Cross-linked metalloproteins: novel systems for the study of intraprotein electron-transfer reactions

Linda M. Peerey
Iowa State University

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Cross-linked metalloproteins—novel systems for the study of intraprotein electron-transfer reactions

Peerey, Linda M., Ph.D.
Iowa State University, 1989
Cross-linked metalloproteins — novel systems for the study of intraprotein electron-transfer reactions

by

Linda M. Peerey

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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ABBREVIATIONS

2-Fpy, 2-fluoropyridine;
A, absorbance;
AcMetH, N-acetyl-L-methionine;
BBQ, 4,4''-bis[(2-butyloctyl)oxy]-1,1':4',1"':'4'',1"':4'',1"':4'''-quaterphenyl;
bpy, 4,4''-bipyridyl;
cyt(III) and cyt(II), ferric and ferrous cytochrome c, respectively;
cyt/pc, either the electrostatic or the covalent complex with the oxidation
states as indicated;
DMF, dimethylformamide;
DSS, sodium salt of 3-(trimethylsilyl)-1-propanesulfonic acid;
DST, disuccinimidyl tartarate;
eaq-, hydrated electron;
EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride;
EDTA, ethylenediaminetetraacetic acid;
EGS, ethylene glycolbis(succinimidylsuccinate);
F, FH', and FH-, fully-oxidized flavin, flavin semiquinone, and fully-
reduced flavin, respectively;
FMN, flavin mononucleotide;
Ll, lumiflavin;
MOPS, 3-(N-morpholino)propanesulfonate buffer;
Mr, molecular mass;
NHE, normal hydrogen electrode;
pc(II) and pc(I), cupric and cuprous plastocyanin, respectively;
Tris, 2-amino-2-hydroxymethyl-1,3-propanediol;
tu, thiourea.
I. OVERVIEW

The initial portion of my work shows, for the first time, that proteins can be cross-linked selectively via transition-metal compounds to form stable protein complexes (1). Incubation of horse heart cytochrome \( c \) (designated cyt) with reagents \( \text{PtCl}_4^{2-} \) and \( \text{trans-}[\text{Pt}(2\text{-Fpy})_2\text{Cl}_2] \) under mild conditions yields the stable diprotein complexes \( \text{trans-}[\text{PtCl}_2(\text{cyt})_2] \) and \( \text{trans-}[\text{Pt}(2\text{-Fpy})_2(\text{cyt})_2] \), respectively (2-Fpy is 2-fluoropyridine). The complexes are purified and characterized chromatographically. The protein molecules are coordinated to the Pt(II) atom through the thioether side chains of their respective Met 65 residues. Spectroscopic and electrochemical measurements indicate that, except for the slightly increased abundance of the high-spin form of ferriheme, the structural and redox properties of the cytochrome \( c \) molecules remain unaltered upon cross-linking. The diprotein complexes are stable indefinitely under ordinary conditions and yet they can be cleaved, and the native protein restored, in a mild reaction. Platinum compounds hold promise as selective and versatile reagents for cross-linking proteins.

Subsequent research focused on oxidoreduction reactions involving the electrostatic and the covalent complex of horse heart cytochrome \( c \) and \textit{Phaseolus vulgaris} plastocyanin, and exploring the importance of protein rearrangement for the intracomplex electron-transfer reaction. Cytochrome \( c \) and plastocyanin are cross-linked one-to-one by a carbodiimide (2), in the same general orientation in which they associate electrostatically (3). The reduction potentials of the Fe and Cu atoms in the covalent diprotein complex are respectively 245 and 385 mV versus NHE; the EPR spectra of the two metals are not perturbed by cross-linking. Four isomers of the covalent diprotein complex, which probably differ slightly from one another in the
manner of cross-linking, are separated efficiently by cation-exchange chromatography. Electron-transfer kinetics of the covalent and electrostatic diprotein complexes are studied using stopped-flow, pulse radiolysis (4), and flash photolysis experiments. The electrostatic complex undergoes intracomplex electron transfer with a rate constant of $1.05 \times 10^3$ s$^{-1}$. However, there appears to be a complete absence of intracomplex electron transfer within all isomers of the covalent complex. The electrostatic and the covalent complexes behave completely differently under identical conditions although the cytochrome $c$ and plastocyanin in them are docked similarly; although significant perturbations of the electronic, structural, and redox properties of the active sites upon cross-linking are ruled out; and although the covalent complex remains reactive in bimolecular electron-transfer reactions with inorganic oxidants, hydrated electrons, and flavin semiquinones. A rearrangement of the proteins for the intracomplex electron-transfer reaction seems to be possible (or unnecessary) in the electrostatic complex but impossible in the covalent complex.

Additionally, this research uses variable-length cross-linkers — DST (6 Å) and EGS (16 Å) — to form covalent cyt/pc complexes. Mild reaction conditions (low protein concentrations and minimal excess of cross-linker) successfully yield the covalent cyt/pc diprotein complex without forming the cyt/cyt complex. Kinetic studies of these cyt/pc diprotein complexes will explore the correlation between the tether length (flexibility) and the intracomplex electron-transfer rate constants. As protein rearrangement becomes feasible within such diprotein complexes, it may be reflected by changes in the intracomplex electron-transfer rate constants.
II. MATERIALS AND METHODS

A. Chemicals

1. **Protein labeling and cross-linking reagents**
   
   Cis-dichloro(2,2'-bipyridyl)platinum(II), Pt(bpy)Cl₂, was obtained from Strem Chemicals, Inc.; 2-fluoropyridine and K₂PtCl₄ were purchased from Aldrich and the latter also borrowed from Johnson Matthey, Inc.; 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC) was obtained from Sigma Chemical Co.; disuccinimidyl tartarate (DST) and ethylene glycolbis-(succinimidylsuccinate) (EGS) were obtained from Pierce Chemical Co.

2. **Amino acids and protein molecular weight standards**
   
   Imidazole was obtained from Aldrich Chemical Co. N-acetyl-L-methionine, aprotinin, trypsinogen inhibitor, trypsinogen, carbonic anhydrase, egg albumin, bovine albumin, and blue dextran were obtained from Sigma Chemical Co.

3. **Electrochemical and redox reagents**
   
   Ferrocene, 4,4'-bipyridine dihydrochloride (bpy), lumiflavin, and flavin mononucleotide (FMN) were obtained from Sigma Chemical Co.; cystamine dihydrochloride was obtained from Aldrich Chemical Co. The oxidants [Fe(C₅H₅)₂]PF₆ (5) and [Co(phen)₃]ClO₄ (6) were synthesized in standard ways.

4. **Chromatography**
   
   The size-exclusion resins Sephadex G-25 (50-mesh, molecular-weight range 1-5 kDa) and Sephadex G-75 (50-mesh, molecular-weight range 5-66 kDa), the anion-exchange resin Sephadex DEAE-
25 (120 mesh), and the cation exchange resin cellulose CM 52 (fine mesh) were obtained from Sigma Chemical Co.; Bio-Gel-2 (molecular-weight range 100-400 Da) was obtained from Bio-Rad Laboratories. Distilled water was demineralized in a Barnstead Nanopure II apparatus.

B. Buffers

1. Reactions and column work

Individual acid and base components were made to the desired final buffer concentration and mixed in the necessary proportions to obtain a buffer solution of the desired pH. The following buffers were used — workable pH ranges are given in parentheses: acetic acid/sodium acetate (3.6-5.6), potassium dihydrogen phosphate/potassium monohydrogen phosphate (6.9-8.0 allows maximum buffering capacity; 5.0-8.2 provides lowered buffering capacity), HCl/trisma base (7.2-9.0), and HMOPS/NaMOPS (ca. 6.5-8.0).

2. Kinetic studies

Ionic strength, $\mu$, is calculated from solution molarities, C, as follows:

$$\mu(\text{K}_2\text{HPO}_4) = (0.5)(C_{\text{K}_2\text{HPO}_4})(3.53)$$

$$\mu(\text{EDTA}) = (0.5)(C_{\text{EDTA}})(11.0)$$

$$\mu(\text{NaCl}) = (C_{\text{NaCl}}).$$
C. Protein Extraction and Purification

1. Purification of cytochrome \( c \)

Ferricytochrome \( c \) from horse heart (preparations of types III and VI) was obtained from Sigma Chemical Co. It was incubated with half an equivalent of \( K_3Fe(CN)_6 \) for several hours and the fully-oxidized protein chromatographed with 85 mM phosphate buffer at pH 7.0 as an eluent, on a column of CM 52 cation exchange resin. Only the major fraction, containing ca. 85% of the commercial protein, was used in subsequent experiments (7).

All dialyses were carried out in Amicon ultrafiltration cells with YM-5 membranes, whose threshold is 5 kDa, at 4 °C and under 50-psi of purified nitrogen. The Centricon, produced by Amicon, relies on centrifugation — rather than gas pressure as do the stirred ultrafiltration cells — and allows protein to be completely removed from the membrane in much smaller volumes of solvent (ca. 0.1 mL) than with the stirred cell. It is a more efficient means for obtaining highly-concentrated stock protein solutions for flash photolysis kinetic runs. These devices will be used in our laboratory in the near future.

2. Isolation of plastocyanin

a. Overview

The procedure for extracting plastocyanin from bean leaves, as described by Milne and Wells (8), and its revision by H. B. Gray, A. M. English and V. Lum (9), was followed with certain procedural changes: the variety of Phaseolus vulgaris was "Tenderpod green snap bush bean" instead of "French bean"; juice extraction was accomplished with a meat grinder rather than a juice extractor; and the desalting and concentrating of juice was carried out in Amicons with YM-5 membranes instead of using Sephadex G-25 and cellulose DEAE resins.

Procedural points to consider for maximizing yields of plastocyanin: 1) be certain all chemicals — especially ammonium
sulfate—are on hand before initiating the procedure; 2) do all operations as far as possible at 4 °C; 3) juice can be stored at -40 °C; 4) clearly label and save all fractions, from ammonium sulfate precipitations and column work, in case you must retrace your steps and find "missing" plastocyanin; 5) use good grade (NH₄)₂SO₄ to avoid heavy-metal contamination; 6) watch that the pH of the ammonium sulfate mixtures does not drop below 4.5—if it does, adjust up with NaOH; 7) finally, treat fractions with Fe(CN)₆³⁻ prior to all column work to ensure visible, blue, oxidized plastocyanin—there is a high tendency for plastocyanin to autoreduce.

b. Planting and harvesting  *Phaseolus vulgaris* (Tenderpod green snap bean) seeds were obtained from Brekke's Town and Country seed supplier in Ames, planted in the ISU Agronomy greenhouse (2 plantings of 550 pots, 2-3 plants per pot), and the leaves harvested after ca. 8 weeks. All subsequent operations were done at 4 °C.

c. Juicing and juice clarification  Bean leaves were washed in cold water, placed in 50 mM acetate buffer at pH 6.0, and juiced in a meat grinder to yield separated juice and pulp. Additional juice was obtained by squeezing the pulp through several layers of cheesecloth. The juice and pulp fractions were stored at -40 °C until all leaves were processed; total yield of juice was ca. 35 L. Thawed juice was centrifuged for 30 minutes at 3000 rpm and the clear, intensely-green supernatant was collected leaving a small amount of pulp. All centrifugation was carried out at 4 °C in a Sorvall machine having a GSA rotor with capacity for six, 250 mL-wide-mouth Nalgene bottles.

d. Ammonium sulfate treatment  The clarified juice was taken to 40% saturation with ammonium sulfate (240 g/L) added slowly with stirring over 1-2 hours, stirred overnight, and centrifuged for 15 minutes at 6000 rpm. The supernatant was collected (leaving yellow-brown pellets), taken to 80% saturation with ammonium sulfate (320
g/L of original solution) added slowly with stirring over 1-2 hours, and the brown solution stirred several more hours. The brown precipitate was collected by centrifugation (15 minutes at 6000 rpm) and resuspended in 50 mM acetate buffer at pH 6.0 (using ca. 1 L of buffer for every 40 L of original solution). After centrifugation (15 minutes at 6000 rpm), the clear brown supernatant was collected, pooled, and adjusted to pH 4.0 with a cold 50:50 mixture of glacial acetic acid in water, over a 15-30 minute period. Centrifugation (15 minutes at 6000 rpm) removed the resulting light brown precipitate and the supernatant was immediately adjusted to pH 6.0 with cold 6 M NaOH.

e. Columns The clear, dark-brown solution was concentrated and desalted by ultrafiltration (using 400 mL Amicons with YM-5 membranes) into 50 mM acetate buffer at pH 6.0. Very concentrated aliquots were treated with Fe(CN)$_6^{3-}$ (ensuring complete oxidation of plastocyanin), loaded onto a column of Sephadex G-75 size-exclusion resin (sized 2.5 x 50 cm), and eluted with 50 mM acetate buffer at pH 6.0. Fractions obtained in order of elution are colored brown, blue, reddish-black-brown, and yellow-brown. The entire blue fraction was collected; fractions from several columns were pooled, concentrated, redialyzed into 50 mM acetate buffer at pH 6.0, treated with Fe(CN)$_6^{3-}$, and loaded onto several columns of Sephadex DEAE-25 anion-exchange resin (sized 1.5 x 15 cm). The entire sample is immobile at 50 mM acetate buffer; a linear gradient to 50 mM buffer containing 500 mM additional NaCl mobilizes a blue band which moves down the column. The blue band is the first mobile fraction, eluting at ca. 300 mM NaCl, and containing plastocyanin with $A_{278}/A_{597} \approx 2$. This plastocyanin was eluted once again through Sephadex G-75 and through Sephadex DEAE-25 for additional purification; fractions with $A_{278}/A_{597} \leq 1.3$ are considered sufficiently pure plastocyanin. Some 30 mg of such plastocyanin was obtained, dialyzed into 50 mM acetate buffer at pH 6.0 (stored at -40 °C), and
used in the DST and EGS modification and cross-linking studies.

f. Additional plastocyanin *Phaseolus vulgaris* (French bean) plastocyanin, a gift from Professor H. B. Gray, was purified by anion-exchange chromatography. The protein was oxidized, dissolved in 55 mM phosphate buffer at pH 6.0, applied to a column of Sephadex DEAE-25 resin, and washed with this buffer. When the buffer was made 150 mM in NaCl, a single band of the pure protein moved down the column. All the experiments were done with plastocyanin having the absorbance quotient $A_{278}/A_{597} \leq 1.30$; this protein was used in all EDC cross-linking studies.

g. Large-scale protein extraction At present, one half acre each of Tenderpod green snap bush bean (for plastocyanin) and red rape seed (for cytochrome $f$) are in the field at Curtiss Farm, ISU College of Agriculture. Approximately 10 lbs of bean seed ($32) and 5-10 lbs of rape seed (50 lbs/$18) were planted. Plants should be harvested at 4 to 6 weeks, long before the edible beans mature. We have received the recommendation to plant more acreage and harvest sooner, rather than trying to increase yields through harvesting older, larger, plants. The group of Prof. H. B. Gray reports yields of 500-1000 mg of plastocyanin from a one-third-acre plot of bean plants; their record yield is 1400 mg.

3. Determination of molecular mass

Size-exclusion gel chromatography was carried out on a column of Sephadex 75-50 resin (sized 1.5 x 70 cm) at 4 °C. The eluent in earlier work was 50 mM Tris-HCl buffer, 100 mM in KCl, at pH 7.5; simply 85 mM phosphate buffer at pH 7.0 was used in later molecular mass determinations. The flow rate of 5.00 mL h$^{-1}$ was maintained with an ISCO WIZ peristaltic pump. Absorbance was measured with an ISCO V-4 detector equipped with a strip-chart recorder. The following proteins, whose molecular weights in kilodaltons are given in parentheses, were used as standards: aprotinin (6.5), horse heart
cytochrome c (12.5), trypsinogen inhibitor (20.1), trypsinogen (24.0),
cARBONIC ANHYDRASE (29.0), egg albumin (45.0), and bovine albumin
(65.0). Each calibration point represents an average of two
measurements. The void time was determined with blue dextran,
whose molecular mass of 2 MDa falls far outside the working range of
the gel, 5-66 kDa. For greater accuracy, elution times at constant flow
rate, rather than elution volumes, were measured. The column was
calibrated before every determination of the unknown molecular
mass.

4. Metal substitution
Substitution of the plastocyanin Cu with the redox inactive Hg is
achieved under fairly mild conditions and is successful with native
plastocyanin or the plastocyanin present in a covalent diprotein
complex; in the latter case (when the complex is cyt/pc) the
cytochrome c remains unperturbed. The buffered protein, ca. 0.15 M
in additional NaCl, is treated with a 20- to 100-fold excess of mercuric
chloride or mercuric acetate for several hours; dialysis then removes
excess reagent and displaced copper. This process is repeated if Cu
substitution is incomplete. Hg-substitution produces a redox inactive
form of plastocyanin which has been structurally characterized (10);
the integrity of the protein matrix is maintained, and complications
arising from heavy-metal contamination — a constant worry with
apoplastocyanin — are easily avoided.

5. Protein concentrations
Concentrations of the separate proteins were determined
spectrophotometrically on the basis of the following absorptivities
(extinction coefficients), ε: 28.5, 106.1, and 11.0 mM⁻¹cm⁻¹ at 360, 410,
and 526 nm respectively, for ferricytochrome c (11); 4.5 mM⁻¹ cm⁻¹
at 597 nm for cupriplastocyanin. The concentration of
ferricytochrome c in diprotein complexes was measured at 410 nm,
where the cupriplastocyanin absorbance is negligible. Concentration of cupriplastocyanin in the complexes was most accurately calculated from the difference between the spectra of cyt(III)/pc(II) and cyt(III), matched at 360 nm.

Alternatively, the respective concentrations of cytochrome c and plastocyanin, mutually present in solution, may be calculated from the net absorbance values at two wavelengths — e.g., the Soret maximum at ca. 410 nm and the blue-copper maximum at 597 nm (using eqs 1 and 2). This is done by measuring the net absorbance at the Soret maximum (provided cytochrome c is fully-oxidized), dissolving one or two grains of Fe(CN)$_6^{3-}$ in the UV-vis sample cuvette, and then measuring the net absorbance at 597 nm. Presence of the Fe(CN)$_6^{3-}$ ensures fully-oxidized plastocyanin while neither interfering with the sample concentration nor the absorbance of the proteins in the 600 nm region.

$$[\text{cyt}] \text{mM} = \frac{A_{\text{Soret max}}}{4.5 \text{mM}^{-1} \text{cm}^{-1}} \quad (1)$$

$$[\text{pc}] \text{mM} = \frac{A_{597 \text{nm}} - (A_{\text{Soret max}}/1.7 \text{mM}^{-1} \text{cm}^{-1})}{4.5 \text{mM}^{-1} \text{cm}^{-1}} \quad (2)$$

Concentration changes in kinetic experiments were calculated from the following absorptivity changes, $\Delta \varepsilon = \varepsilon_{\text{red}} - \varepsilon_{\text{ox}}$: -13.8, 44.5, 31.1, 18.5, and 0 mM$^{-1}$cm$^{-1}$ at 360, 417, 425, 550, and 557 nm, respectively, for cytochrome c; -2.05 and 2.4 mM$^{-1}$cm$^{-1}$ at 550 and 557 nm, respectively, for plastocyanin.
D. Spectroscopic and Electrochemical Techniques

1. Absorption spectroscopy
   Electronic spectra were recorded with an IBM 9430 UV-vis spectrophotometer, equipped with a two-grating monochromator. Vibration spectra in the far-IR region were recorded with an IBM IR98 FT instrument, whose sample chamber was flushed with purified nitrogen. Nujol mulls were prepared from lyophilized proteins.

2. EPR spectroscopy
   The X-band spectra were recorded at 5 K with a Bruker ER200-SRC instrument equipped with an Oxford Instruments ESR900 cryostat. A double rectangular cavity had a nominal frequency of 9.56 GHz; modulation frequency was 100 kHz. The samples were fully oxidized: the native cytochrome c with K₃Fe(CN)₆; the modified cytochrome c and the protein dimer with [Co(phen)₃(C104)₃. The oxidants were removed by dialysis into 85 mM phosphate buffer at pH 7.0.

3. NMR spectroscopy
   The ¹H NMR spectra at 300 MHz and the ¹⁹F spectra at 282 MHz were recorded with the Bruker WM300 spectrometer. Residual protons (in D₂O) were the internal standard in the former; a 10 mM solution of trifluoroacetic acid in 85 mM phosphate buffer at pH 6.8, kept in a coaxial capillary tube, served as the external standard in the latter. The protein-containing samples for ¹H NMR measurements (ca. 0.5 mM) were dialyzed into D₂O by ultrafiltration and then lyophilized with successive portions of D₂O to remove the NH resonances (12-14); protein samples for ¹⁹F measurements (ca. 0.2 mM) were dissolved in 150 mM phosphate buffer at pH 5.5, containing 20% D₂O.
4. Voltammetry

Differential-pulse and cyclic voltammograms were obtained with an IBM EC225 electrochemical analyzer equipped with a Houston Instruments Omnigraph 200XY recorder. A BAS cell assembly consisted of an Ag/AgCl couple as reference, a Pt wire as auxiliary, and a 1.6 mm Au disk as working electrode. The composition of the sample solutions was as follows: ca. 0.5 mM in protein and 10 mM in the mediator (15) (4,4'-bipyridyl used with cytochrome c; cystamine dihydrochloride used with plastocyanin), dissolved in 85 mM phosphate buffer at pH 7.0. A small jacketed cell of 5 mL allowed experiments with 2-mL samples at constant temperature. The solutions were deoxygenated by gentle bubbling of argon and a blanket of this gas was maintained during the measurements.

5. Redox titration

The use of redox titrations allows determination of reduction potentials by a method other than voltammetry. Oxidized protein is titrated with varying amounts of [Fe(CN)₆]³⁻, allowed to come to equilibrium, and the resulting concentrations of oxidized and reduced species are determined using UV-vis spectroscopy. The reduction potential is obtained by applying eqs 3 and 4.

Eq 3 is a general expression, for a system at equilibrium, containing ferri- and ferrocyanide, and cupri- and cuproplastocyanin. It simplifies to the expression in eq 4 when [pc(I)] equals [pc(II)]. A plot is made of log ([Fe(CN)₆]⁴⁻)/[Fe(CN)₆]³⁻) versus log ([pc(I)]/[pc(II)]) (as the ordinate and abscissa, respectively) for various concentrations of added Fe(CN)₆⁴⁻. The value of log ([Fe(CN)₆]⁴⁻)/[Fe(CN)₆]³⁻) where log ([pc(I)]/[pc(II)]) equals zero, is read from this plot; error bars are estimated from the uncertainty in the slope. The value of log ([Fe(CN)₆]⁴⁻)/[Fe(CN)₆]³⁻) is then used in eq 4 to calculate the
reduction potential of plastocyanin; the reduction potential of \([\text{Fe(CN)}_6]^{3-/4-}\) is 0.424 V, and the pre-log term has the value of 0.05915 V versus NHE.

\[
E_{\text{cell}} = 0 = E^o_{\text{Fe(CN)}_6^{3-/4-}} - E^o_{\text{pc(II)/pc(I)}} +
\]

\[
\frac{2.3RT}{nF} \log \left[ \frac{[\text{Fe(CN)}_6^{3-}][\text{pc(I)}]}{[\text{Fe(CN)}_6^{4-}][\text{pc(II)}]} \right]
\]

\[
E^o_{\text{pc(II)/pc(I)}} = E^o_{\text{Fe(CN)}_6^{3-/4-}} - \frac{2.3RT}{nF} \log \left[ \frac{[\text{Fe(CN)}_6^{4-}]}{[\text{Fe(CN)}_6^{3-}]} \right]
\]

E. Kinetic Techniques

1. **Stopped-flow spectrophotometry**

   All experiments were done at room temperature, with an apparatus made by Kinetic Instruments, Inc. and equipped with software by OLIS, Inc. K3[Fe(CN)_6] and [Fe(C_5H_5)_2]PF_6 were the oxidants, and the covalent complex cyt(II)/pc(I) (a mixture of the four isomers) was the reductant. The procedures were tested by reproducing the well-known rate constants for the oxidation of the native ferrocytochrome c (16). The protein samples were reduced with ascorbic acid, dialyzed against 10 mM phosphate buffer at pH 7.0 that was 90 mM in NaCl, and stored under nitrogen. All buffers were deaerated. The syringes were filled under external pressure of
nitrogen. Concentrations were measured spectrophotometrically before each kinetic experiment. Oxidation of ferrocytochrome $c$ was monitored at both 417 and 550 nm.

Pseudo-first-order conditions were achieved with a 5-20-fold excess of the inorganic oxidant over the diprotein complex. Each point on the plot of $k_{\text{obsd}}$ versus concentration was an average of at least five separate measurements under identical conditions. The correlation coefficient ($r^2$) for a line through five or six such points was always 0.99. Oxidation of the cuproplastocyanin moiety in cyt(II)/pc(I) was so much slower than the oxidation of the ferrocytochrome $c$ moiety — ca. 6 times in the case of $[\text{Fe(C}_5\text{H}_5\text{)}_2]^+$ (17, 18) and ca. 100 times in the case of $[\text{Fe(CN)}_6]^3-$ (16, 19) — that it did not affect significantly the kinetic measurements and calculations. Indeed, in control reactions in which cyt(II)/pc(I) was in excess over $[\text{Fe(CN)}_6]^3-$ the amount of cyt(III)/pc(I) formed was equal to the amount of $[\text{Fe(CN)}_6]^3-$ available.

2. Pulse radiolysis

The proteins were dissolved in, or dialyzed into, phosphate buffer at pH 7.0, whose ionic strength, $\mu$, was 1, 2, 40, or 200 mM. Tertiary butanol (2% by volume) was added to scavenge the OH-radicals. The samples were deoxygenated by gentle bubbling with nitrogen and transferred anaerobically to a quartz cell with optical path of 1.0 or 2.0 cm. Spectrophotometric measurements ruled out any appreciable reduction of the proteins during the deoxygenation, before the radiolysis. Fresh samples were used in all experiments.

A 250-ns pulse of 35-mA current from a Van de Graaf electron accelerator delivered ca. 5.3 krad of 4-MeV electrons and produced ca. 2 $\mu$M solution of $e_{aq}^-$ in the sample (20). Concentrations of protein complex generally ranged from 12 to 35 $\mu$M. Under these conditions, double reduction of cyt(III)/pc(II) to cyt(II)/pc(I) could safely be
ignored. Enough cyt(II)/pc(II) was produced to allow accurate spectrophotometric monitoring of the subsequent electron-transfer reaction, when this reaction occurred. At least four traces were averaged for the calculation of each observed rate constant.

The initial reduction of ferricytochrome c by eaq− was observed on the microsecond time scale, and the subsequent oxidation of ferrocyanochrome c by cuproplastocyanin was followed on the millisecond time scale. Although 557 nm is an isosbestic point for cytochrome c, the delta absorbance of this protein did contribute somewhat to the delta absorbance of plastocyanin at this nominal wavelength because a wide slit had to be used. Oxidation of plastocyanin was quantitated by careful subtraction of the contribution from cytochrome c.

The observed rate constants were the same, within the error limits, regardless of the wavelengths monitored. In the covalent complex and in the fully-formed electrostatic complex, first-order conditions for the interprotein electron-transfer reaction prevailed; in the mixture of partially-associated proteins, pseudo-first-order conditions prevailed. Indeed, all the plots of log A versus time were linear for at least three half-lives. The cupriplastocyanin concentrations used for the kinetic calculations were averages of the values before and long after the pulse. The rate constants for the protein association (so-called preequilibrium) and for the subsequent electron-transfer reaction were calculated from the observed rate constants with the program provided to us by Professors Gordon Tollin and Michael A. Cusanovich.

3. Flash photolysis

The laser flash photolysis technique and method of data collection and analysis were as generally described (21, 22). FMN and Lf were purified under dim light by passage through a Bio-Gel-2 size-
exclusion column equilibrated with double distilled water, and
lyophilized. Standard buffer contained 70 μM flavin (Lf or FMN), 0.5
mM EDTA, and 3 mM phosphate buffer at pH 7.0 (μ = 10mM).
Addition of 100 μL of phosphate buffer (0.5 M, pH 7.2) directly to the
sample cuvette increased μ to ca. 170 mM.

One milliliter of the standard buffer was deoxygenated in the
stoppered cuvette by vigorous bubbling with purified Ar for 15 minutes
prior to the addition of protein. Following addition of a 5-50 μL aliquot
of protein (in 3 mM phosphate buffer at pH 7.0) the solution was
blanketed with Ar. All solutions containing flavins, especially in the
presence of EDTA, were kept in the dark.

Flash photolysis was carried out with a PRA LN100 nitrogen
laser-pumped dye solution [2.5mM 4,4″-bis[(2-butyloctyl)oxy]-
1,1′:4′,1″:4″,1‴-quaterphenyl, (BBQ), in 50:50 toluene:ethanol, in a 1-cm
pathlength cuvette. The photogenerated flavin triplet-state and its
subsequent reduction by EDTA produces the flavin semiquinone; this
radical species provides the reducing equivalent for the reduction of
cytochrome c and plastocyanin. Fully-reduced flavin (FH⁻) is
subsequently generated, as shown in eq 5, by disproportionation of the
semiquinone. This slower process occurs in the absence of a suitable
oxidizing agent such as dissolved oxygen, ferricytochrome c, or
cupriplastocyanin. Less than 1% of the total flavin initially present

\[
FH⁻ + FH⁻ \rightarrow F + FH⁻ + H⁺
\]  

was excited with each laser flash and protein concentrations ranged
from 5-50 μM — thus the reduction of both proteins was pseudo-first-
order. The cytochrome c and plastocyanin chromophores were
monitored independently at 550 and 557 nm respectively;
superposition of the flavin chromophore necessitated that the
observation wavelength be greater than 500 nm. All measurements
were done under anaerobic conditions; reactions were monitored over three to four half-lives; and typically three to five concentrations of protein were used to determine the second-order rate constants. Under the experimental conditions used, the time resolution of the apparatus was 0.3-1.0 μs (limited mostly by fluorescence and scattering artifacts).

4. Comparison of fast kinetic techniques

The two fast kinetic techniques of pulse radiolysis and flash photolysis both afforded sufficient and rapid production of ferrocyanochrome c in the presence of cupriplastocyanin, and thus allowed subsequent reactions to be cleanly followed. Our kinetic studies — with limited amounts of protein, that we wished to monitor over several seconds — encountered the following limitations in the technique of pulse radiolysis: a sample volume of 20-30 mL prohibiting measurements at higher protein concentrations; the necessity of bubbling the protein solution prior to each run, resulting in possible reduction of plastocyanin; the use of such a highly-reducing species as eaq⁻ (E° = -2.7 V) which might react with the protein matrix as well as with the desired metal centers; and lastly, a detection system with a maximum observation time of only 1 to 2 seconds. The ease of acquiring pulse radiolysis data is likewise complicated by: the need for the constant presence of the operator of the electron generator; the inconvenience of the sample and the operators being in separate, isolated rooms; and the limited number and availability of operational facilities. The technique of flash photolysis, on the other hand, incorporates the following advantages: sample volumes of only 1 mL; protein solutions that were never subjected to bubbling; the use of various flavins allowing for flexibility in altering the charge, size, and reduction potential of the reducing species; observation times of up to
10 seconds with excellent baseline stability; and finally, the affordable nature of such a kinetic set up and its inevitable purchase by our group.
II. TRANSITION-METAL COMPOUNDS AS NEW REAGENTS FOR SELECTIVE CROSS-LINKING OF PROTEINS — SYNTHESIS AND CHARACTERIZATION OF TWO BIS(CYTOCHROME C') COMPLEXES OF PLATINUM

A. Introduction

Covalent cross-linking of proteins to other proteins, membranes, and smaller biomolecules is used widely in enzymology, structural biochemistry, biophysics, and chemical biology (23-27). Numerous bifunctional organic reagents have been developed for this purpose and their properties and applications cataloged (28). To be useful for cross-linking, a reagent should be soluble in water, selective toward particular amino acid side chains, and reactive at mild, preferably physiological, conditions. The cross-linked aggregate should be stable and yet cleavable, so that the original partners can be recovered from it. Most of the commonly-used reagents are selective toward amino and sulfhydryl groups; others, such as photogenerated nitrenes, are nonselective.

This study introduces an inorganic approach to the cross-linking of proteins. Various spectroscopic and chemical properties of transition metals render their complexes uniquely suited for specific binding to biological macromolecules, the ultimate multifunctional ligands (29, 30). Strong, easily detectable charge-transfer absorption bands of metal complexes are sensitive to the ligands and to the more distant environment; paramagnetic ions can serve as EPR probes and NMR relaxation agents; electron density renders the metals useful as tags for X-ray crystallography and electron microscopy; affinity toward ligands can be controlled by the oxidation state, by the hardness or softness of the metal atom, and by the structure and charge of the complex as a whole. Properties of platinum make it especially suitable
for use in labeling reagents: it forms a myriad of stable complexes, undergoes various substitution reactions at convenient rates, and possesses an abundant and receptive isotope, $^{195}$Pt, whose NMR chemical shifts span some 15000 ppm (31-33). A recent study from this laboratory has demonstrated the fitness of platinum for protein labeling (34). The complex chloro(2,2':6',2"-terpyridine)platinum(II), Pt(trpy)Cl+, proved to be a sensitive spectroscopic tag for cytochrome c. Its specificity (at pH 5) toward the imidazole side chain of histidine is caused by an interesting interplay between the steric and electronic effects of the aromatic terpyridine ligand.

Whereas the tridentate ancillary ligand ensures that only the Cl$^-$ ion is displaced from Pt(trpy)Cl+, and hence that only one protein molecule binds to the Pt atom, more than one Cl$^-$ ion can, in principle, be displaced by proteins in the unidentate PtCl$_4^{2-}$ complex. Although PtCl$_4^{2-}$ has been used widely for protein tagging (35-37), such cross-linking has not been observed because the reactions have commonly been carried out with crystals, in which the protein molecules are immobile. This report shows that proteins in solution, exemplified by cytochrome c, can be cross-linked selectively, under mild condition, by the PtCl$_4^{2-}$ complex.

B. Cytochrome c

Horse heart cytochrome c is a 12.5 kDa, single-chain, iron heme protein (38, 39); it is well characterized and commercially available. Its physiological role, in the eukaryotic mitochondrial respiratory chain, is as a one-electron shuttle between the membrane-bound complexes cytochrome c reductase and cytochrome c oxidase; the latter is the terminal component which reduces molecular oxygen to water. The single, covalently-attached heme contains a six-coordinate
iron atom — ligated by the four porphyrin nitrogens, the imidazole nitrogen of histidine 18 and the thioether sulfur of methionine 80. The heme iron exists in the oxidation states of III and II, with a reduction potential of 0.26 V, versus NHE. The heme is only accessible to solvent and to external redox partners at the "heme crevice" — located on the "front" side of the protein molecule (40, 41). Electrostatic interactions are dominated by an abundance of 19 lysine residues; ferricytochrome c has a net charge of +8 at pH 7.0. The majority of these positive lysine residues are on the front face roughly encircling the heme crevice; all are exposed to solvent.

C. Preparation of Linking Reagents and of Model Complexes

1. \textit{Cis- and trans-}[Pt(2-Fpy)$_2$Cl$_2$]

   The procedure was analogous to the published one (42), except that 2-fluoropyridine, (2-Fpy), was used instead of pyridine. To 100 mg (0.24 mmol) of K$_2$PtCl$_4$ in 2 mL of water was added 47 mg (0.48 mmol) of 2-Fpy. A precipitate, formed over several hours, was washed with cold water. The $^{19}$F NMR spectrum of the solid dissolved in DMF showed it to consist of more than 95% of the cis isomer and of a very small amount of the trans isomer, exactly as reported originally (38). The subsequent treatment of [Pt(2-Fpy)$_4$]$^{2+}$ with HCl yielded not the pure trans-[Pt(2-Fpy)$_2$Cl$_2$], as was the case with the pyridine ligand, but a mixture of cis- and trans-[Pt(2-Fpy)$_2$Cl$_2$]. Since only the trans isomer proved soluble in benzene, it was extracted from the mixture with this solvent.

2. \textit{Cis- and trans-}[Pt(2-Fpy)$_2$(DMF)$_2$]$^{2+}$

   These linking reagents, containing labile DMF ligands, were prepared by addition of 6.8 mg (40 μmol) of AgNO$_3$ to 9.2 mg (20 μmol)
of the mixture of cis- and trans-[Pt(2-Fpy)2Cl2] dissolved in 1 mL of DMF. After the mixture was allowed to stand in the dark for 1 day, the precipitated AgCl was removed by centrifugation. The DMF complexes remained in clear solution.

3. cis-[Pt(bpy)(DMF)2]+

A solution of 8.4 mg (20 μmol) of cis-[Pt(bpy)Cl2] in 1 mL of DMF was treated with 6.8 mg (40 μmol) of AgNO3, as described above. After the removal of AgCl by centrifugation, a clear solution of cis-[Pt(bpy)(DMF)2]2+ remained. Aliquots of this solution were used for incubation with cytochrome c.

4. Model complexes trans-[Pt(2-Fpy)L2]+ with imidazole (n = 2) and N-acetylmethionine (n = 0) as L.

The syntheses were carried out by a modified method of Pearson et al. (43). To 9.2 mg (20 μmol) of trans-[Pt(2-Fpy)2Cl2] in 1 mL of solvent were added 6.8 mg (40 μmol) of AgNO3 and 40 μmol of ligand L (2.7 mg of imidazole or 7.6 mg of N-acetylmethionine, respectively). The reaction mixture was stirred, in the dark, for 5 days; during the first day it was also heated at 60 °C. After the AgCl precipitate was removed by centrifugation, the 19F NMR spectra of the resulting solutions confirmed that the model complexes were formed completely.

D. Preparation of Diprotein Complexes

1. Trans-[PtCl2(cyt)2]

Ferricytochrome c was incubated, in the dark, with K2PtCl4 in 150 mM phosphate buffer at pH 5.5. The protein concentration was 2 mM, usually 25 mg (2 μmol) in 1 mL of the buffer. The concentration of the linking reagent was varied systematically from 1 to 12 mM, i.e.,
from 0.42 to 5.0 mg (1 to 12 μmol) in 1 mL. The optimal yield of the diprotein complex, 15%, was achieved with equimolar amounts of the protein and PtCl$_4^{2-}$ in a 1-day incubation. The unconsumed K$_2$PtCl$_4$ was removed by dialysis into 150 mM phosphate buffer at pH 5.5. The protein solution was then subjected to cation-exchange chromatography on a CM 52 column, sized 1.5 x 25 cm, with the same 150 mM buffer as an eluent, or to size-exclusion chromatography, as described in section II part C3. The first exploratory experiments were carried out with ferrocytochrome c. The protein was treated with 1.5 times the equivalent amount of ascorbic acid, dialyzed, and incubated with K$_2$PtCl$_4$ under an atmosphere of nitrogen. Since ferricytochrome c proved entirely stable under the reaction conditions, the main work was done with it.

Cation-exchange chromatography yielded three well-separated bands. The first two bands, native cytochrome c and its derivative labeled with PtCl$_3^-$, were eluted with the 150 mM buffer. The third band remained immobile atop the column, but was eluted easily when the buffer was made 100 mM in NaCl.

Samples for size-exclusion chromatography were dialyzed into the Tris-HCl buffer. The raw product of incubation, a mixture of protein derivatives, yielded two fractions. The first one, with molecular mass of 30.7 ± 0.8 kDa, contains the diprotein complex, i.e., the protein dimer. The second band, with molecular mass of 13.0 kDa, evidently consists of monomeric cytochrome c. Size-exclusion chromatography of the separate fractions from the CM 52 column confirmed that the two mobile bands contained monomeric cytochrome c and that the third one contained the protein dimer.

2. $\text{Trans-}[\text{Pt}(2\text{-Fpy})_2(\text{cyt})_2]$

The reagent $\text{trans-}[\text{Pt}(2\text{-Fpy})_2(\text{DMF})_2]^{2+}$ was used in a procedure analogous to the one described above for PtCl$_4^{2-}$. The
solution of the linking reagent in DMF was concentrated so that the
required amount of it was contained in an 80-μL aliquot. Since the
volume of the aqueous (buffered) protein solution was 2 mL, the
resulting reaction mixture contained only 4% DMF. Higher
concentrations of DMF were avoided lest cytochrome c become
denatured.

3. Attempted preparation of cis-[Pt(bpy)(cyt)2]

Incubation of cytochrome c with cis-[Pt(bpy)(DMF)2]2+ was
carried out as with trans-[Pt(2-Fpy)2(DMF)2]2+. Cation-exchange
chromatography on a CM 52 column yielded three major and two
minor fractions. The first major one exhibits the UV-vis spectrum of
the native protein. The Pt(bpy) chromophores in the other four
fractions were easily detected and quantitated on the basis of their
characteristic absorption maximum at 320 nm and a shoulder at ca.
308 nm. The second and third major fractions each consist of
multiply-labeled protein derivatives. Size-exclusion chromatography
of the reaction mixture not subjected to cation-exchange
chromatography yielded a single band, corresponding to the molecular
mass of cytochrome c. Evidently, all the fractions separated on the CM
52 column consist of monomers — of cytochrome c and of labeled
derivatives. Incubation of singly-labeled protein derivatives, which
may be formulated cis-[Pt(bpy)(cyt)L] wherein L is DMF or H2O, with
an equivalent amount of the native cytochrome c also failed to produce
any diprotein complexes. Size-exclusion chromatography showed the
reaction mixture to consist solely of monomeric protein species.

4. Cleavage of diprotein complexes

Complex [PtCl2(cyt)2] was incubated with an excess of thiourea
in phosphate buffer for 2 days. Size-exclusion chromatography
showed that the reaction mixture contained monomeric cytochrome c,
i.e., that the Pt-protein bonds had been cleaved.

**E. Formation and Structure of the Diprotein Complexes**

1. **Linking reactions and the molecular mass**

   The reactions between equimolar amounts of cytochrome \( c \) and complexes \( \text{PtCl}_4^{2-}, \text{trans-}[\text{Pt}(2-\text{Fpy})_2(\text{DMF})_2]^{2+}, \text{cis-}[\text{Pt(bpy)}_{\text{DMF}})_2]^{2+} \), and \( \text{cis-}[\text{Pt(bpy)}_2(\text{cyt})_L] \) take place under mild conditions, namely at 4 °C or at room temperature. The yield of the diprotein complex \( [\text{PtCl}_2(\text{cyt})_2] \) is 15%. Incubation for 1 day is sufficient; the yield is not improved after 3 months at room temperature. Ferricytochrome \( c \) proved entirely stable under the reaction conditions, so the ferrous form of the protein, which has a tighter conformation \( (44) \), did not need to be used.

   The first two bands obtained by cation-exchange chromatography of the mixture of cytochrome \( c \) and \( \text{PtCl}_4^{2-} \) contain native cytochrome \( c \) and labeled derivative, \( [\text{PtCl}_3(\text{cyt})] \). The third band, which adhered to the top of the column, was eluted easily when \( \text{NaCl} \) was added to the buffer. This chromatographic behavior is identical with that of the cytochrome \( c \) from baker's yeast, a protein that contains free cysteine 102 and dimerizes by the formation of a disulfide bond \( (37,42) \). This great similarity in the chromatographic mobility was an early indication that the third fraction obtained from the cation-exchange column contains a diprotein complex.

   The molecular mass of \( 30.7 \pm 0.8 \text{ kDa} \), determined by size-exclusion chromatography as shown in Figure 1, confirmed this hypothesis. The value is greater than that expected of \( [\text{PtCl}_2(\text{cyt})_2] \) by about 18%, but this discrepancy becomes understandable in view of the mechanism of gel-filtration chromatography. A strict proportionality between the elution rate and the \( \log M_R \), (itself an approximate
measure of the biopolymer size) is obtained only with protein molecules of similar shapes. The seven globular, one-chain proteins that are used as standards indeed fall on a straight line, as Figure 1 shows. Linking of two spheroidal molecules of cytochrome c via a PtCl₂ bridge, however, results in an elongated diprotein complex, which, to Sephadex gel, appears a little larger than a spheroidal protein of the same molecular mass.

2. The binding sites

The amino acid side chains through which the protein molecules coordinate to the Pt atom are identified on the basis of unambiguous crystallographic and NMR findings by others. Dickerson and co-workers introduced, and many others applied, PtCl₄²⁻ as a specific reagent for the methionine residue in proteins (43-45). It labels Met 65 in ferricytochrome c from horse virtually exclusively although incubation at pH 6.2 resulted in very slight additional labeling of His 33 (43). Studies by ¹H NMR spectroscopy showed that, at pH 5.5, PtCl₄²⁻ binds exclusively to methionine: to Met 29 in ribonuclease (46) and, again, to Met 65 in cytochrome c from tuna (47). We carried out the incubation at pH 5.5, so that the imidazole side chain, whose pKₐ is 6.4 (48), is protonated and unreactive. Labeling of Met 80, an axial ligand to the Fe atom in the heme, is ruled out on the basis of the aforementioned crystallographic and NMR studies, and because the spectroscopic and redox properties of the cytochrome c are not altered in the diprotein complexes. To conclude, the proteins are coordinated to the Pt atom through the S atoms in their respective Met 65 residues.

The new protein dimers are remarkably stable owing to the well-known affinity of thioether ligands for the Pt(II) atom. The [PtCl₂(cyt)₂] complex, for example, remains intact even after several months in the buffered solution at room temperature. Size-exclusion
Figure 1. A plot of log molecular weight versus $t_e/t_0$ from size-exclusion chromatography; determination of the molecular mass of $\text{trans-}[\text{PtCl}_2(\text{cyt})_2]$, a complex containing cytochrome $c$ from horse heart. The molecular mass of 30.7 ± 0.8 kDa is determined by the procedure described in section II, part C3. Elution and void times, designated $t_e$ and $t_0$ respectively, are measured more accurately than the corresponding volumes.
chromatography of the aged solution indicates a complete absence of the monomeric cytochrome c, i.e., of dissociation. The covalent bonds between the Pt atom and its binding sites in the two proteins can be cleaved, however, by highly nucleophilic ligands. Thiourea, designated tu, can displace the proteins and extrude the platinum link, as shown in eq 6. This combination of stability under ordinary conditions and easy

\[ [\text{PtCl}_2(\text{cyt})_2] + 4\text{tu} \rightarrow 2\text{cyt} + [\text{Pt(tu)}_4]\text{Cl}_2 \quad (6) \]

removability under conditions harmless to the protein is a particularly useful feature of the PtL₂ links.

The question of stereochemistry at the Pt atom in [PtCl₂(cyt)₂] was addressed by experiments in which cytochrome c was incubated with cis-[Pt(bpy)(DMF)₂]²⁺, a compound whose disubstituted derivative would have to adopt a cis configuration. As eq 7 shows, only monoprotein complexes are obtained. The lability of DMF ligands

\[
\begin{align*}
\text{cis-Pt} & \quad \text{cyt} \\
\text{DMF} & \quad \text{cyt} \\
\end{align*}
\]

notwithstanding, the isolated monoprotein complex cannot bind the second molecule of cytochrome c. Since two cytochrome molecules apparently cannot occupy cis positions, the diprotein complexes probably have trans configurations, as would be expected in view of the protein size.

The shortest interprotein distance in trans-[PtL₂(cyt)₂] complex
can be estimated at 4.6 Å, twice the length of a typical Pt-SR₂ bond (49). The shortest interatomic contacts in the crystal between the two molecules of tuna cytochrome c in the unit cell fall in the range 4.5-5.0 Å (50). Although inexact, this comparison indicates that trans linking through a PtL₂ fragment does not bring the protein molecules prohibitively close to each other. Coordination in the cis positions is ruled out, for it would place the S donor atoms about 3.2 Å from each other.

Formation of the bis(cytochrome c) complexes is shown schematically in equation 8. The first protein ligand, bonded to the Pt atom through the S atom of Met 65, facilitates the second substitution by a kinetic trans effect, a well-known property of thioether ligands

\[
\text{Cl} \quad \text{L-Pt-L} \quad \text{cyt} \quad -\text{Cl}^- \quad \text{L-Pt-L} \quad \text{cyt} \quad -\text{Cl}^- \quad \text{L-Pt-L}
\]

where L is: Cl⁻ or

and

\[
\text{Fe} \quad \text{S-CH₃} \quad \text{Fe}
\]

is cytochrome c (cyt)
F. Structural and Redox Properties of the Diprotein Complexes

The diprotein complexes \textit{trans}-[PtCl\textsubscript{2}(cyt)\textsubscript{2}] and \textit{trans}-[Pt(2-Fpy)\textsubscript{2}(cyt)\textsubscript{2}] were compared with one another and with the native cytochrome \textit{c} by various physical methods in order to determine whether linking alters the protein structure and properties of its active site. Most of the findings are presented in Table 1.

The EPR spectra confirm that the electronic structure of the ferriheme is virtually unperturbed upon linking of the proteins; the slight differences among the \(g\) values fall within the error limits of the measurement (51-53). A small amount (less than ca. 10\%) of the protein exists in the high-spin Fe\textit{III} form, characterized by values of about 2.0 and 6.0. Since the UV-vis absorption maxima are not moved significantly, whereas the Soret and other bands are known to move to shorter wavelengths in high-spin heme proteins (54), this form must represent a very minor constituent of the protein dimers.

Particularly diagnostic is the absorption band at 695 nm, whose intensity depends in part on the interactions between the Fe atom and its axial ligands, Met 80 and His 18 (55-57). Although its absorptivity of ca. 1.0 mM\textsuperscript{-1}cm\textsuperscript{-1} is too low to permit accurate comparisons of the band intensity among proteins, the presence of this band in the spectra of the dimer indicates that the Fe atoms remain hexacoordinate. Dissociation of the Met 80 ligand, a process detectable even in the native cytochrome \textit{c} (58) and responsible for conversion to the high-spin form, is slightly enhanced in the protein dimer.

The reduction potential of the diprotein complex, ca. 220 mV, is lower by ca. 30 mV than that of the native protein because of the presence of some high-spin form. Since the reduction potential of the high-spin cytochrome \textit{c} from \textit{Rhodospirillum rubrum} is 10 mV at pH 7 (59), 240 mV below that of the low-spin proteins, the observed difference of 30 mV can be taken as further evidence that only a minor fraction of the diprotein complex exists in the high-spin form.
Table 1. Electronic and structural characteristics of native cytochrome c from horse heart and of its two bis(protein)platinum complexes

<table>
<thead>
<tr>
<th>property</th>
<th>cyt</th>
<th>trans-[PtCl$_2$-(cyt)$_2$]</th>
<th>trans-[Pt(2-Fpy)$_2$-(cyt)$_2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPR $g$ values$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$g_x$</td>
<td>1.26</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>$g_y$ $^b$</td>
<td>2.339</td>
<td>2.291</td>
<td>2.299</td>
</tr>
<tr>
<td>$g_z$</td>
<td>3.018</td>
<td>2.976</td>
<td>2.986</td>
</tr>
<tr>
<td>$g_{//}$ $^c$</td>
<td>2.167$c$</td>
<td>1.991$d$</td>
<td>1.989$d$</td>
</tr>
<tr>
<td>$g_\perp$ $^d$</td>
<td>6.045$c$</td>
<td>5.948$d$</td>
<td>5.893$d$</td>
</tr>
<tr>
<td>UV-vis abs max, nm$^e$</td>
<td>409</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>535</td>
<td>535</td>
<td>534</td>
</tr>
<tr>
<td></td>
<td>695</td>
<td>695</td>
<td>695</td>
</tr>
<tr>
<td>reduction pot., mV vs NHE$^f$</td>
<td>257 ± 5</td>
<td>220 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ In 150 mM phosphate buffer at pH 7.0.
$^b$ Measured at the peak maximum.
$^c$ High-spin ferriheme in less than 2% of the native protein.
$^d$ High-spin ferriheme in less than 10% of the diprotein complex.
$^e$ In 85 mM phosphate buffer at pH 5.5.
$^f$ In 85 mM phosphate buffer at pH 7.0 and at 25°C, with 4,4'-bipyridyl as mediator.
Table 1 (Continued)

<table>
<thead>
<tr>
<th>hyperfine-shifted $^1$H NMR resonances of CH$_3$ groups, ppm $^g$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$ in ring IV</td>
<td>35.68</td>
<td>35.7</td>
</tr>
<tr>
<td>CH$_3$ in ring II</td>
<td>32.53</td>
<td>32.53</td>
</tr>
<tr>
<td>S-CH$_2$CH$_3$ in ring II or CH 2 and CH 4 of His 18</td>
<td>24.5</td>
<td>25.4</td>
</tr>
<tr>
<td>-CH$_2$ of propionate in ring IV</td>
<td>19.24</td>
<td>19.3</td>
</tr>
<tr>
<td>$\beta$-CH$_2$ of His 18</td>
<td>14.63</td>
<td>14.65</td>
</tr>
<tr>
<td>unassigned</td>
<td>12.71</td>
<td>12.8</td>
</tr>
<tr>
<td>-CH$_2$ of propionate in ring IV</td>
<td>11.41</td>
<td>11.4</td>
</tr>
<tr>
<td>CH$_3$ of Met 80</td>
<td>-24.49</td>
<td>-24.49</td>
</tr>
<tr>
<td>$\gamma$-CH$_2$ of Met 80</td>
<td>-28.3</td>
<td>-28.3</td>
</tr>
<tr>
<td>far-IR bands, cm$^{-1}$</td>
<td>345$^h$</td>
<td>343</td>
</tr>
</tbody>
</table>

$^g$ With respect to DSS as standard.

$^h$ Mixture of native cytochrome $c$ and K$_2$PtCl$_4$ in the molar ratio of 2:1.

The protein NMR spectrum of the active site in heme proteins depends markedly on the interactions between the paramagnetic Fe(III) atom and its axial ligands and between the heme periphery and the side chains of neighboring amino acid residues (60-65). Particularly sensitive to the protein conformation are the hyperfine $^1$H shifts, which have been assigned (66-70). They remain unperturbed in the diprotein complex.

The PtCl$_2$ link between the protein molecules is directly detectable by IR spectroscopy. The complex $trans$-[PtCl$_2$(cyt)$_2$]
Table 2. $^{19}$F NMR spectra of model complexes and of a bis(cytochrome $c$)platinum(II) complex

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$^{19}$F Chem Shift, ppm</th>
<th>$^{19}$F Chem Shift from CF$_3$COOH</th>
<th>$3J(^{195}$Pt-$^{19}$F) Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-[Pt(2-Fpy)$_2$Cl$_2$]</td>
<td>DMF</td>
<td>13.14</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>14.10</td>
<td>165</td>
</tr>
<tr>
<td>trans-[Pt(2-Fpy)$_2$Cl$_2$]</td>
<td>DMF</td>
<td>15.58</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>H$_2$O</td>
<td>15.91</td>
<td>160</td>
</tr>
<tr>
<td>trans-[Pt(2-Fpy)$_2$(Im)$_2$]$^{2+}$</td>
<td>H$_2$O</td>
<td>13.38</td>
<td>214</td>
</tr>
<tr>
<td>trans-[Pt(2-Fpy)$_2$(AcMetH)$_2$]$^c$</td>
<td>H$_2$O</td>
<td>12.61</td>
<td>172</td>
</tr>
<tr>
<td>trans-[Pt(2-Fpy)$_2$(cyt$^{II}_2$)$^d$</td>
<td>H$_2$O</td>
<td>14.7</td>
<td>-</td>
</tr>
<tr>
<td>trans-[Pt(2-Fpy)$_2$(cyt$^{III}_2$)$^d$</td>
<td>H$_2$O</td>
<td>14.7</td>
<td>-</td>
</tr>
</tbody>
</table>

a 80% liquid indicated and 20% D$_2$O.

b Im is imidazole.

c AcMetH is N-acetyl-L-methionine.
d The oxidation state of iron is indicated.

exhibits a single band in the 650-200 cm$^{-1}$, as expected of a trans-PtCl$_2$ fragment. The position of this Pt-Cl stretching band is virtually the same as that of the band exhibited by a mixture of cytochrome $c$ and K$_2$PtCl$_4$ in the ratio of 2:1.

The presence of ancillary 2-fluoropyridine ligands permits easier detection of the link by $^{19}$F NMR spectroscopy. As Table 2 shows, the $^{19}$F chemical shift and the $^{195}$Pt-$^{19}$F coupling constant in model complexes depend on the other two ligands coordinated to the Pt
atom. The $^{19}$F chemical shift of $\text{trans-}[\text{Pt}(2\text{-Fpy})_2(\text{cyt})_2]$ falls in the expected interval, but the width of the resonance band, caused by the slow nuclear relaxation in the macromolecular complex, limits the usefulness of $^{19}$F NMR spectroscopy for structural studies. Since direct methods are preferable, the binding site on the protein was identified unambiguously on the basis of the known selectivity of the PtCl$_4^{2-}$ complex toward cytochrome $c$, as explained in section III part E.

G. Advantages and Prospective Applications of Cross-Linking via Platinum Complexes

This report introduces inorganic complexes as reagents for cross-linking of proteins. In addition to the general properties of transition metals, mentioned in section III part A, platinum exhibits several advantages. Whereas the common organic reagents react with amino and thiol groups, PtCl$_4^{2-}$ seems to be specific for thioether groups. The reagent is stable in aqueous solution and reacts with proteins under physiological conditions. Behavior of the prototypal bis(cytochrome $c$) complexes indicates that the cross-links are stable indefinitely under ordinary conditions and yet cleavable in a mild reaction. Especially attractive is the prospect of altering the reagent properties by varying the ancillary ligands; in this study they are Cl$^-$ and 2-Fpy. Bimetallic complexes in which the metal atoms are tethered with cyclic and acyclic ligands of different length promise to combine the advantages of the existing organic and new inorganic reagents.
IV. OXIDOREDUCTION REACTIONS INVOLVING THE ELECTROSTATIC AND THE COVALENT COMPLEX OF CYTOCHROME C AND PLASTOCYANIN — ZERO-LENGTH TETHERING WITH THE HETEROBIFUNCTIONAL CROSS-LINKING REAGENT EDC

A. Introduction

Pairwise associations between various redox proteins have been simulated by computer graphics and examined by chromatographic, spectroscopic, and other methods. These complexes owe their stability to electrostatic and hydrophobic interactions between the protein molecules involved (71). Most of the studies to date have dealt with complexes containing cytochrome c as one component and any of the following metalloproteins as the other: cytochrome c peroxidase (72, 73), cytochrome b5 (74, 75), cytochrome c reductase (76), cytochrome c oxidase (77), flavodoxin (69), and plastocyanin (3, 78, 79). Although a true structure for none of the complexes is known, the first crystallographic success probably is not far in the future.

Diprotein complexes and hybrid hemoglobins are well suited to the study of biological electron-transfer reactions. The distance between the donor and the acceptor and their mutual orientation can be estimated reliably from the crystal structures and from the computed models. The driving force and the intervening medium can be varied purposefully by metal substitution and by site-specific mutagenesis. Although the investigation of intracomplex redox reactions began only several years ago, interesting findings have been made, and important new questions have been raised (80-91). The kinetics of these reactions is one aspect of the present study.

When dissociation of a protein complex must be avoided its constituents are cross-linked. Besides the standard bifunctional
reagents, transition-metal complexes are beginning to be used for this purpose (1, 92); refer to section III. Additionally, the use of homobifunctional cross-linkers as variable-length tethers in covalent cytochrome $c$/plastocyanin complexes is described in section V. In any case, it is necessary to determine whether the cross-linked aggregate is a correct model for the natural one. Such a determination is another aspect of the present study.

This seems to be only the second report on an electron-transfer reaction within a complex containing an iron protein and a copper protein; see King et al. (3). Although cytochrome $c$ and plastocyanin do not participate in the same electron-transfer chain, their association is similar to the natural associations of the former with cytochrome $b_5$ (93) and with cytochrome $c$ peroxidase (94) and of the latter with cytochrome $f$ (95). Much is known about the redox reactions of cytochrome $c$ (44, 96) and plastocyanin (19) with each other and with inorganic reagents. These previous studies set the stage for the present one, whose aim is to examine whether cross-linking of these proteins affects the following reactions: 1) the oxidation of ferrocytochrome $c$ by inorganic reagents (eq 9); 2) the reduction of ferricytochrome $c$ by hydrated electrons (eq 10); 3) the reduction of ferricytochrome $c$ and cupriplastocyanin by flavin semiquinones — where FH$^*$ is lumiflavin or FMN — (eq 11 and 12); and 4) the oxidation of ferrocytochrome $c$ by cuproplastocyanin (eq 13). The first reaction, involving $[\text{Fe(CN)}_6]^3-$ and $[\text{Fe(C_5H_5)2}]^{2+}$ as oxidants, is studied by stopped-flow spectrophotometry. Reactions 10 through 13 are studied by pulse radiolysis (eq 10 and 13) and by flash photolysis (eq 11, 12, and 13). Kinetics studies of reactions 9 through 13 involved both the electrostatic (natural) and the covalent (cross-linked) diprotein complexes.
B. Plastocyanin

Plastocyanin is a well-characterized, 10.5 kDa, single-chain, blue copper protein. It is unavailable commercially but is routinely isolated from the leaves of higher plants. It is a mobile, one-electron shuttle between photosystems II and I in the photosynthetic electron-transfer pathway. Plastocyanin contains a single Cu atom which is ligated by residues His 37, Cys 84, His 87, and Met 92 (in *Phaseolus vulgaris* and French bean plastocyanin) in a distorted tetrahedral coordination. The metal is buried near the "top" of the molecule, in a hydrophobic region; the imidazole ring of His 87 is exposed on the surface, however (97-99). The copper exists in oxidation states of II and I, with a standard reduction potential of 0.36 V versus NHE.

An excess of negatively-charged aspartate and glutamate residues, with only five positively-charged lysines, gives cupriplastocyanin a net charge of -9, at pH 7.0. Asymmetric distribution of these residues on the surface of the protein results in a negatively-charged "east" patch. The lysines are scattered over the surface of the lower half of the protein. Interactions of plastocyanin
with redox partners occur in two general regions: the hydrophobic "north" patch and the hydrophilic "east" patch. Partitioning between these two sites is largely controlled by the hydrophilic/hydrophobic character of the external redox partner (40, 100-107).

C. Preparation of the cyt/pc Complexes

The electrostatic complex was made by mixing equimolar amounts of the two proteins in the same buffer; the solvent and ionic strength of the mixture is conveniently changed using ultrafiltration. The covalent complex was made by a published method (2). The reaction mixture was 80 µM in each protein and 1 mM in EDC; the solvent was 5 mM MOPS at pH 6.5, with 0 and 100 mM additional NaCl; and the incubation lasted from 16 to 29 hours at room temperature. The product was purified by size-exclusion chromatography on a Sephadex G-75 (50 mesh) gel — with the yield of the diprotein fraction always being greater than 95%.

This heterogeneous covalent complex (37 mg or 1.6 µmol) was dialyzed into 10 mM phosphate buffer at pH 7.0, concentrated, and applied to a CM 52 column (sized 2.5 x 8.5 cm). For the reaction whose solvent contained no additional NaCl, the first three bands were eluted by this 10 mM buffer, whereas the fourth one required a shallow gradient from 10 mM to ca. 30 mM buffer. The relative amounts of the cyt/pc isomers were virtually independent of the incubation time with EDC. The average chromatographic yields from several syntheses were as follows: 19, 26, 29, and 26% for the isomers numbered C1, C2, C3 and C4 in the order of elution. All kinetic studies of EDC cross-linked diprotein complexes performed thus far involve these four isomers — synthesized in 5 mM MOPS buffer with no additional NaCl.

A second set of reaction conditions (having 100 mM additional
NaCl) have also been explored. The yield of heterogeneous diprotein complex has now decreased from greater than 95% to only 30%. Separation of the diprotein complexes on CM 52 again produces four distinct bands whose elution character is similar to, but whose relative yields are different from, those obtained at lower ionic strength. Chromatographic yields were, approximately: 40, 30, and 30% for isomer C1, isomers C2 and C3 combined, and isomer C4, respectively (numbered in the order of elution).

D. Properties of the cyt/pc Complexes

1. Electrostatic and covalent binding

Cytochrome c and plastocyanin associate strongly with each other because their respective charges (in the oxidized state at pH 7.0) are approximately +8 and -9. The docking probably occurs near the exposed heme edge on the "front" side of the former protein, where the basic lysine residues are concentrated, and on the "east" side of the latter one, where the acidic glutamate and aspartate residues are clustered (78, 79). This view of the attachment is supported by much experimental evidence and by a preliminary simulation with computer graphics (80). The copper-iron distance in the complex is ca. 18 Å. Perhaps more relevant to the electron-transfer reaction is the shortest distance between the respective coordination spheres; the sulfur atom of Cys 84 in plastocyanin and the nearest edge of the heme group in cytochrome c are ca. 12 Å apart (80).

Cross-linking by the water soluble EDC (Scheme I) amounts to formation of amide bonds between allegedly the same lysine side chains in cytochrome c and the same aspartate or glutamate side chains in plastocyanin that are implicated in the electrostatic docking. The former residues probably include Lys 13 and 86 (73, 108). The latter residues occur mainly in the segment 42-45 but also at the
positions 59-61 and 68 (109, 110). The close structural similarity between the electrostatic and covalent complexes of cyt/pc has been claimed (2, 3), but this claim can be examined only when the cross-linked residues in the latter complex are identified.

In view of the number of potential cross-linking sites in the two proteins, the heterogeneity of the covalent cyt/pc complex appeared to us inevitable. Indeed, chromatography on CM 52 yielded four distinct
fractions whose elution behavior reveals some of their properties. The diprotein complex probably interacts with the cation exchanger through cytochrome c, which is positively charged. This interaction is weak — a dilute buffer is sufficient for elution — because the cyt/pc complex as a whole is nearly electroneutral. The isomers probably differ only slightly from one another in the net charge or in the distribution of local charges. The net charge should not depend on the number of cross-links because formation of each amide bond neutralizes one cationic and one anionic side chain. The distribution of charges should depend on the number of cross-links, on their location, and on possible modification (without cross-linking) of the two proteins.

Heterogeneity of the covalent complex between cytochrome c and cytochrome c peroxidase has recently been investigated (111), but the present study seems to be the first one in which such isomers are separated from one another. They were mostly characterized together but were examined one by one for the intracomplex electron-transfer reaction. The four isomers of the covalent cyt/pc complex are henceforth referred to as C1, C2, C3, and C4, in the order in which they elute.

2. Composition and the metal sites in the covalent complex

Geren et al. (2) purified the cyt/pc complex and determined its composition. We corroborated their findings. Size-exclusion chromatography of the combined isomers yielded a single, homogeneous band. The unperturbed visible spectra of the four separate isomers confirmed the chromophore ratio of 1.00 ± 0.08 in each one. The covalent complex proved completely stable upon repeated oxidations, reductions, dialyses, elutions from Sephadex G-75 and CM 52 columns, stopped-flow experiments, pulse-radiolysis experiments and flash photolysis experiments.

The apparent molecular mass of 27.0 ± 1.2 kDa, an average result of several determinations, is ca. 17% greater than the actual
value. This error is understandable in view of the mechanism of size-exclusion chromatography. A strict proportionality between the elution time and log $M_r$ — molecular mass itself is only an approximate measure of the biopolymer size — is obtained only with protein molecules of similar shapes. Several globular, single-chain proteins defined linear calibration plots, but the diprotein complex eluted as if it were somewhat larger than a spheroidal protein of the same molecular mass. This phenomenon has been studied semiquantitatively with diprotein complexes whose elongation varied with the size of the inorganic complex used for cross-linking (1, 92); refer to Table A-1 in the appendix.

We further characterized the cyt(III)/pc(II) complex by EPR spectroscopy; the findings are in Table 3. The overlapping $g_y$ component for cytochrome $c$ and $g_{11}$ component for plastocyanin were nevertheless identified; other signals were assigned straightforwardly. Covalent cross-linking does not seem to cause significant electronic perturbation of the iron and copper sites.

3. **Reduction potentials of the covalent complex**

Differential-pulse voltammograms contained a clear wave for cytochrome $c$ but only a shoulder on the anodic side of this wave for plastocyanin. These findings are consistent with the report that cross-linking of these proteins hampers their electroactivity at the surface of an electrode (112). An alternative method for determining the reduction potential of plastocyanin was through a redox titration with $[\text{Fe(CN)}_6]^{4-}$, monitored with UV-vis spectroscopy; this succeeded easily.

The reduction potentials of the cross-linked cytochrome $c$ and plastocyanin are slightly lower and slightly higher, respectively, than the corresponding potentials of the native proteins. A very similar increase occurs when plastocyanin is modified with an amine (109,
Table 3. Properties of the covalent complex between cytochrome c and plastocyanin, cyt/pc, and of the native proteins

<table>
<thead>
<tr>
<th>property</th>
<th>cyt/pc</th>
<th>cyt</th>
<th>pc</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPR g values</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$g_x$</td>
<td>1.3</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td>$g_y$</td>
<td>2.3</td>
<td>2.25</td>
<td></td>
</tr>
<tr>
<td>$g_z$</td>
<td>3.09</td>
<td>3.02</td>
<td></td>
</tr>
<tr>
<td>$g_{\perp}$</td>
<td>2.3</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>$g_{\perp}$</td>
<td>2.056</td>
<td>2.053</td>
<td></td>
</tr>
<tr>
<td>reduction potential at 25°C (mV vs. NHE)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>245 ± 5\textsuperscript{c}</td>
<td>256 ± 2\textsuperscript{c,d}</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>385 ± 5\textsuperscript{e}</td>
<td></td>
<td>360 ± 4\textsuperscript{e,f}</td>
</tr>
<tr>
<td>molecular mass (kDa)\textsuperscript{g}</td>
<td>27.0 ± 1.2</td>
<td>12.5</td>
<td>10.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} In 85 mM phosphate buffer at pH 7.0.
\textsuperscript{b} Measured at peak maximum.
\textsuperscript{c} Determined by differential-pulse voltammetry with 4,4'-bipyridine as mediator.
\textsuperscript{d} References 16 and 113.
\textsuperscript{e} Determined by spectrophotometric titration with [Fe(CN)₆]⁴⁻.
\textsuperscript{f} References 19 and 113.
\textsuperscript{g} Determined by size-exclusion chromatography.

110). The "divergence" of the reduction potentials upon cross-linking probably is caused more by the neutralization of the charged side...
chains than by the proximity of the protein molecules to each other. The directions in which these potentials shift are consistent with the charges that are neutralized; since the charges in the two proteins are opposite, the shifts are opposite.

E. Extracomplex Oxidoreduction Reactions

Geren et al. (2) showed qualitatively that cytochrome c in the covalent cyt/pc complex is reducible by small reagents but that it is virtually unreactive toward cytochrome c oxidase. We confirmed the reducibility of cytochrome c and of plastocyanin in cyt(III)/pc(II) with ascorbate and \([\text{Fe(CN)}_6]^{4-}\) ions.

1. Oxidation by iron reagents

In order to examine quantitatively the possible effect of cross-linking on the redox reactions of cytochrome c we used stopped-flow spectrophotometry to study the kinetics of the reaction in eq 9 with

\[
\text{Fe(III) complex + cyt(II)/pc(I)} \quad \rightarrow \quad \text{cyt(III)/pc(I)} + \text{Fe(II) complex} \quad (9)
\]

Two different ferric compounds: \([\text{Fe(CN)}_6]^3-\), a hydrophilic anion (\(E^o = 0.42 V\)), and \([\text{Fe(C}_5\text{H}_5\text{)}_2]^+\), a hydrophobic cation (\(E^o = 0.40 V\)). The findings are listed in Table 4. The presence of plastocyanin somewhat inhibits the oxidation of ferrocytochrome c by \([\text{Fe(CN)}_6]^3-\) and somewhat promotes its oxidation by \([\text{Fe(C}_5\text{H}_5\text{)}_2]^+\). Both changes are attributable to the negative charge of plastocyanin. Its electrostatic effect may be direct (on the oxidants), or indirect (on the positive charge of cytochrome c), or both. A steric effect may operate, too. Hexacyanoferrate (III) anion reacts mainly near the exposed heme
edge (68), in the same general area on the cytochrome c surface to which plastocyanin is attached. Therefore, the inhibition may be due partially to a shielding, by plastocyanin, of the heme edge from the inorganic oxidant. For a more detailed discussion of the promotion of the reaction with ferricenium cation, it should be known whether it interacts with cytochrome c specifically and, if so, which region of the protein surface is involved (17). Without this knowledge, it can only be conjectured that any steric shielding of the heme is overcome by the electrostatic effect of plastocyanin.

Table 4. Rate constants for the bimolecular oxidation of ferroheme in the native cytochrome c and in the covalent complex between cytochrome c and plastocyanin

<table>
<thead>
<tr>
<th>oxidant</th>
<th>reductant</th>
<th>$k \times 10^{-6}$ (M$^{-1}$s$^{-1}$)</th>
<th>ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{Fe(CN)}_6]^{3-}$</td>
<td>cyt(II)</td>
<td>8.0</td>
<td>_b</td>
</tr>
<tr>
<td>$[\text{Fe(CN)}_6]^{3-}$</td>
<td>cyt(II)/pc(I)</td>
<td>2.1</td>
<td>this work</td>
</tr>
<tr>
<td>$[\text{Fe(C}_5\text{H}_5\text{)}_2]^+$</td>
<td>cyt(II)</td>
<td>6.4</td>
<td>_c</td>
</tr>
<tr>
<td>$[\text{Fe(C}_5\text{H}_5\text{)}_2]^+$</td>
<td>cyt(II)/pc(I)</td>
<td>9.0</td>
<td>this work</td>
</tr>
</tbody>
</table>

a Determined by stopped-flow spectrophotometry, in 85 mM phosphate buffer at pH 7.0, at 25°C.
b References 68 and 114.
c Reference 17.

2. Reduction by hydrated electrons
Since the visible absorption bands of e$_{aq}^-$ and of blue copper overlap and since the reduction of cupriplastocyanin in cyt(III)/pc(II)
yields the thermodynamically stable cyt(III)/pc(I), pulse-radiolysis studies of this process were not carried out in detail. Reduction of ferricytochrome c (eq 10), however, was examined quantitatively

\[ \text{eaq}^- + \text{cyt(III)} / \text{pc(II)} \rightarrow \text{cyt(II)} / \text{pc(II)} \]  

(10)
because it yields the unstable cyt(II)/pc(II), in which the interprotein electron-transfer reaction is thermodynamically allowed. The rate of initial reduction of the ferriheme in the native cytochrome c and in the electrostatic and covalent complexes cyt(III)/pc(II) is diffusion-controlled; representative traces are presented in Figure 2. The average rate constant for the three cases is \((1.2 \pm 0.2) \times 10^{10} \text{M}^{-1}\text{s}^{-1}\), in accord with the previous determination for the native protein (115-118). The representative traces are divided approximately equally between ferricytochrome c and cupriplastocyanin, whereas in the electrostatic complex there is a slight preference for the reduction of ferricytochrome c. Ferriheme in each of the four isomers of the covalent complex proved reducible by eaq⁻.

a. Native proteins  Bimolecular rate constants for the lumiflavin and FMN semiquinone reduction of native ferricytochrome c and native cupriplastocyanin are given in Table 6. The FMN reduction of cytochrome c and plastocyanin experiences attractive and repulsive coulombic interactions, respectively, owing to the negative charge on FMN. Accordingly, the rate constants are some 20-times larger for the reduction of cytochrome c than for plastocyanin. In comparison, the rate constants for the lumiflavin reduction of cytochrome c and plastocyanin are of comparable magnitude, as expected for a neutral semiquinone.

b. Electrostatic cyt/pc complex  A one-to-one mixture of cytochrome c and plastocyanin at low ionic strength contains the electrostatically-associated cyt/pc complex. Addition of a subequivalent
Figure 2. Absorbance versus time traces monitoring the reduction of ferricytochrome $c$ by $e_{aq}^-$ at 360 nm, in phosphate buffer at pH 7.0.

(a) Native cyt(III), 20.5 $\mu$M, at $\mu = 40$ mM.
(b) Electrostatic complex cyt(III)/pc(II), 20.5 $\mu$M, at $\mu = 40$ mM.
(c) Covalent complex cyt(III)/pc(II), 12.1 $\mu$M, at $\mu = 2$ mM.
**3. Reduction by flavin semiquinones**

**Table 5. The structure and selected physical characteristics of lumiflavin and flavin mononucleotide**

<table>
<thead>
<tr>
<th>Physical Characteristics</th>
<th>Lumiflavin (Llf)</th>
<th>Flavin mononucleotide (FMN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-CH₃</td>
<td>-CH₂-(HCOH)&lt;sub&gt;3&lt;/sub&gt;-CH₂-OPO₃²⁻</td>
</tr>
<tr>
<td>Net charge at pH 7.0</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>442</td>
<td>446</td>
</tr>
<tr>
<td>ε (mM⁻¹cm⁻¹)</td>
<td>11.0</td>
<td>11.0</td>
</tr>
<tr>
<td>E&lt;sup&gt;°&lt;/sup&gt;F/FH (mV vs NHE)</td>
<td>-231&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-238&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>k&lt;sub&gt;2&lt;/sub&gt; (M⁻¹s⁻¹) for:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FH⁻ + FH → F + FH⁻</td>
<td>9.4(10&lt;sup&gt;9&lt;/sup&gt;)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.1(10&lt;sup&gt;9&lt;/sup&gt;)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Refer to the structure in Scheme II.
<sup>b</sup> Reference 119.
<sup>c</sup> Reference 120.
<sup>d</sup> Reference 121.
Scheme II

\[ \text{F (oxidized)} \]

\[ \text{FH}^- \text{ (semiquinone)} \]

\[ \text{FH}^- \text{ (fully-reduced)} \]

Where \(-R\) is:
- \(-\text{CH}_3\) for lumiflavin, or
- \(-\text{CH}_2-(\text{HCOH})_3\text{-CH}_2\text{-OPO}_3^2^-\) for FMN.
Table 6. Bimolecular rate constants for the semiquinone reduction of cytochrome \( c \) and plastocyanin\(^a\)

<table>
<thead>
<tr>
<th>reaction</th>
<th>( k_2 \times 10^{-7} \text{M}^{-1}\text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FMN(^b)</td>
</tr>
<tr>
<td>( \text{FH}^{-} + \text{cyt(III)} \rightarrow \text{cyt(II)} )(^d)</td>
<td>6.8</td>
</tr>
<tr>
<td>( \text{FH}^{-} + \text{pc(II)} \rightarrow \text{pc(I)} )</td>
<td>0.314</td>
</tr>
</tbody>
</table>

\(^a\) Where \( \mu \) is 10 mM phosphate buffer at pH 7.0; protein is in pseudo-first-order excess over the flavin semiquinone.

\(^b\) FMN reacts at the exposed heme edge; ref. 122.

\(^c\) General information on Lf is in ref. 123.

\(^d\) The reducing semiquinone, \( \text{FH}^{-} \), is FMN or Lf as indicated.

of flavin semiquinone results in the pseudo-first-order reduction of a small amount of each protein. Reduction of each protein is monitored independently and quantitated; the partitioning of the semiquinone between eq 11 and eq 12 was thus determined (see Table 7). Reduction of plastocyanin is thermodynamically favored over that of cytochrome \( c \); cuproplastocyanin does not participate in subsequent electron transfer reactions. Reduction of ferricytochrome \( c \), however, produces \( \text{cyt(II)}/\text{pc(II)} \) which subsequently undergoes electron transfer to yield
cyt(III)/pc(I) (eq 13). The desired semiquinone reduction product, ferrocytochrome c, is produced in greater yield with the negatively-charged FMN than with the neutral lumiflavin species; FMN was thus the flavin of choice for use with the electrostatic and the covalent cyt/pc complexes.

Both the attractive and repulsive electrostatic interactions inherent, respectively, in the reactions FMN + cyt(III) and FMN + pc(II) are greatly minimized in the nearly electroneutral cyt/pc electrostatic complex. Indeed, the FMN semiquinone reductant partitions itself equally between ferricytochrome c and cupriplastocyanin. Bimolecular rate constants were not determined for FMN reduction of either protein in the electrostatic complex. These reactions could not be followed for a sufficient number of half-lives before the fast subsequent reaction, eq 13, interfered with the trace through production of additional cuproplastocyanin and consumption of ferrocytochrome c.

c. Covalent cyt/pc complex  The majority of kinetics were done using the FMN semiquinone as reductant in order to maximize the production of ferrocytochrome c, i.e., the intermediate cyt(II)/pc(II). Formation of this kinetic intermediate allows one to monitor the subsequent intracomplex electron-transfer, eq 13. As shown in Table 7, partitioning of the reducing semiquinone between plastocyanin (eq 11) and cytochrome c (eq 12) is similar in the electrostatic and covalent diprotein complexes. Reduction of plastocyanin is slightly favored in the C1, C2, and C3 covalent isomers, whereas partitioning between cytochrome c and plastocyanin is equimolar in covalent isomer C4. The lower yield of ferrocytochrome c in the first three isomers reflects a decrease in the accessibility of the heme edge, and/or a lessening of
Table 7. Partitioning in the semiquinone reduction between cytochrome c and plastocyanin in the electrostatic and covalent cyt/pc complexes

\[
\begin{align*}
FH^- + \text{cyt(III)/pc(II)} & \rightleftharpoons \text{cyt(II)/pc(II)} & (11) \\
& \rightleftharpoons \text{cyt(III)/pc(I)} & (12)
\end{align*}
\]

<table>
<thead>
<tr>
<th>complex cyt/pc</th>
<th>FH(^b)</th>
<th>[cyt(II)/pc(II)]:[cyt(III)/pc(I)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>electrostatic</td>
<td>Lf</td>
<td>1:3</td>
</tr>
<tr>
<td></td>
<td>FMN</td>
<td>1:1</td>
</tr>
<tr>
<td>covalent:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>isomers C1, C2, C3</td>
<td>FMN</td>
<td>1:1.5</td>
</tr>
<tr>
<td>isomer C4</td>
<td>FMN</td>
<td>1:1</td>
</tr>
</tbody>
</table>

\(^a\) Where \(\mu\) is 10 mM at pH 7.0 phosphate buffer; cyt(II) and pc(II) are in pseudo-first-order excess over the flavin semiquinone.

\(^b\) The reducing semiquinone, FH\(^-\), is FMN or Lf as indicated.

The presence of the positively-charged cytochrome c consistently facilitates the reaction between FMN and plastocyanin — both of which are negatively charged. Likewise, the presence of
negatively-charged plastocyanin consistently inhibits the reaction between oppositely-charged FMN and cytochrome c (eq 12). Note also, the range of reactivities the four covalent isomers exhibit with FMN — revealing significant diversity in probably both the respective protein orientations and in the overall electrostatic character of these heterodiprotein complexes — all of which contain covalently-tethered cytochrome c and plastocyanin.

Table 8. Bimolecular rate constants, in order of decreasing magnitude, for the FMN semiquinone reduction of cytochrome c and plastocyanin in the covalent diprotein complex

<table>
<thead>
<tr>
<th>µ = 10 mM;</th>
<th>FMN + cyt(III)/pc(II) → cyt(II)/pc(II)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein species</td>
<td>cyt</td>
</tr>
<tr>
<td>k 2 x 10⁻⁷ M⁻¹s⁻¹</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>µ = 10 mM;</th>
<th>FMN + cyt(III)/pc(II) → cyt(III)/pc(I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein species</td>
<td>C4</td>
</tr>
<tr>
<td>k 2 x 10⁻⁷ M⁻¹s⁻¹</td>
<td>7.5</td>
</tr>
</tbody>
</table>

⁴ Solvent is phosphate buffer at pH 7.0 and the indicated ionic strength.

⁵ Boldface indicates the protein whose chromophore was monitored.
Table 8 (Continued)

<table>
<thead>
<tr>
<th>Protein species</th>
<th>C4</th>
<th>cyt</th>
<th>C3</th>
<th>C2</th>
<th>C1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_2 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$</td>
<td>9.7</td>
<td>7.0</td>
<td>6.6</td>
<td>3.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$\mu \approx 170 \text{ mM}; \quad \text{FMN} + \text{cyt(III)/pc(II)} \rightarrow \text{cyt(II)/pc(II)}$

<table>
<thead>
<tr>
<th>Protein species</th>
<th>C4</th>
<th>C3</th>
<th>C2</th>
<th>Pc</th>
<th>C1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_2 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$</td>
<td>9.7</td>
<td>5.4</td>
<td>3.4</td>
<td>1.3</td>
<td>0.73</td>
</tr>
</tbody>
</table>

$\mu \approx 170 \text{ mM}; \quad \text{FMN} + \text{cyt(III)/pc(II)} \rightarrow \text{cyt(III)/pc(III)}$

F. Intracomplex Electron-Transfer Reactions

1. Overview of the reactions

The reactions that can occur in our pulse-radiolysis and flash photolysis experiments (where $e^-$ is hydrated electrons, $e_{aq}^-$, or flavin semiquinones, FH$^-$) are summarized in Scheme III. The initial concentrations of ferricytochrome $c$ and of cupriplastocyanin are equal. Both the electrostatic and the covalent diprotein complexes are designated cyt/pc. Both the separate and the associated proteins can be reduced; the respective rate constants are designated $k$ and $k'$. Since the association constant $K_A$ depends more on the complementary docking of ionic residues than on the overall charges of the proteins and since the oxidation state of each metal atom changes by only one unit, the approximation in eq 14 is justified. Any cuproplastocyanin that was formed (in the reactions $k_{pc}$ and $k_{pc}'$) remained unreactive.
Figure 3. UV-vis spectra of ferricytochrome *c* (solid) and cupriplastocyanin (dashed). Arrows indicate the direction of the change in absorbance, at selected wavelengths, resulting from a one-electron reduction of each protein.
because its oxidation by ferricytochrome \( c \) is thermodynamically unfavorable. When, however, ferrocytochrome \( c \) was formed (in reactions \( k_{cyt} \) and \( k'_{cyt} \)), the subsequent electron-transfer reaction between the proteins was favorable. The shortage of the reducing species (i.e., \( e_{aq}^- \) or \( FH^- \)) ensured that the concentration of ferrocytochrome \( c \) always was much lower than the concentration of cupriplastocyanin. Therefore, the bimolecular electron-transfer
reactions, when the proteins were not cross-linked and the ionic strength was relatively high, occurred under pseudo-first-order conditions. Most of the reactions were followed at the heme chromophore because its change in absorptivity ($\Delta \varepsilon$) was the greatest; some reactions were also followed at the copper chromophore. Cytochrome $c$ and plastocyanin are well suited for the study of interprotein electron-transfer reactions because changes in the oxidation states of the two metals can be monitored and quantitated separately; refer to Figure 3.

The most interesting process in Scheme III is the interprotein electron-transfer reaction. Under unimolecular conditions, i.e., when the diprotein complex is fully formed, the simple relation in eq 15 applies. Under bimolecular conditions, the protein association (so-called preequilibrium) must also be taken into account; then the first-order rate constant is given by eq 16. Although the same expression is consistent with the so-called dead-end mechanism, this latter possibility is generally disfavored (17). Both the unimolecular and the bimolecular reactions are analyzed below.

2. Electrostatic complex: pulse radiolysis experiments

A comparison between Figure 4a on the one hand and Figure 4b, c on the other shows that the initial reduction of ferricytochrome $c$ in the electrostatic complex cyt(III)/pc(II) is followed by reoxidation. The corresponding absorbance change, which obeys the first-order law, is attributed to the reaction $k_{et}$ in Scheme III. All the
ferrocytochrome \( c \) formed by the electron pulse is subsequently 
reoxidized by the cupriplastocyanin; the ratio between the 
ferricytochrome \( c \) and cuproplastocyanin formed was \( 1.05 \pm 0.05 \)
regardless of the concentration of the diprotein complex. This 
equivalence between the reductant and the oxidant was established by
monitoring the iron and the copper chromophore separately.

As Table 9 shows, the observed rate constant at the low ionic
strength remained unchanged over a 12-fold range of the complex
concentration; according to eq 11, \( k_{et} = (1.05 \pm 0.12) \times 10^3 \text{s}^{-1} \).
Moreover, the plots of log \( A \) versus time were linear. These facts prove
that the electron-transfer reaction occurs unimolecularly, within the
complex. A bimolecular reaction between diprotein complexes was
suppressed at the micromolar concentrations used.

According to eq 16, the rate constant \( k_{et} \) with its independence of
concentration in the given range, requires that \( K_A \geq (5 \pm 2) \times 10^6 \text{M}^{-1} \)
at \( \mu = 1 \text{mM} \). Association constants of similar magnitudes (at low ionic
strengths) have been reported for electrostatic complexes of
cytochrome \( c \) with cytochrome \( b_5 \) (75), cytochrome \( c \) peroxidase (83),
and cytochrome \( c \) oxidase (124).

The observed rate constant at higher ionic strength depends
markedly on the protein concentration, as Table 9 shows. This
dependence was fitted to the two pertinent reactions in Scheme III.
Only one rate constant was fixed: \( k_{-2} = 1.0 \times 10^3 \text{s}^{-1} \), as determined by
King et al. (3). The other three rate constants were optimized as
follows: \( k_2 = 1.1 \times 10^8 \text{M}^{-1}\text{s}^{-1} \), \( k_{et} = 1.1 \times 10^3 \text{s}^{-1} \), and \( k_{rev} = 0 \). This
last finding denotes irreversibility of the electron-transfer step, a
common characteristic of redox metalloproteins (96). The two
different determinations yielded the same value of \( k_{et} \).

The association constant at \( \mu = 40 \text{mM} \), obtained from eq 14, is
\( 1.1 \times 10^5 \text{M}^{-1} \). It is alternatively calculated, with eq 16, from the rate
Figure 4. Absorbance versus time traces monitoring the fates of the species formed by reduction of different samples by $e_{aq}^-$, in phosphate buffer at pH 7.0.

(a) Native cyt(II), 13.8 $\mu$M, at $\mu = 40$ mM, monitored at 550 nm.
(b) Electrostatic complex cyt(II)/pc(II), 35 $\mu$M, at $\mu = 1$ mM, monitored at 425 nm.
(c) Electrostatic complex cyt(II)/pc(II), 14.5 $\mu$M, at $\mu = 40$ mM, monitored at 557 nm.
(d) Covalent complex cyt(II)/pc(II), isomer 2, 4.8 $\mu$M, at $\mu = 200$ mM, monitored at 417 nm.
(e) Covalent complex cyt(II)/pc(II), isomer 3, 12.1 $\mu$M, at $\mu = 2$ mM, monitored at 360 nm.
Table 9. Observed first-order rate constants for the interprotein electron-transfer reaction involving the electrostatic complex between cytochrome c and plastocyanin\textsuperscript{a}

<table>
<thead>
<tr>
<th>Concentration\textsuperscript{b} (\textmu{}M)</th>
<th>$k_{\text{obsd}} \times 10^{-2}$ (s\textsuperscript{-1})</th>
<th>$\mu = 1$ mM</th>
<th>$\mu = 40$ mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9</td>
<td>10.6 ± 2.3</td>
<td></td>
<td>3.03 ± 0.10</td>
</tr>
<tr>
<td>5.6</td>
<td>10.9 ± 0.7</td>
<td></td>
<td>5.87 ± 0.77</td>
</tr>
<tr>
<td>8.0</td>
<td>10.9 ± 0.7</td>
<td></td>
<td>5.22 ± 0.49</td>
</tr>
<tr>
<td>14.5</td>
<td>10.5 ± 1.1</td>
<td></td>
<td>7.38 ± 0.28</td>
</tr>
<tr>
<td>15.0</td>
<td></td>
<td></td>
<td>6.79 ± 0.30</td>
</tr>
<tr>
<td>17.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35.0</td>
<td>10.2 ± 1.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined by pulse radiolysis, in phosphate buffer at pH 7.0, and ambient temperature.

\textsuperscript{b} Concentration of each cyt(III) and pc(II) in the equimolar mixture.

constant $k_{\text{et}}$. An average of the $K_A$ values for the protein concentrations listed in Table 9 is $(8.1 \pm 1.4) \times 10^4$ M\textsuperscript{-1}, at $\mu = 40$ mM. The equality, within the margin of error, of the $K_A$ values obtained by these two methods confirms the applicability of eq 16 to the system under consideration.

An increase in the ionic strength of the solution can accelerate, decelerate, or not affect the electron-transfer reaction within an
electrostatic protein complex; all three possibilities, in cases of different proteins, are discussed in the works cited in the introduction (section IV part A). In the case of cytochrome \( c \) and plastocyanin, an increase in the ionic strength from 1 to 40 mM affects the complex stability in the expected way but does not influence the intracomplex electron-transfer rate constant. The rate constant of \((1.05 \pm 0.12) \times 10^3 \text{ s}^{-1}\) seems to be the optimal one in this electrostatic complex.

3. Electrostatic complex: flash photolysis experiments

After the semiquinone reduction of cytochrome \( c \) (the reactions designated \( k_{\text{Cyt}} \) and \( k'_{\text{Cyt}} \) in Scheme III, i.e., the reaction in eq 11), the ferrocytochrome \( c \) transfers an electron to cupriplastocyanin (the reactions designated \( k_{\text{PC}} \) and \( k'_{\text{PC}} \) in Scheme III, i.e., the reaction in eq 13). The latter reaction is pseudo-first-order in cupriplastocyanin, is

\[
\begin{align*}
\text{FH}^- + \text{cyt(III)}/\text{pc(II)} & \rightarrow \text{cyt(II)}/\text{pc(II)} \quad (11) \\
\text{cyt(II)}/\text{pc(II)} & \rightarrow \text{cyt(III)}/\text{pc(I)} \quad (13)
\end{align*}
\]

cleanly monitored on the millisecond time scale, and may be followed independently at either the Fe or Cu chromophore. At an ionic strength of 10 mM, the observed rate constant for the reaction in eq 13 is dependent on protein concentration. It was thus necessary to obtain values for \( k_{\text{et}} \left( \text{s}^{-1} \right) \) and \( K_A \left( \text{M}^{-1} \right) \) — given in Table 10 — by fitting the plot of \( k_{\text{obsd}} \) versus \([\text{pc(II)}]\) to a biphasic reaction scheme consisting of a second-order reaction followed by a first-order reaction (Scheme IV). The rate of intracomplex electron transfer unobscured by associative effects is \( k_{\text{et}} = (1.25 \pm 0.15) \times 10^3 \text{ s}^{-1} \); this value is independent of both the species of flavin (producing the ferrocytochrome \( c \)) and of the chromophore monitored. This latter observation confirms that indeed ferrocytochrome \( c \) is the species reducing cuproplastocyanin. All
ferrocytochrome $c$ is reoxidized in the reaction in eq 13; there is a complete return of the baseline to the prepulse position when monitoring exclusively cytochrome $c$. Also, there is a one-to-one ratio between the moles of ferrocytochrome $c$ which are reoxidized and the moles of cupriplastocyanin which become reduced.

The value of $K_A$ for the electrostatic cyt/pc complex (when $\mu = 10$ mM at pH 7.0) is ca. $5 \times 10^4$ M$^{-1}$. This value, likewise, is independent

**Table 10. Association constants and intracomplex electron-transfer rate constants for the electrostatic cyt/pc complex**

<table>
<thead>
<tr>
<th>monitored reactions</th>
<th>$K_A \times 10^{-4}$ M$^{-1}$</th>
<th>$k_{et} \times 10^{-3}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>Lf</td>
<td>FMN</td>
</tr>
<tr>
<td>cyt(II)/pc(II)</td>
<td>5.1 ± 0.1</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>cyt(III)/pc(I)$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt(II)/pc(II)</td>
<td>6.2 ± 0.5</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>cyt(III)/pc(I)$^d$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Where $\mu$ is 10 mM phosphate buffer at pH 7.0.

$^b$ Boldface indicates the protein whose chromophore was monitored. The mixed-valent species cyt(II)/pc(II) is the kinetic product of the following flavin semiquinone reduction, where FH$^-$ is either lumiflavin (Lf) or FMN:

\[
\text{FH}^- + \text{cyt(III)/pc(II)} \rightarrow \text{cyt(II)/pc(II)}
\]

$^c$ Monitored at 550 nm.

$^d$ Monitored at 557 nm.
of the species of flavin semiquinone and of the monitored chromophore.

4. **Covalent complex: pulse radiolysis**

Figure 4 (d and e) and Figure 5 show that the mixed-valence species, cyt(II)/pc(II), is stable for at least 1.0 s after the electron pulse that produced it; the reaction designated $k_{et}$ was not detected. All four isomers of the complex, in which the proteins presumably are cross-linked in slightly different orientations, proved unreactive in repeated experiments at ionic strengths of both 2 and 200 mM. Similar horizontal plots were obtained by monitoring absorbance at different wavelengths. A trivial cause of the unreactivity, namely, reduction of cyt(III)/pc(II) into cyt(II)/pc(I) before the electron pulse, was ruled out by spectrophotometric examination of the covalent complex before and after thorough bubbling with nitrogen; the reduction was negligible. No chemical degradation of the covalent complex was ever observed. If the electron-transfer reaction within the covalent complex occurs at all, its rate constant must be less than ca. 0.2 s$^{-1}$.

5. **Covalent complex: flash photolysis experiments**

In confirmation of the previously-obtained pulse radiolysis findings, no intracomplex electron transfer (eq 13) is seen in any of the four covalent isomers, cyt(II)/pc(II), even though the observation time is here extended to 10 s. This allows us to reduce the possible upper
Figure 5. Absorbance versus time traces showing the absence of the electron-transfer reaction in different isomers of the covalent complex cyt(II)/pc(II), formed by reduction of the corresponding samples of cyt(III)/pc(II) by $e_{aq}^-$, in phosphate buffer at pH 7.0.

(a) Isomer 2, 7.0 μM, at $\mu = 2$ mM, monitored at 417 nm.
(b) Isomer 2, 7.0 μM, at $\mu = 200$ mM, monitored at 417 nm.
(c) As in b, but monitored at 550 nm.
limit of $\lambda_{et}$ to 0.05 s$^{-1}$, a measurable rate constant on the 10 s time scale. The ferrocytochrome $c$ does prove itself a viable redox protein however; in all four of the covalent complexes ferrocytochrome $c$ indeed does reduce cuproplastocyanin — but this reaction occurs through an intercomplex mechanism (eq 17), with $\lambda_{obsd}$ being

$$
\text{cyt(III)/pc(II)} \quad \xrightarrow{\text{eq 17}} \quad \text{cyt(III)/pc(I)}
$$

dependent on the concentration of covalent complex. Bimolecular rate constants for such intercomplex electron transfer reactions are given in Table 11. The diverse reactivity among the four isomers is readily apparent, as was seen before, in the reaction of these same isomers with FMN (Table 8). The range of the bimolecular rate constants, though, is notably greater for the inter-diprotein-complex reactions in Table 11.

G. Reactivity of the Covalent cyt/pc Complexes

In each of the four isolated cyt/pc covalent isomers, cytochrome $c$ and plastocyanin are fully and reversibly able to be oxidized and reduced. A correlation between the rate of reaction of ferrocytochrome $c$ with cuproplastocyanin and the size of the redox partners is shown in Table 12. The driving force for these reactions is essentially constant, although the electrostatic character of the native and complexed proteins does differ.

The trends in reactivity among the covalent isomers are presented in Table 13 and prove very consistent — remaining unaltered by the size of the reactant (FMN versus the diprotein
complex), and by the ionic strength (10 or 170 mM). It also becomes apparent that for those isomers in which cytochrome c is more reactive (in bimolecular electron-transfer reactions), so also is the plastocyanin of that diprotein complex more reactive. These trends reflect, in part, the accessibility of the metal centers to the various external redox partners; this is perhaps more pertinent for cytochrome c (where the exposed heme edge is the primary site of interaction with redox partners) than for plastocyanin (having two regions, the "north" hydrophobic patch and the "east" hydrophilic patch), for interacting with external redox partners. Steric accessibility may be playing a greater role than electrostatic

Table 11. Bimolecular rate constants for intercomplex electron transfer in the covalent cyt/pc complex

<table>
<thead>
<tr>
<th>Covalent isomer:</th>
<th>C4</th>
<th>C3</th>
<th>C2</th>
<th>C1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k \times 10^{-4}$ M$^{-1}$s$^{-1}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\mu = 10$ mM$^b$</td>
<td>46</td>
<td>7.7</td>
<td>3.1</td>
<td>1.8</td>
</tr>
<tr>
<td>$\mu \approx 170$ mM</td>
<td>19</td>
<td>4.6</td>
<td>3.1</td>
<td>0.64</td>
</tr>
</tbody>
</table>

$^a$ Boldface indicates those proteins involved in electron transfer; the cyt(II)/pc(II) reactant was produced from the FMN semiquinone:

$\text{FMN + cyt(II)/pc(II)} \rightarrow \text{cyt(II)/pc(II)}$.

$^b$ Solvent is phosphate buffer at pH 7.0 and indicated ionic strength.
Table 12. Bimolecular rate constants for the electron-transfer between ferrocytochrome $c$ and cuproplastocyanin for protein partners of varying size

<table>
<thead>
<tr>
<th>reaction monitored $^a$</th>
<th>$k_2(M^{-1}s^{-1})^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu = 10 \text{ mM}$</td>
</tr>
<tr>
<td>pc(II) + cyt(II) $\rightarrow$ pc(I) + cyt(III)</td>
<td>$5.1(10^6)c$</td>
</tr>
<tr>
<td>pc(II) + cyt(II)/pc(II) $\rightarrow$ pc(I) + cyt(III)/pc(II) $^d$</td>
<td>$2.3(10^5)$</td>
</tr>
<tr>
<td>cyt(III)/pc(II) + cyt(II)/pc(II) $\rightarrow$ cyt(III)/pc(I) + cyt(III)/pc(II) $^d$</td>
<td>$7.7(10^4)$</td>
</tr>
</tbody>
</table>

$^a$ Boldface indicates those proteins involved in electron transfer; cyt/pc represents covalent isomer C3.

$^b$ Solvent is phosphate buffer at pH 7.0 and indicated ionic strength.

$^c$ Determined at $\mu = 100 \text{ mM}$ at pH 7.5; ref 3.

$^d$ The cyt(II)/pc(II) reactant was produced from the FMN semiquinone reaction: FMN + cyt(III)/pc(n) $\rightarrow$ cyt(II)/pc(n).

interactions in determining the above reactivity trends, however; this is supported because the relative orders are unaffected by an increase in ionic strength, i.e., a decrease in electrostatic interactions.
Table 13. Trends in reactivity of covalent isomers, cyt/pc, as ionic strength and size of redox partners are varied

<table>
<thead>
<tr>
<th>reaction&lt;sup&gt;a&lt;/sup&gt;</th>
<th>μ (mM)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>decreasing bimolecular rate constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN + cyt(III)/pc(II) → cyt(II)/pc(II)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 170</td>
<td>C4 &gt; C3 &gt; C2 &gt; C1</td>
</tr>
<tr>
<td>FMN + cyt(III)/pc(II) → cyt(III)/pc(I)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 170</td>
<td>C4 &gt; C3 &gt; C2 &gt; C1</td>
</tr>
<tr>
<td>cyt(III)/pc(II) + cyt(II)/pc(II) → cyt(III)/pc(I) + cyt(III)/pc(II)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10 170</td>
<td>C4 &gt; C3 &gt; C2 &gt; C1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Boldface indicates the protein whose chromophore was monitored.

<sup>b</sup> Solvent is phosphate buffer at pH 7.0 and indicated ionic strength.

<sup>c</sup> The cyt(II)/pc(II) reactant was produced from the FMN semiquinone reaction: FMN + cyt(III)/pc(II) → cyt(II)/pc(II).

H. Conclusions

The electrostatic and the covalent complexes behave completely differently under identical conditions although the cytochrome c and plastocyanin in them are docked similarly; although significant perturbations of the electronic, structural, and redox properties of the active sites upon cross-linking are ruled out; and although the covalent complex remains reactive in bimolecular electron-transfer reactions.
with inorganic oxidants, hydrated electrons, flavin semiquinones, and monomeric and dimeric protein complexes.

Recent experimental and theoretical studies of protein complexes provide some clues about this contrast. Calculations of Brownian dynamics showed how an intracomplex electron-transfer reaction requires particular orientations of the protein partners and how these orientations are achieved by rotational diffusion within the nonoptimal aggregates (125). In the electrostatic cyt/pc complex such adjustment of the structure may be facile or not required at all. In the covalent complex, however, the adjustment probably is impeded by the multiple amide bonds. These tight cross-links seem to prevent cytochrome c and plastocyanin from exploring each other; these proteins seem to be locked in unproductive orientations.

Only in one previous study known to us have electron-transfer reactions within an electrostatic and a covalent complex (in that case, involving cytochrome c and cytochrome c peroxidase) been compared. Although both of these complexes proved reactive, some kinetic differences between them were noted (89). An even greater difference between the two types of cyt/pc complexes, the very subjects of our study, was observed electrochemically at an electrode (112). These researchers attributed the facile electroactivity of the electrostatic complex to the rapid electron exchange between the two proteins, in accord with our pulse-radiolysis measurements. They observed the diminished electroactivity of the covalent complex, a property consistent with our finding of no electron-transfer reaction within it. Further comparative studies are needed to elucidate the effects of covalent cross-linking on the intracomplex electron-transfer reactions.

The easy separation of four isomers of the covalent cyt/pc complex, reported herein, may prompt similar chromatographic analyses and fuller characterization of other protein complexes. The contrast between the reactive electrostatic complex and the similar but
unreactive covalent complex illustrates the intricacy of biological electron-transfer reactions and shows that properties of a protein aggregate can be changed completely by covalent cross-linking. The present kinetic analysis calls for a computational analysis of the association between cytochrome c and plastocyanin.
V. NOVEL COMPLEXES OF CYTOCHROME C AND PLASTOCYANIN — VARIABLE-LENGTH TETHERING WITH THE FLEXIBLE, HOMOBIFUNCTIONAL REAGENTS DST AND EGS

A. Introduction

Homobifunctional $N$-hydroxysuccinimide (NHS) esters are a class of variable length tethers which range from 6 to 16 Å in spacer length, and are specifically reactive toward primary amines — such as the epsilon amine groups of lysines, or available N-terminus amines. Such cross-linkers have been used for covalent complexation of cytochrome $c$ with flavodoxin (126), and with cytochrome $c$ peroxidase (127, 128). At neutral to alkaline pH, the amino group on a particular ligand will undergo nucleophilic attack of the NHS ester to form a stable amide bond and release $N$-hydroxysuccinimide as a by-product (Scheme V). In aqueous solutions the NHS ester is consumed by two competing reactions: one is the reaction with primary amines, and the other is hydrolysis of the NHS ester (129-131). The latter reaction inactivates the NHS ester and decreases the efficiency of the cross-linking reaction. Hydrolysis is favored in dilute protein solutions; in more concentrated protein solutions, the acylation reaction is favored. NHS esters have a half-life of 4-5 hours at pH 7.0 in an aqueous solution free of extraneous amines. As pH is increased, the rates of both the hydrolysis and the acylation reaction are increased. Reaction conditions such as the pH, the excess of cross-linker, and the concentration of proteins, should thus be chosen that will maximize the yield of the desired cross-linked product, while avoiding excessive single-ended, non-cross-linking, lysine modification (favored in dilute protein solutions as well as by large excesses of cross-linker).

The two cross-linkers disuccinimidyl tartarate (DST) and
Scheme V.

Generalized reaction between an NHS ester and a primary amine:

\[ \text{O} - \text{O} - \text{N}^{ullet} \quad + \quad \text{R} - \text{NH}_2 \quad \xrightarrow{7 < \text{pH} < 9} \quad \text{O} - \text{N} - \text{R} - \text{H} + \text{HO} - \text{N}^{ullet} \]

Structure of the DST and EGS cross-linkers:

(DST, 6 Å spacer)

(EGS, 16 Å spacer)

ethylene glycolbis(succinimidylsuccinate) (EGS) were chosen because they are the shortest and longest NHS ester cross-linkers available (6 and 16 Å, respectively), and their spacers are of comparable composition; refer to Scheme V. In our cross-linking studies, we wish to maximize the yield of the heterodiprotein complex cyt/pc, while
avoiding formation of homodiprotein complexes (especially cyt/cyt),
and also while minimizing nonproductive, single-ended, lysine
modifications.

B. Preparation of Diprotein Complexes

Cross-linking was done under two sets of reaction conditions. The earlier reaction mixtures were 0.55 mM in both cytochrome c and plastocyanin and 6.8 mM (i.e., a 12.5-fold excess) in either the DST or EGS cross-linkers. These reactions are designated A and B, respectively, in Table 14. The solvent was 5 mM MOPS at pH 7.0; incubation lasted for 16 hours at room temperature. The pH was maintained at 7.0 ± 0.3 by repeated additions of 5 to 10 μL aliquots of base (a pH 12.5 solution of NaOH in 5 mM MOPS) during the first hour; total amount of added base solution was ca. 6% of the reaction volume.

In later reactions, designated C in Table 14, concentrations of the proteins and the cross-linker were decreased; each protein was 50 μM and the cross-linker (only EGS was used) was 400 μM (i.e., an 8-fold excess). The reaction solvent, time, and temperature were unchanged from the earlier reactions; the pH, however, remained at 7.0 ± 0.2 without addition of base.

All reaction mixtures were concentrated by ultrafiltration, applied to a column of Sephadex G-75 size-exclusion resin, and eluted with 85 mM phosphate buffer at pH 7.0. Yields of the monomeric, dimeric, and polymeric protein fractions are presented in Table 14. The isolated dimeric and monomeric protein fraction were subsequently concentrated and passed through a column of Sephadex DEAE anion-exchange resin. Fractions were eluted with 85 mM phosphate buffer at pH 7.0 containing additional amounts of NaCl.
Table 14. Cross-linking with DST and EGS: synthesis and initial reaction-mixture separation using size-exclusion chromatography

<table>
<thead>
<tr>
<th>reaction designation</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction conditions:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cross-linker</td>
<td>DST</td>
<td>EGS</td>
<td>EGS</td>
</tr>
<tr>
<td>[cyt] = [pc] (µM)</td>
<td>550</td>
<td>550</td>
<td>50</td>
</tr>
<tr>
<td>excess of cross-linker</td>
<td>12-fold</td>
<td>12-fold</td>
<td>8-fold</td>
</tr>
<tr>
<td>Percentages of indicated proteins in fractions obtained from size-exclusion chromatography:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt</td>
<td>pc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>monomeric</td>
<td>39 81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dimeric</td>
<td>29 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>polymeric</td>
<td>32 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt</td>
<td>pc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47 81</td>
<td>88 90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 11</td>
<td>12 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 8</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Buffer was 5 mM MOPS at pH 7.0.
b Refers to the covalent diprotein complexes cyt/pc and cyt/cyt; pc/pc is a very minor product, if it forms at all.
c Fractions with molecular mass greater than the diprotein complexes.
Table 14 (Continued)

**Composition of dimeric protein fraction:**

<table>
<thead>
<tr>
<th>percentage of indicated dimeric complex:</th>
<th>cyt/pc</th>
<th>cyt/cyt</th>
<th>cyt/cyt</th>
<th>net yields:</th>
<th>cyt/pc</th>
<th>cyt/cyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt/pc</td>
<td>53 ± 6</td>
<td>57 ± 2</td>
<td>88</td>
<td>cyt/pc</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>cyt/cyt</td>
<td>47 ± 6</td>
<td>43 ± 2</td>
<td>12</td>
<td>cyt/cyt</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 15. Ion-exchange chromatography of the dimeric and the monomeric protein fractions (presented in Table 14)

<table>
<thead>
<tr>
<th>reaction designation^a</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>cross-linker</td>
<td>DST</td>
<td>EGS</td>
</tr>
</tbody>
</table>

**Chromatography of the dimeric protein complex:**

<table>
<thead>
<tr>
<th>fraction number (in order of elution)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>eluent: 85 mM phosphate buffer at pH 7.0, with indicated amount of NaCl (mM)</td>
<td>50</td>
<td>225</td>
<td>400</td>
<td>100</td>
<td>365</td>
<td>400</td>
</tr>
</tbody>
</table>

^a Reaction specifics are given in Table 14.
Table 15 (Continued)

Composition of the fractions from ion-exchange:

<table>
<thead>
<tr>
<th>[cyt]/[pc]</th>
<th>4.6</th>
<th>1.1</th>
<th>0.22</th>
<th>1.4</th>
<th>0.93</th>
<th>0.54</th>
</tr>
</thead>
<tbody>
<tr>
<td>percent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>distribution of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt</td>
<td>44</td>
<td>48</td>
<td>8</td>
<td>16</td>
<td>79</td>
<td>5</td>
</tr>
<tr>
<td>pc</td>
<td>10</td>
<td>47</td>
<td>43</td>
<td>11</td>
<td>81</td>
<td>8</td>
</tr>
<tr>
<td>percentage of pc that is autoreduced&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31</td>
<td>67</td>
<td>53</td>
<td>6</td>
<td>58</td>
<td>35</td>
</tr>
</tbody>
</table>

Chromatography of the monomeric protein fractions:

<table>
<thead>
<tr>
<th>fraction number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(in order of elution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eluent: 85 mM phosphate buffer at pH 7.0, with indicated amount of NaCl (mM)</td>
<td>0</td>
<td>50</td>
<td>200</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>percentage distribution of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt</td>
<td>54</td>
<td>--</td>
<td>46</td>
<td>19</td>
<td>79</td>
</tr>
<tr>
<td>pc</td>
<td>--</td>
<td>~100</td>
<td>100</td>
<td>--</td>
<td>~100</td>
</tr>
<tr>
<td>percentage of pc that is autoreduced&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>13</td>
<td>--</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>b</sup> The percentage of plastocyanin in the reduced state after chromatography and ultrafiltration; protein samples are fully oxidized with Fe(CN)₆³⁻ before every chromatographic run.
Specific elution conditions and yields of the resulting protein fractions are detailed in Table 15.

C. Formation and Isolation of the Diprotein Complexes

Size-exclusion chromatography efficiently separates the reaction mixture into its polymeric, dimeric and monomeric protein fractions (see Table 14). The yield of desired cyt/pc heterodiprotein complex is ca. 11% in each of the reaction conditions in Table 14. The yield is virtually independent of the length of cross-linker and of the concentration of proteins in the reaction mixture, under the given conditions. However, at higher protein concentration (reaction conditions A and B), there is also a 9% yield of the unwanted cyt/cyt homodiprotein complex; it is exclusively the yield of this diprotein complex that is lowered to ca. 1% in reaction C — where the protein concentration is diluted 11-fold with respect to reactions A and B.

Isolated dimeric and monomeric protein fractions obtained from size-exclusion chromatography were additionally chromatographed on anion-exchange resin. Heterogeneity in the charged surface amino acids of the monomeric cytochrome c and the diprotein complexes (obtained from either the DST or EGS reactions) is especially evident from their mobility; the samples elute over a wide range of eluent molarities but display few defined bands. Heterogeneity is largely due to single-ended modification of a number of different lysine side chains in cytochrome c (both monomeric and in diprotein complexes). Single-ended modification arises when lysyl groups react with a cross-linker whose remaining succinimidyl end group undergoes hydrolysis instead and fails to form a diprotein cross-link. The result elongates the lysyl group by 6 or 16 Å, for DST and EGS respectively, and creates a -1 charge in place of the original +1 charge. The altered mobility of monomeric cytochrome c can only result from such single-ended
modification; similar modifications are likewise inevitable in the covalently cross-linked cytochrome c.

Single-ended modifications are more likely in cytochrome c than in plastocyanin (the former protein having 19 lysines and the latter only 5), and are responsible for the similar mobilities of the cyt/pc and the cyt/cyt diprotein complexes on ion-exchange resins. One is unable to separate the desired heterodiprotein complex, cyt/pc, from the homodiprotein complex, cyt/cyt, either by ion-exchange or by size-exclusion chromatography. Kinetic studies of the heterodiprotein complex are only possible on a sample that is free of the homodiprotein complex. This can thus best be accomplished through properly-chosen reaction conditions which fail to allow the formation of the homodiprotein complex in the first place.

Heterogeneity in the diprotein complex may also arise from differing relative orientations of the cross-linked cytochrome c and plastocyanin — an exclusive docking orientation is not expected. The availability of surface electrostatic patches (for interaction with ion-exchange resins) indeed depends on the docking orientation of cytochrome c and plastocyanin. One must note, however, that the EDC cross-linked cyt/pc diprotein complex elutes from ion-exchange resin in clearly-defined bands. Also note that EDC does not produce single-ended modification of lysines, and thus leaves the original positive electrostatic patches intact. These differences indeed emphasize the role of DST- and EGS-single-ended lysine modification in producing heterogeneously-modified cytochrome c. The number of unproductive, non-cross-linking modifications would be decreased through the use of a minimal excess of cross-linker during the reaction. The present results show that a drop from 12- to 8-fold excess of cross-linker has no effect on the yield of the desired heterodiprotein complex, but certainly must produce fewer extraneous lysine modifications, and better maintain the integrity of the native protein.

DST and EGS reaction mixtures chromatograph similarly on
size-exclusion resin; yields of cytochrome c and plastocyanin in the various fractions are virtually independent of the length of cross-linker. However, the length does significantly affect the mobility of these reaction mixtures on anion-exchange resin. Modification of lysine residues with either cross-linker reduces the existing +1 charge to neutrality (for cross-linked lysine residues) or to -1 (for lysines modified through a single-ended attack). This decreases the overall positive charge of the protein and increases its retention on anion-exchange resins, relative to the native monomeric proteins and to the EDC cross-linked diprotein complexes. Proteins modified with EGS are consistently retained more strongly on anion-exchange resin than are those modified with DST. Indeed, the length of modified lysine (with elongated side chain and a negative charge) is greater in the case of EGS modification, than for DST modification, allowing for increased interaction with anion-exchange resins.

These results show that for reaction mixtures containing oppositely-charged cytochrome c and plastocyanin, high protein concentrations result in the formation of both cyt/pc and cyt/cyt covalent diprotein complexes. Although the latter requires that two proteins of like charge come in close proximity, the numerous lysine residues in cytochrome c increase the probability of its formation. At lower protein concentrations, however, formation of the electrostatic cyt/pc complex is made more favorable, and the likelihood of the cyt/cyt complex decreases dramatically. Thus it appears that protein concentrations at least as low as 50 μM are needed to prevent formation of unwanted cyt/cyt diprotein complexes; also, reactions using even less than an 8-fold excess of cross-linker should be carried out — attempting to even further minimize extraneous, single-ended lysine modifications in the covalent diprotein complex.
D. Continuation of Research

1. Characterization

Additional characterizations of the purified, lysine-cross-linked heterodimers, such as acquiring their reduction potentials and EPR spectra, will address the integrity of the metal centers. Voltammetry at a stationary gold disc electrode should yield $E^\circ$ of cytochrome $c$. For plastocyanin, the success or failure in resolving a voltametric signal will reveal its accessibility to the mediated electrode. If necessary, redox titrations will instead be used to determine the $E^\circ$ of plastocyanin. The heme iron of cytochrome $c$ in cyt/pc is now in a more negatively-charged environment, resulting both from single-ended lysine modifications and from the close proximity of negatively-charged plastocyanin. A decrease in the reduction potential of cytochrome $c$ was observed in the cyt/pc complex formed using EDC, and was attributed to the close presence of plastocyanin. Cytochrome $c$ experiences an even greater amount of negative charge in the cyt/pc complexes formed here, using DST and EGS; the decrease in its reduction potential should be even more pronounced. It would perhaps also be of interest to determine the reduction potentials for the various modified, monomeric cytochrome $c$ and plastocyanin fractions obtained from the cross-linking reactions and purified on anion-exchange resin. In this way, the effects of single-ended lysine modification on reduction potential may be observed independent of the effects of cross-linking, and the close proximity of the partner protein. The EPR spectra of the fully-oxidized heterodimers, and also the modified monomeric fractions, is another fairly simple and informative technique of characterizing the iron and copper centers.

2. Kinetics

Flash photolysis kinetic studies will be carried out similarly to those performed on the EDC cross-linked cyt/pc complex.
Photogenerated semiquinones will rapidly reduce a subequivalent of the covalently-complexed cytochrome c, forming the cyt(II)/pc(II) intermediate. The most intriguing question to be answered is: will $k_{et}$ for reaction 9 be independent of the concentration of diprotein and of the ionic strength — thus showing that intracomplex electron transfer is indeed feasible in these more loosely-tethered covalent complexes.

\[
\text{cyt(II)/pc(II)} \rightarrow \text{cyt(III)/pc(I)} \quad (9)
\]

If intracomplex electron transfer is observed, its magnitude may correlate with the length and flexibility of the existing covalent tether. Whereas negatively-charged FMN was the flavin of choice in the EDC cross-linked cyt/pc complexes, the more negatively-charged DST and EGS cross-linked cyt/pc complexes will probably require the use of the neutrally-charged lumiflavin.
The number and location of existing cross-links and the sites of modification (without cross-linking) should be determined for the EDC, DST, and EGS cross-linked cyt/pc complexes mentioned thus far. Digests of the covalent cyt/pc complexes will be compared to that of the one-to-one mixture of cytochrome c and plastocyanin using HPLC. Cross-linking and single-ended modifications should produce fractions in the covalent diprotein digest that are unparalleled in the digest of the mixture of native proteins. These fractions will then be sequenced, and the number, type, and location of the modifications will be addressed. Care must be taken to ensure that existing cross-links and modifications are not cleaved in the above procedures, however.

In conjunction with additional physical characterization of the covalent cyt/pc complexes, computer graphic modeling of various cyt/pc complexes might be carried out. This would utilize the existing crystal structures of the two native proteins, an energy minimization program for exploring favorable electrostatic docking orientations, any definitive sites of cross-linking and/or modification as they become available, and the maximum span of the cross-linker. Estimation of the metal-metal separation (center-to-center and edge-to-edge), and plausible protein-protein orientations can thus be explored.

To date, there exists no crystal structure of a covalent diprotein complex, although this is the ultimate method for determining protein-protein orientation, as well as the distance and medium over which electron transfer may occur. In the near future when plastocyanin will be in greater supply in this laboratory, I feel it is well worth the attempt to grow suitable crystals for an X-ray crystal structure determination, especially of the EDC cross-linked cyt/pc complex. There is a far greater homogeneity within each of the EDC diprotein fractions (isomers) isolated from cation-exchange chromatography,
than was evidenced in the DST and EGS cross-linked diprotein complexes. Homogeneous interaction of the covalent diprotein complex with the ion-exchange resin may likewise be reflected in more readily attaining an ordered crystal.

Numerous variations can be applied to the covalent cyt/pc complexes studied thus far. The driving force for interprotein electron-transfer (either inter- or intracomplex) may be purposefully altered through metal substitution. The medium through which electron transfer occurs may be varied through site-specific mutagenesis prior to cross-linking, or existing residues in the native proteins may be modified.

Additional variation may be had in the choice of proteins which are to be cross linked. It will prove interesting to compare the kinetics exhibited by the covalently-complexed cytochrome f/plastocyanin system to those of the cytochrome c/plastocyanin complex. Cytochrome f and plastocyanin are physiological redox partners, and their specificity of electrostatic association (132-134) (possibly also realized in the covalent complex) may reflect an enhanced protein-protein recognition. This may be result in different relative orientations of the two proteins in the covalent complex — possibly now permitting intracomplex electron transfer within this covalent, nondissociating precursor complex.
VII. REFERENCES


135. Lukes, A. J.; Kostić, N. M. research in progress.
ACKNOWLEDGEMENTS

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**IX. APPENDIX**

Table A-1: Apparent increase in $M_r$ of covalent diprotein complexes\(^a\) as a function of the length of covalent tether

<table>
<thead>
<tr>
<th>approximate protein-protein separation (Å)</th>
<th>apparent % increase in $M_r$</th>
<th>cross-linking reagent</th>
<th>cross-linked proteins</th>
<th>apparent $M_r$ (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17</td>
<td>EDC</td>
<td>cyt/pc</td>
<td>27 ± 1(^b)</td>
</tr>
<tr>
<td>4.6</td>
<td>23</td>
<td>PtCl(_4^{2-})</td>
<td>cyt/cyt</td>
<td>31 ± 1(^c)</td>
</tr>
<tr>
<td>7.1</td>
<td>36</td>
<td>Rh(_2)(OAc)(_4)(_2)</td>
<td>cyt/cyt</td>
<td>35 ± 1(^d)</td>
</tr>
<tr>
<td>7.3</td>
<td>40</td>
<td>Ru(_3)O(OAc)(_6)(_3)</td>
<td>cyt/cyt</td>
<td>35 ± 4(^e)</td>
</tr>
<tr>
<td>9</td>
<td>52</td>
<td>DST</td>
<td>cyt/pc</td>
<td>35 ± 3(^f)</td>
</tr>
<tr>
<td>19</td>
<td>52</td>
<td>EGS</td>
<td>cyt/pc</td>
<td>35 ± 3(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Determined using size-exclusion chromatography.
\(^b\) Refer to section IV.
\(^c\) Refer to section III.
\(^d\) Reference 92.
\(^e\) Reference 135.
\(^f\) Refer to section V.