger = (\Delta G^\circ + \lambda)^2/4\lambda$ is the free energy of activation and $A = |H_{DA}|^2/(2\hbar\lambda R)$. The nonradiative rates obtained from the two lifetime components of zwitterionic tryptophan[10,11] are plotted according to eq 4. Figure 5 indicates that the slopes are parallel and thus yield the same activation energies. For zwitterionic tryptophan, therefore, $\Delta G^\ddagger$ is the same for each of the conformers present in solution. Consequently, it is likely that the charge-transfer process for each conformer is characterized by the same ΔG° and λ.

Now let us consider the tryptophan conformer whose charge-transfer rate in water is measured to be $(600\text{ ps})^{-1}$. We assume that the analogous conformer in 7-azatryptophan has the same values of λ and H_{DA}. *If this is the case, then as we suggested earlier, the 46 nm (9.8 kcal/mol) difference in the emission maxima of tryptophan and 7-azatryptophan may be attributed to* ΔG°. The drastic reduction in charge-transfer rate in 7-azatryptophan with respect to tryptophan may be interpreted in terms of a much reduced reaction exothermicity. In other words, the activated nature of the charge-transfer process from tryptophan demonstrated in Figure 5 puts the reaction in the "normal Marcus region". Because of its decreased singlet energy with respect to that of tryptophan, 7-azatryptophan will lie even further into the normal region and hence have a smaller charge-transfer rate (Figure 6).

The Tryptophan and 7-Azatryptophan Population in Nonaqueous Solvents. The interpretation of the fluorescence lifetime data for 7-azatryptophan proposed above depends not only on the intrinsic electrophilicity of the side chain to be maintained in the solvents being compared but also on the homogeneity of the sample. If there are specific changes in protonation in going from water to DMSO or CH_3CN, then the conclusions drawn above may be called into question. For example, at neutral pH tryptophan and 7-azatryptophan exist as zwitterions. We must ask whether in DMSO or CH_3CN they are also zwitterionic or what the relative populations of uncharged, cationic, anionic, and zwitterionic species are.

Ware and co-workers[33] have argued, based on trends of dissociation constants as a function of dielectric constant, that in DMSO tryptophan exists exclusively in the zwitterionic form. Greenstein and Winitz[34] have tabulated data for a water–ethanol mixture that is 65% ethanol (dielectric constant of 40). With respect to pure water, the pK_a of the acid groups investigated is never raised by more than about 1.5 units, and the pK_a of the basic groups investigated is raised or lowered on the average by about half a unit. We note that these studies were not performed on tryptophan, presumably because of its limited solubility. For

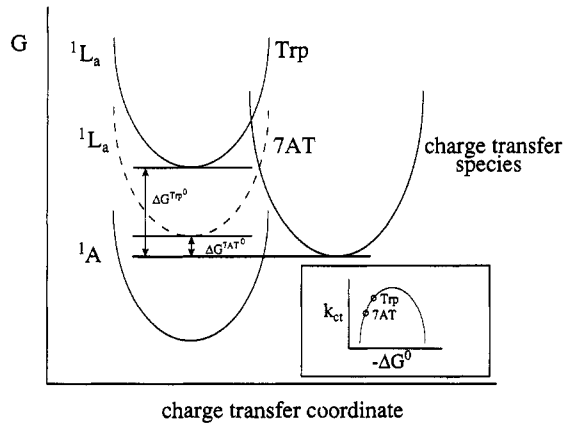

Figure 6. Schematic energy diagrams for tryptophan and 7-azatryptophan in water illustrating the role of reaction exothermicity on the rate of charge transfer. In problems dealing with charge (electron) transfer, it is customary to equate the energy, E, with the free energy, G, because the entropy and the product of pressure and volume are expected to remain constant between the reactants and the products. In the main part of the figure, the solid ^1L_a surface represents tryptophan. The dashed ^1L_a surface represents 7-azatryptophan and is consequently lower in energy by an amount corresponding to its excited-state singlet energy difference with respect to tryptophan. The two ^1L_a states intersect the charge-transfer species. The reaction coordinate is referred to as a "charge-transfer coordinate" and is assumed to incorporate the presence of both the amino acid side chain and the solvent. For further details concerning these surfaces, see ref 8. The inset depicts dependence of the charge-transfer rate on reaction exothermicity, $-\Delta G°$, the difference between the minima of the ^1L_a surfaces and that of the charge-transfer state. The solid inverted parabola represents the behavior of the rate predicted from eqs 2 and 3. The "normal Marcus region", corresponding to activated processes, lies to the left of the maximum. The lower singlet energy of 7-azatryptophan places it below tryptophan on this curve. In other words, if the 7-azatryptophan potential surface is similar to that of tryptophan, decreasing $-\Delta G°$ will also raise the barrier to charge transfer.

purposes of comparison, the dielectric constants of DMSO and CH_3CN are 36.7 and 36.0, respectively.[35]

Wada et al.[36] measured the equilibrium constant of zwitterionic and uncharged forms a glycine in mixtures of water containing varying proportions of a nonaqueous component: $K_D = [NH_3^+\text{-}CH_2CO_2^-]/[NH_2CH_2CO_2H]$. They found that while in pure water $K_D = 2.36 \times 10^5$, in solutions where the mole fraction of DMSO and CH_3CN were 0.100 and 0.300, respectively, the values of K_D decreased to 5.76×10^4 and 2.28×10^4. The extrapolated data of Wada et al. for *methanol* suggest that for pure solution in the nonaqueous component an upper limit for K_D is likely to be 10^2.

For tryptophan in methanol, Ware and co-workers observe a triple-exponential fluorescence decay, the third component of which has an amplitude of 10% and a lifetime of 7 ns. They attribute this third component to an anionic species. Neither they nor we, however, observe a third component in DMSO (Table 3). Furthermore, we do not observe a third component in CH_3CN.

Previously, we had argued that a criterion for nonexponential fluorescence decay in tryptophan was a protonated amino group, which functions to activate the carboxylate and render it a good electron acceptor.[9,10] This conclusion was drawn from noting that anionic tryptophan yields a single-exponential fluorescence decay. In tryptophan, the carboxylate group is essential for nonexponential decay since both protonated and deprotonated tryptamine yield single-exponential fluorescence decays. In order to exhibit nonexponential decay in DMSO and CH_3CN, both protonation of the amino group and deprotonation of the acid group are thus essential. We conclude that the tryptophan and 7-azatryptophan populations in DMSO and CH_3CN are largely zwitterionic.

It is interesting to note that the fluorescence lifetime of tryptophan ethyl ester in DMSO is double exponential with lifetimes and amplitudes similar to that in water. Both protonated and deprotonated tryptophan ethyl ester (pH 5 and 9) exhibit nonexponential fluorescence decay because the ester group is a sufficiently good electrophile in each case.[10] This observation, *which does not depend upon the state of ionization of the solute*, is consistent with the conclusion drawn earlier that the most important factor in inducing nonexponential fluorescence decay in 7-azatryptophan upon changing solvent from water to DMSO or CH_3CN is the change in free energy.

Conclusions

We have proposed 7-azatryptophan as a powerful alternative to tryptophan as an optical probe of protein structure and dynamics. We have investigated the nonradiative processes of 7-azatryptophan and explored why its fluorescence decay in water is so different from that of tryptophan. Despite the similarities between the 7-azaindole and the indole chromophores such as the presence of two closely spaced excited states[6,37] and the importance of monophotonic ionization as a pathway of nonradiative decay,[8] this difference is most strongly manifested in the single-exponential fluorescence decay of 7-azatryptophan and its spectroscopic distinguishability with respect to tryptophan[1-5] and the other nonnatural amino acid probe that has been proposed, 5-hydroxytryptophan (Figure 2). We have suggested that the key to understanding this difference lies in the photolability of the proton attached to N_1.[7] In 7-azaindole, the reactivity of this proton has been demonstrated by the excited-state tautomerization of its dimers[38] and of its complexes with alcohols.[2,29] Although in water this excited-state double-proton transfer is prohibited owing to the peculiarities of the solute–solvent interactions, this does not imply that the N_1 proton is not reactive. Our proton inventory work on 7-azaindole[7] and on 7-azatryptophan indicates that although this proton is involved in a fundamentally different type of excited-state process in water than in alcohols, it is still a participant in the most important nonradiative process—after the monophotonic ionization event that occurs immediately upon photon absorption.[4,8]

The rate of this nonradiative pathway, which we have postulated to be the abstraction of the N_1 proton by water, is at least comparable to, and most likely greater than, the rate of charge transfer to the side chain in 7-azatryptophan. We propose that the relative insignificance of charge transfer in 7-azatryptophan in water (and hence the absence of nonexponential fluorescence decay) in terms of an unfavorable free energy of reaction that results from the low energy of the excited state (Figure 6). This explanation is confirmed by the appearance of nonexponential fluorescence decay of 7-azatryptophan in DMSO and CH_3CN, where the excited-state energy is raised from that in water by 10 and 19 nm, respectively.

Acknowledgment. J.W.P. is an Office of Naval Research Young Investigator. F.G. is the recipient of a fellowship from Phillips Petroleum. Additional support was provided by the Iowa State University Biotechnology Council and IPRT. We thank R. L. Rich for providing a critical reading of the manuscript and for helpful comments.

References and Notes

(1) Négrerie, M.; Bellefeuille, S. M.; Whitham, S.; Petrich, J. W.; Thornburg, R. W. *J. Am. Chem. Soc.* **1990**, *112*, 7419.
(2) Négrerie, M.; Gai, F.; Bellefeuille, S. M.; Petrich, J. W. *J. Phys. Chem.* **1991**, *95*, 8663.
(3) Rich, R. L.; Négrerie, M.; Li, J.; Elliott, S.; Thornburg, R. W.; Petrich, J. W. *Photochem. Photobiol.* **1993**, *58*, 28.
(4) Gai, F.; Chen, Y.; Petrich, J. W. *J. Am. Chem. Soc.* **1992**, *114*, 8343.
(5) Chen, Y.; Rich, R. L.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1993**, *97*, 1770.
(6) Rich, R. L.; Chen, Y.; Neven, D.; Négrerie, M.; Gai, F.; Petrich, J. W. *J. Phys. Chem.* **1993**, *97*, 1781.
(7) Chen, Y.; Gai, F.; Petrich, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 10158.
(8) Gai, F.; Rich, R. L.; Petrich, J. W. *J. Am. Chem. Soc.*, in press.

(9) Chang, M. C.; Petrich, J. W.; McDonald, D. B.; Fleming, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 3819.
(10) Petrich, J. W.; Chang, M. C.; McDonald, D. B.; Fleming, G. R. *J. Am. Chem. Soc.* **1983**, *105*, 3824.
(11) Petrich, J. W. Thesis, The University of Chicago, 1985.
(12) Szabo, A. G.; Rayner, D. M. *J. Am. Chem. Soc.* **1980**, *102*, 554.
(13) Szabo, A. G.; Rayner, D. M. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 909.
(14) Werner, T. C.; Forster, L. S. *Photochem. Photobiol.* **1979**, *29*, 905.
(15) Ross, J. B. A.; Rousslang, K. W.; Brand, L. *Biochemistry* **1981**, *20*, 4361.
(16) Chen, L. X.-Q.; Petrich, J. W.; Fleming, G. R.; Perico, A. *Chem. Phys. Lett.* **1987a**, *139*, 55.
(17) Cockle, S. A.; Szabo, A. G. *Photochem. Photobiol.* **1981**, *34*, 23.
(18) Bain, J. D.; Glabe, C. G.; Dix, T. A.; Chamberlin, A. R. *J. Am. Chem. Soc.* **1989**, *111*, 8013. Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301.
(19) Donzel, B.; Gauduchon, P.; Wahl, Ph. *J. Am. Chem. Soc.* **1974**, *96*, 801.
(20) Engh, R. A.; Chen, L. X.-Q.; Fleming, G. R. *Chem. Phys. Lett.* **1986**, *126*, 365.
(21) Tilstra, L.; Sattler, M. C.; Cherry, W. R.; Barkley, M. D. *J. Am. Chem. Soc.* **1990**, *112*, 9176.
(22) Creed, D. *Photochem. Photobiol.* **1984**, *39*, 537.
(23) Lakowicz, J. R.; Cherek, H.; Grycaynski, I.; Joshi, N.; Johnson, M. L. *Biophys. Chem.* **1987**, *28*, 35. James, D. R.; Ware, W. R. *Chem. Phys. Lett.* **1985**, *120*, 445. Siemiaruk, A.; Ware, W. R. *Chem. Phys. Lett.* **1987**, *140*, 277. Tanaka, F.; Mataga, N. *Biophys. J.* **1987**, *51*, 487. Livesey, A. K.; Brochon, J.-C. *Biophys. J.* **1987**, *52*, 693.
(24) Senear, D. F.; Laue, T. M.; Ross, J. B. A.; Waxman, E.; Eaton, S.; Rusinova, E. *Biochemistry* **1993**, *32*, 6179. Ross, J. B. A.; Senear, D. F.; Waxman, E.; Kombo, B. B.; Rusinova, E.; Huang, Y. T.; Laws, W. R.; Hasselbacher, C. A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 12023. Hogue, C. W. V.; Rasquinha, I.; Szabo, A. G.; MacManus, J. P. *FEBS Lett.* **1992**, *310*, 269. Kishi, T.; Tanaka, M.; Tanaka, J. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1267.
(25) Crooks, J. E. In *Comprehensive Chemical Kinetics*; Bamford, C. H., Tipper, C. H. F., Eds.; Elsevier: Amsterdam, 1977; Vol. 8, p 197.
(26) Schowen, K. B. J. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; p 225. Limbach, H.-H. In *Electron and Proton Transfer in Chemistry and Biology*; Müller, A., Ratajczak, H., Junge, W., Diemann, E., Eds.; Elsevier: Amsterdam, 1992; p 329.
(27) Chou, P.-T.; Martinez, M. L.; Cooper, W. C.; Collins, S. T.; McMorrow, D. P.; Kasha, M. *J. Phys. Chem.* **1992**, *96*, 5203.
(28) McMahon, L. P.; Colucci, W. J.; McLaughlin, M. L.; Barkley, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 8442. Yu, H.-T.; Colucci, W. J.; McLaughlin, M. L.; Barkley, M. D. *J. Am. Chem. Soc.* **1992**, *114*, 8449.
(29) Moog, R. S.; Maroncelli, M. *J. Phys. Chem.* **1991**, *95*, 10359. McMorrow, D.; Aartsma, T. J. *Chem. Phys. Lett.* **1986**, *125*, 581. Konijnenberg, J.; Huizer, A. H.; Varma, C. A. G. O. *J. Chem. Soc., Faraday Trans. 2* **1988**, *84*, 1163.
(30) See, for example, the following and references therein: Ulstrup, J. *Charge Transfer Processes in Condensed Media*; Springer: Berlin, 1979. DeVault, D. *Quantum Mechanical Tunnelling in Biological Systems*; Cambridge University Press: Cambridge, 1984.
(31) Rehm, D.; Weller, A. *Isr. J. Chem.* **1970**, *8*, 259.
(32) Miller, J. R.; Peeples, J. A.; Schmitt, M. J.; Closs, G. L. *J. Am. Chem. Soc.* **1982**, *104*, 6488.
(33) Gudgin, E.; Lopez-Delgado, R.; Ware, W. R. *J. Phys. Chem.* **1983**, *87*, 1559.
(34) Greenstein, J. P.; Winitz, M. *Chemistry of the Amino Acids*; Wiley: New York, 1961; Vol. 1, Chapter 4, p 511.
(35) Laughton, P. M.; Robertson, R. E. In *Solute-Solvent Interactions*; Coetzee, J. F., Ritchie, C. D., Eds.; Marcel Dekker: New York, 1969; p 399.
(36) Wada, G.; Tamura, T.; Okina, M.; Nakamura, M. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 3064.
(37) Négrerie, M.; Gai, F.; Lambry, J.-C.; Martin, J.-L.; Petrich, J. W. *J. Phys. Chem.* **1993**, *97*, 5046.
(38) Taylor, C. A.; El-Bayoumi, M. A.; Kasha, M. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 253. Hetherington, W. M., III; Micheels, R. M.; Eisenthal, K. B. *Chem. Phys. Lett.* **1979**, *66*, 230.