Lee, Y. C., Stowell, C. P., & Krantz, M. J. (1976) *Biochemistry 15*, 3956–3963.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M., & Lönn, H. (1983) *J. Biol. Chem. 258*, 199–202.
Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., & Bock, K. (1984) in *Biochemical and Biophysical Studies of Proteins and Nucleic Acids* (Lo, T. B., Lin, T. Y., & Li, C. H., Eds.) pp 349–360, Elsevier, New York.
McKelvy, J. F., & Lee, Y. C. (1969) *Arch. Biochem. Biophys. 132*, 99–110.
Mencke, A. J., & Wold, F. (1982) *J. Biol. Chem. 257*, 14799–14805.
Pinto, M., & Bundle, D. R. (1983) *Carbohydr. Res. 124*, 313–318.
Rogers, J. C., & Kornfeld, S. (1971) *Biochem. Biophys. Res. Commun. 45*, 622–629.
Seglen, P. O. (1976) *Methods Cell Biol. 13*, 29–83.
Shephard, V. L., Lee, Y. C., Schlesinger, P. H., & Stahl, P. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1019–1022.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Stowell, C. P., & Lee, Y. C. (1980) *Adv. Carbohydr. Chem. Biochem. 37*, 225–281.
Stowell, C. P., Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 4904–4908.
Townsend, R. R., Hardy, M. R., Wong, T. C., & Lee, Y. C. (1986) *Biochemistry 25*, 5716–5725.
Uchida, Y., Tsukada, Y., & Sugimori, T. (1977) *J. Biochem. (Tokyo) 82*, 1425–1433.
Weigel, P. H., Naoi, M., Roseman, S., & Lee, Y. C. (1979) *Carbohydr. Res. 70*, 83–91.
Yan, S. B., & Wold, F. (1984) *Biochemistry 23*, 3759–3765.

Oxidoreduction Reactions Involving the Electrostatic and the Covalent Complex of Cytochrome *c* and Plastocyanin: Importance of the Protein Rearrangement for the Intracomplex Electron-Transfer Reaction†

Linda M. Peerey and Nenad M. Kostić*
Department of Chemistry and Ames Laboratory, Iowa State University, Ames, Iowa 50011
Received August 29, 1988; Revised Manuscript Received October 18, 1988

ABSTRACT: Horse heart cytochrome *c* and French bean plastocyanin are cross-linked one-to-one by a carbodiimide [Geren, L. M., Stonehuerner, J., Davis, D. J., & Millett, F. (1983) *Biochim. Biophys. Acta 724*, 62] in the same general orientation in which they associate electrostatically [King, G. C., Binstead, R. A., & Wright, P. E. (1985) *Biochim. Biophys. Acta 806*, 262]. The reduction potentials of the Fe and Cu atoms in the covalent diprotein complex are respectively 245 and 385 mV vs 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. Stopped-flow spectrophotometric experiments with the covalent diprotein complex show that the presence of plastocyanin somewhat inhibits oxidation of ferrocytochrome *c* by $[Fe(CN)_6]^{3-}$ and somewhat promotes oxidation of this protein by $[Fe(C_5H_5)_2]^+$. These changes in reactivity are explained in terms of electrostatic and steric effects. Pulse-radiolysis experiments with the electrostatic diprotein complex yield association constants of $\geq 5 \times 10^6$ and 1×10^5 M^{-1} at ionic strengths of 1 and 40 mM, respectively, and the rate constant of 1.05×10^3 s^{-1}, regardless of the ionic strength, for the intracomplex electron-transfer reaction. Analogous pulse-radiolysis experiments with each of the four isomers of the covalent diprotein complex, at ionic strengths of both 2 and 200 mM, show an absence of the intracomplex electron-transfer reaction. A rearrangement of the proteins for this reaction seems to be possible (or unnecessary) in the electrostatic complex but impossible in the covalent complex.

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 (Tam & Williams, 1985). 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 (Poulos & Finzel, 1984; Waldmeyer & Bosshard, 1985), cytochrome b_5 (Salemme, 1976; Mauk et al., 1986), cytochrome *c* reductase (Bosshard et al., 1979), cytochrome *c* oxidase (Michel & Bosshard, 1984), flavodoxin (Hazzard et al., 1986), and plastocyanin (Augustin et al., 1983; Chapman et al., 1984; King et al., 1985). 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

†This work was financed by the U.S. Department of Energy, Chemical Sciences Division, under Contract W-7405-ENG-82.

0006-2960/89/0428-1861$01.50/0

investigation of intracomplex redox reactions began only several years ago, interesting findings have been made, and important new questions have been raised (McLendon & Miller, 1985; Peterson-Kennedy et al., 1985; McLendon et al., 1986, 1987; Liang et al., 1986, 1988; Gingrich et al., 1987; Cheung & English, 1988; Conklin & McLendon, 1988; McLendon, 1988; Hazzard et al., 1988a,b,c). 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 (Peerey & Kostič, 1987; Chen & Kostič, 1988; Kostič, 1988). In any case, it is necessary to determine whether the synthetic 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. (1985). Although cytochrome *c* and plastocyanin do not participate in the same electron-transport chain, their association is similar to the natural associations of the former with cytochrome b_5 (Eley & Moore, 1983) and with cytochrome *c* peroxidase (Gupta & Yonetani, 1973) and of the latter with cytochrome *f* (Beoku-Betts et al., 1985). Much is known about the redox reactions of cytochrome *c* (Moore et al., 1984; Cusanovich et al., 1987) and plastocyanin (Sykes, 1985) 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 three reactions. First, the oxidation of ferrocytochrome *c* by inorganic reagents (eq 1);[1] second, the reduction of ferricytochrome *c* by hydrated electrons (eq 2); and third, the oxidation of ferrocytochrome *c* by cupriplastocyanin (eq 3). The first reaction, involving $[Fe(CN)_6]^{3-}$ and $[Fe(C_5H_5)_2]^+$ as oxidants, is studied by stopped-flow spectrophotometry. The second and the third reactions, involving both the electrostatic (natural) and the covalent (cross-linked) diprotein complexes, are studied by pulse radiolysis.

$$\text{Fe(III) complex} + \text{cyt(II)/pc(I)} \rightarrow \text{cyt(III)/pc(I)} + \text{Fe(II) complex} \quad (1)$$

$$e_{aq}^- + \text{cyt(III)/pc(II)} \rightarrow \text{cyt(II)/pc(II)} \quad (2)$$

$$\text{cyt(II)/pc(II)} \rightarrow \text{cyt(III)/pc(I)} \quad (3)$$

Materials and Methods

Chemicals. Ferrocene, 4,4′-bipyridine dihydrochloride, Sephadex G-75 (50 mesh), Sephadex DEAE A-25, cellulose CM-52, and EDC were obtained from Sigma Chemical Co.; cystamine dihydrochloride was obtained from Aldrich Chemical Co. The ferricenium salt, $[Fe(C_5H_5)_2]PF_6$, was synthesized in a standard way (Yang et al., 1975). Distilled water was demineralized to a resistance greater than 10 MΩ cm.

Protein Purification. Horse heart ferricytochrome *c* (type III from Sigma) was purified by cation-exchange chromatography (Brautigan et al., 1978). French bean plastocyanin, a gift from Professor Harry 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 A-25, and washed with this buffer. When the buffer was made 150 mM in NaCl, a single blue band of the pure protein moved down the column. All the experiments were done with plastocyanin having the absorbance quotient $A_{278}/A_{597} \leq 1.20$. Dialyses were done in Amicon ultrafiltration cells with YM-5 membranes, at 4 °C, under 45-psi pressure of purified nitrogen.

Protein Concentrations. The separate proteins were quantitated spectrophotometrically on the basis of the following absorptivities (extinction coefficients), ϵ: 28 500, 106 100, and 11 000 M^{-1} cm^{-1} at 360, 410, and 526 nm, respectively, for ferricytochrome *c*; 4500 M^{-1} cm^{-1} at 597 nm for cupriplastocyanin. 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 calculated from the difference between the spectra of cyt(III)/pc(II) and cyt(III), matched at 360 nm. Concentration changes in kinetic experiments were calculated from the following absorptivity changes, $\Delta\epsilon = \epsilon_{red} - \epsilon_{ox}$: −13 800, 44 500, 31 100, 18 500, and 0 M^{-1} cm^{-1} at 360, 417, 425, 550, and 557 nm, respectively, for cytochrome *c*; −2050 and −2400 M^{-1} cm^{-1} at 550 and 557 nm, respectively, for plastocyanin.

The absorbance of cytochrome *c* at 360 nm, which was not obscured by that of hydrated electrons, permitted the kinetic study of the initial reduction event in the pulse-radiolysis experiments. Absorbance in the Soret region (at 417 and 425 nm) reflected the oxidation state of the iron atom only. Absorbance in the α region (at 550 nm) reflected such states of both the iron and copper atoms; oxidation of the one and reduction of the other caused changes in the same direction, and the two contributions proved separable.

Preparation of the cyt/pc Complexes. The electrostatic complex was made by mixing equimolar amounts of the two proteins in the same buffer. The covalent complex was made by a published method (Geren et al., 1983). The reaction mixture was 80 μM in each protein and 1 mM in EDC; the solvent was 5 mM MOPS buffer at pH 6.5; and the incubation lasted from 16 to 29 h at room temperature. The product was purified by size-exclusion chromatography on a column of Sephadex G-75 (50 mesh) gel.

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 (2.5 × 8.5 cm). 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 1 through 4 in the order of elution.

Properties of the cyt/pc Complexes. A fuller account of the procedures is given in our previous report (Peerey & Kostič, 1987). Molecular mass was determined on a standardized column of Sephadex G-75 (50 mesh) gel (1.5 × 70 cm) with 85 mM phosphate buffer at pH 7.0 as eluent. For greater accuracy, elution times at constant flow rate, rather than elution volumes, were measured. Absorption spectra were recorded with an IBM 9430 UV–vis spectrophotometer, equipped with a double-grating monochromator; protein concentrations in the pulse-radiolysis studies were measured with an HP 8450 UV–vis spectrophotometer. Differential-pulse voltammetry was done with a stationary gold electrode;

[1] Abbreviations: EDC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride; MOPS, 3-(*N*-morpholino)propanesulfonate buffer; cyt(III), ferricytochrome *c*; cyt(II), ferrocytochrome *c*; pc(II), cupriplastocyanin; pc(I), cuproplastocyanin; cyt/pc, either the electrostatic or the covalent complex with the oxidation states as indicated; NHE, normal hydrogen electrode; M_r, molecular mass; *A*, absorbance.

4,4′-bipyridine and cystamine were the mediators for cytochrome *c* and plastocyanin, respectively (Haladjian et al., 1983; Hill et al., 1985). The EPR spectra were recorded at 4.2 K.

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. $K_3[Fe(CN)_6]$ 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* (Moore et al., 1984). 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 of ferrocytochrome *c* and of cuproplastocyanin 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_{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 $[Fe(C_5H_5)_2]^+$ (Carney et al., 1984; Pladziewicz et al., 1985) and ca. 100 times in the case of $[Fe(CN)_6]^{3-}$ (Moore et al., 1984; Sykes, 1985)—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 $[Fe(CN)_6]^{3-}$ the amount of cyt(III)/pc(I) formed was equal to the amount of $[Fe(CN)_6]^{3-}$ available.

Pulse Radiolysis. The proteins were dissolved in phosphate buffer at pH 7.0, whose ionic strength (μ) was 1, 2, 40, or 200 mM. Tertiary butanol (2% by volume) was added to scavenge the $OH^\bullet$ radicals. The samples were deoxygenated gently with nitrogen and transferred anaerobically to a quartz cell with optical path of 10 or 20 mm. 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 Graaff accelerator delivered ca. 5.3 krad of 4-MeV electrons and produced a ca. 2 μM solution of e_{aq}^- in the sample (Foyt, 1981). 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 e_{aq}^- was observed on the microsecond time scale, and the subsequent oxidation of ferrocytochrome *c* by cupriplastocyanin was followed on the millisecond time scale. Although 557 nm is an isosbestic point for cytochrome *c*, absorbance of this protein did contribute somewhat to the absorbance of plastocyanin at this nominal wavelength because a wide slit had to be used. Oxidoreduction 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 wavelength 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.

Properties of the Cyt/Pc Complexes

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 (Augustin et al., 1983; Chapman et al., 1984). This view of the attachment is supported by much experimental evidence and by a preliminary simulation with computer graphics (King et al., 1985). 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 (King et al., 1985).

Cross-linking by the water-soluble EDC 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 Lys 86 (Waldmeyer & Bosshard, 1985; Nishimoto, 1986). The latter residues occur mainly in the segment 42–45 but also at the positions 59–61 and 68 (Burkey & Gross, 1981, 1982). The close structural similarity between the electrostatic and covalent complexes cyt/pc has been claimed (Geren et al., 1983; King et al., 1985), 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 (Erman et al., 1987), 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.

Composition and the Metal Sites in the Covalent Complex. Geren et al. (1983) purified the cyt/pc complex and determined its composition. We corroborated their findings.

Table I: Properties of the Covalent Complex between Cytochrome *c* and Plastocyanin and of the Native Proteins

property[a]	cyt/pc	cyt	pc
EPR *g* values			
g_x	1.3	1.26	
g_y[b]	2.3	2.25	
g_z	3.09	3.02	
$g_\parallel$	2.3		2.2
$g_\perp$	2.056		2.053
reduction potential at 25 °C (mV vs. NHE)			
Fe	245 ± 5[c]	256 ± 2[c,d]	
Cu	385 ± 5[e]		360 ± 4[e,f]
molecular mass (kDa)[g]	27.0 ± 1.2	12.5	10.5

[a] In 85 mM phosphate buffer at pH 7.0. [b] Measured at peak maximum. [c] Determined by differential-pulse voltammetry with 4,4′-bipyridine as mediator. [d] Moore et al. (1984); Taniguchi et al. (1980). [e] Determined by spectrophotometric titration with $[Fe(CN)_6]^{4-}$. [f] Sykes (1985); Taniguchi et al. (1980). [g] Determined by size-exclusion chromatography.

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 the Sephadex and CM-52 columns, stopped-flow experiments, and pulse-radiolysis experiments.

The apparent molecular mass of 27.0 ± 1.2 kDa, an average result of several determinations, is by 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 the log M_r—molecular mass itself is only an approximate measure of the biopolymer size—obtains 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 (Peerey & Kostić, 1987; Chen & Kostić, 1988; Kostić, 1988).

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

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 two proteins hampers their electroactivity (Barker & Hill, 1987). Redox titration of the plastocyanin moiety with $[Fe(CN)_6]^{4-}$ 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 EDC and an amine (Burkey & Gross, 1981, 1982). 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 these charges in the two proteins are opposite, the shifts are opposite.

Table II: Rate Constants for the Bimolecular Oxidation of Ferroheme in the Native Cytochrome *c* and in the Covalent Complex between Cytochrome *c* and Plastocyanin[a]

oxidant	reductant	$k \times 10^{-6}$ (M^{-1} s^{-1})	ref
$[Fe(CN)_6]^{3-}$	cyt(II)	8.0	*b*
$[Fe(CN)_6]^{3-}$	cyt(II)/pc(I)	2.1	this work
$[Fe(C_5H_5)_2]^+$	cyt(II)	6.4	*c*
$[Fe(C_5H_5)_2]^+$	cyt(II)/pc(I)	9.0	this work

[a] Determined by stopped-flow spectrophotometry, in 85 mM phosphate buffer at pH 7.0, at 25 °C. [b] Moore et al. (1984); Cassat et al. (1974). [c] Carney et al. (1984).

Extracomplex Oxidoreduction Reactions

Geren et al. (1983) showed qualitatively that the covalent cyt/pc complex is reducible by small reagents but that it is virtually unreactive toward cytochrome *c* oxidase and toward succinate-dependent cytochrome *c* reductase. We confirmed the reducibility of cyt(III)/pc(II) with ascorbate and $[Fe(CN)_6]^{4-}$ ions.

Oxidation by Iron Reagents. In order to examine quantitatively the possible effect of cross-linking on the redox reactions of cytochrome *c*, we studied the kinetics of the reaction in eq 1 with two different ferric compounds: $[Fe(CN)_6]^{3-}$, a hydrophilic anion, and $[Fe(C_5H_5)_2]^+$, a hydrophobic cation. The findings are listed in Table II. The presence of plastocyanin somewhat inhibits the oxidation of ferrocytochrome *c* by $[Fe(CN)_6]^{3-}$ and somewhat promotes its oxidation by $[Fe(C_5H_5)_2]^+$. 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 (Moore et al., 1984), 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 (Carney et al., 1984). Without this knowledge, it can only be conjectured that any steric shielding of the heme is overcome by the electrostatic effect of plastocyanin.

Reduction by 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 2), however, was examined quantitatively 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. The average rate constant for the three cases is $(1.2 \pm 0.2) \times 10^{10}$ M^{-1} s^{-1}, in accord with the previous determination for the native protein (Koppenol & Butler, 1984). The representative traces are shown in Figure 1. In the covalent complex the electrons 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 e_{aq}^-.

Intracomplex Electron-Transfer Reactions

Overview of the Reactions. The reactions that can occur in our pulse-radiolysis experiments are summarized in Scheme

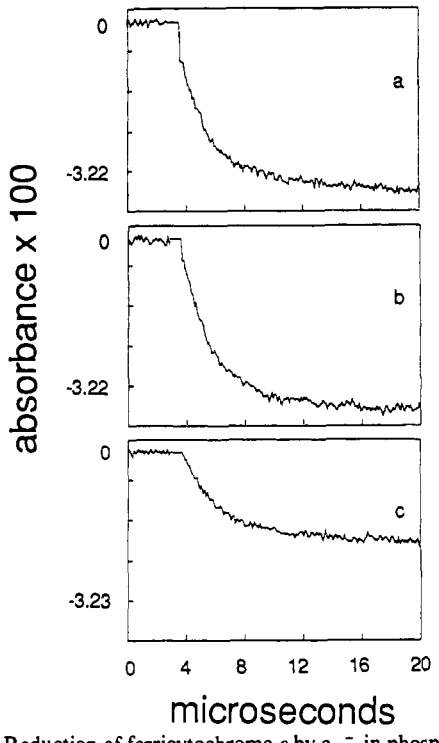
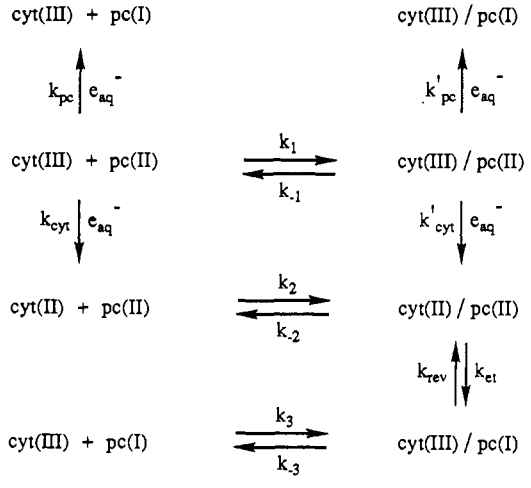


FIGURE 1: Reduction of ferricytochrome *c* by e_{aq}^-, in phosphate buffer at pH 7.0, monitored at 360 nm. (a) Native cyt(III), 20.5 μM, at μ = 40 mM. (b) Electrostatic complex cyt(III)/pc(II), 20.5 μM, at μ = 40 mM. (c) Covalent complex cyt(III)/pc(II), 12.1 μM, at μ = 2 mM.

Scheme I



I. 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 by electrons; 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 4 is justified. Any cuproplastocyanin that was formed (in

$$K_A = \frac{k_2}{k_{-2}} \approx \frac{k_1}{k_{-1}} \qquad (4)$$

the reactions k_{pc} and k'_{pc}) remained unreactive because its oxidation by ferricytochrome *c* is thermodynamically unfavorable. When, however, ferrocytochrome *c* was formed (in the reactions k_{cyt} and k'_{cyt}), the subsequent electron-transfer

Table III: Observed First-Order Rate Constants for the Interprotein Electron-Transfer Reaction Involving the Electrostatic Complex between Cytochrome *c* and Plastocyanin[a]

	$k_{obsd} \times 10^{-2}$ (s^{-1})	
concentration[b] (μM)	μ = 1 mM	μ = 40 mM
2.9	10.6 ± 2.3	
5.6		3.03 ± 0.10
8.0	10.9 ± 0.7	
14.5		5.87 ± 0.77
15.0		5.22 ± 0.49
17.0	10.5 ± 1.1	
24.0		7.38 ± 0.28
25.2		6.79 ± 0.30
35.0	10.2 ± 1.0	

[a] Determined by pulse radiolysis, in phosphate buffer at pH 7.0, at ambient temperature. [b] Concentration of each cyt(III) and pc(II) in the equimolar mixture.

reaction between the proteins was favorable. The shortage of e_{aq}^- 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 ($\Delta\epsilon$) 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.

The most interesting process in Scheme I is the interprotein electron-transfer reaction. Under unimolecular conditions, i.e., when the diprotein complex is fully formed, the simple relation in eq 5 obtains. Under bimolecular conditions, the protein

$$k_{obsd} = k_{et} \qquad (5)$$

association (so-called preequilibrium) must also be taken into account; then the first-order rate constant is given by eq 6.

$$k_{obsd} = \frac{k_{et}K_A[pc(II)]}{1 + K_A[pc(II)]} \qquad (6)$$

Although the same expression is consistent with the so-called dead-end mechanism, this latter possibility is generally disfavored (Sykes, 1985). Both the unimolecular and the bimolecular reactions are analyzed below.

Electrostatic Complex. A comparison between Figure 2a on the one hand and Figure 2b,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 I. 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 ± 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 III shows, the observed rate constant at the low ionic strength remained unchanged over a 12-fold range of the complex concentration; according to eq 5, k_{et} = (1.05 ± 0.12) × 10^3 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.

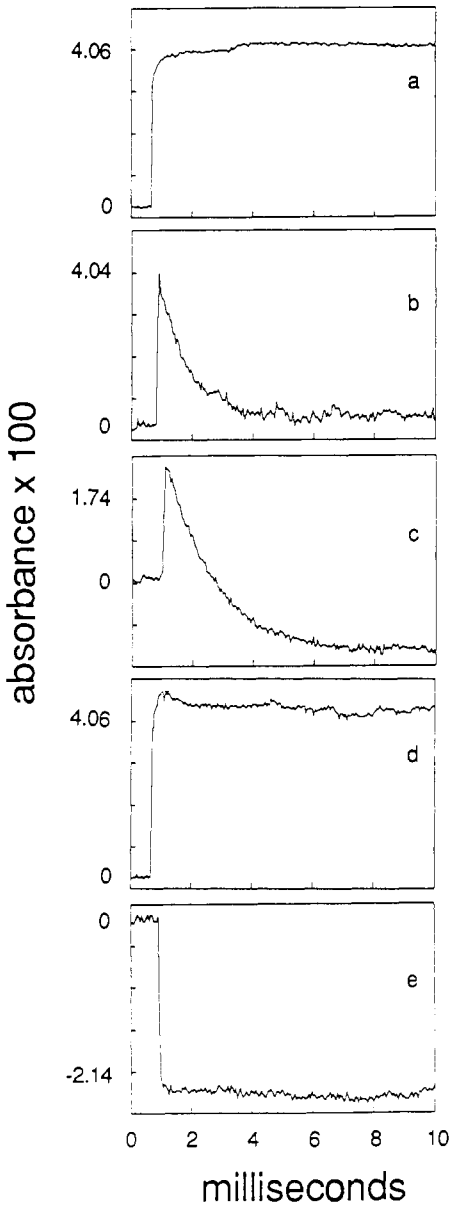


FIGURE 2: Fates of the species formed by reduction of different samples by e_{aq}^-, in phosphate buffer at pH 7.0. (a) Native cyt(II), 13.8 μM, at μ = 40 mM, monitored at 550 nm. (b) Electrostatic complex cyt(II)/pc(II), 35 μM, at μ = 1 mM, monitored at 425 nm. (c) Electrostatic complex cyt(II)/pc(II), 14.5 μM, at μ = 40 mM, monitored at 557 nm. (d) Covalent complex cyt(II)/pc(II), isomer 2, 4.8 μM, at μ = 200 mM, monitored at 417 nm. (e) Covalent complex cyt(II)/pc(II), isomer 3, 12.1 μM, at μ = 2 mM, monitored at 360 nm.

According to eq 6, the rate constant k_{et}, and its independence of concentration in the given range require $K_A \geq (5 \pm 2) \times 10^6$ M^{-1} at μ = 1 mM. Association constants of similar magnitudes (at low ionic strengths) have been reported for electrostatic complexes of cytochrome *c* with cytochrome b_5 (Mauk et al., 1986), cytochrome *c* peroxidase (Cheung & English, 1988), and cytochrome *c* oxidase (Osheroff et al., 1980).

The observed rate constant at a higher ionic strength depends markedly on the protein concentration, as Table III shows. This dependence was fitted to the two pertinent reactions in Scheme I. Only one rate constant was fixed: $k_{-2} = 1.0 \times 10^3$ s^{-1}, as determined by King et al. (1985). The other three rate constants were optimized as follows: $k_2 = 1.1 \times 10^8$ M^{-1} s^{-1}, $k_{et} = 1.1 \times 10^3$ s^{-1}, and $k_{rev} = 0$. This last finding denotes irreversibility of the electron-transfer step, a common characteristic of redox metalloproteins (Cusanovich et al., 1987). The two different determinations yielded the same value of k_{et}.

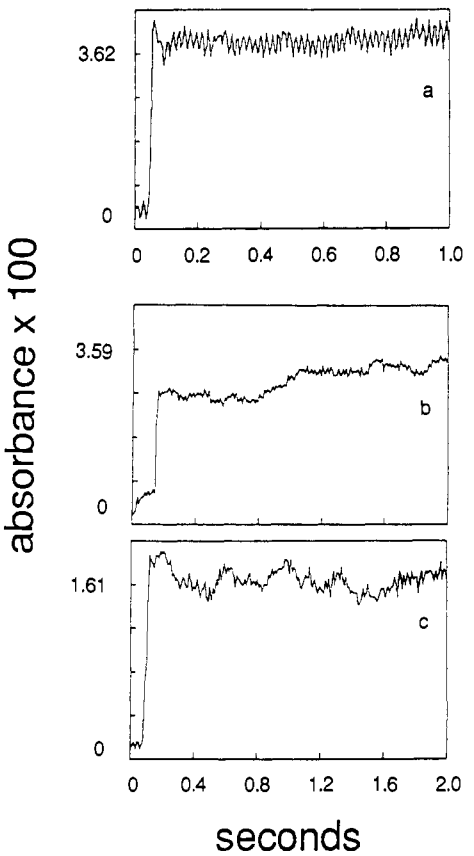


FIGURE 3: Absence of the electron-transfer reaction in different isomers of the covalent complex cyt(II)/pc(II), formed by reduction of the corresponding samples of cyt(III)/pc(II) by e_{aq}^-, in phosphate buffer at pH 7.0. (a) Isomer 2, 7.0 μM, at μ = 2 mM, monitored at 417 nm. (b) Isomer 1, 6.9 μM, at μ = 200 mM, monitored at 417 nm. (c) As in (b), but monitored at 550 nm.

The association constant at μ = 40 mM, obtained from eq 4, is 1.1×10^5 M^{-1}. It is alternatively calculated, with eq 6, from the rate constant k_{et}. An average of the K_A values for the protein concentrations listed in Table III is $(8.1 \pm 1.4) \times 10^4$ M^{-1} at μ = 40 mM. The equality, within the margin of error, of the K_A values obtained by the two methods confirms the applicability of eq 6 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. 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 reaction. The rate of $(1.05 \pm 0.12) \times 10^3$ s^{-1} seems to be the optimal one in this electrostatic complex.

Covalent Complex. Figures 2d,e and 3 show that the mixed-valence species, cyt(II)/pc(II), is stable for at least 2.0 s after the electron pulse that created 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 ways, 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(III)/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}.

The electrostatic and the covalent complexes behave completely differently under identical conditions although the cytochrome *c* and plastocyanin in them are claimed to be 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 and with hydrated electrons.

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 electrostatic aggregates (Northrup et al., 1988). 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 (Hazzard et al., 1988c). An even greater difference between the two types of cyt/pc complexes, the very subjects of our study, was observed electrochemically (Barker & Hill, 1987). 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.

Conclusions

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.

Acknowledgments

Stephen J. Atherton and Stephan Hubig helped us with the pulse-radiolysis experiments at the Center for Fast Kinetics Research, which is supported jointly by the Biomedical Research Technology Program of the Division of Research Resources, NIH (Grant RR00886), and by the University of Texas at Austin. Harry B. Gray gave us the plastocyanin, and Michael A. Cusanovich and Gordon Tollin gave us a program for kinetic calculations. Ann M. English, James H. Espenson, James K. Hurst, George L. McLendon, Norman Sutin, Gordon Tollin, and Ralph G. Wilkins commented on various aspects of the study. We thank them all.

Registry No. $[Fe(CN)_6]^{3-}$, 13408-62-3; $[Fe(C_5H_5)_2]^+$, 12125-80-3.

References

Augustin, M. A., Chapman, S. K., Davies, D. M., Sykes, A. G., Speck, S. H., & Margoliash, E. (1983) *J. Biol. Chem. 258*, 6405.

Barker, P. D., & Hill, H. A. O. (1987) *Recl. Trav. Chim. Pays-Bas 106*, 296.

Beoku-Betts, D., Chapman, S. K., Knox, C. V., & Sykes, A. G. (1985) *Inorg. Chem. 24*, 1677.

Bosshard, H. R., Zürrer, M., Schägger, H., & von Jagow, G. (1979) *Biochem. Biophys. Res. Commun. 89*, 250.

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978) *Methods Enzymol. 53*, 129.

Burkey, K. O., & Gross, E. L. (1981) *Biochemistry 20*, 5495.

Burkey, K. O., & Gross, E. L. (1982) *Biochemistry 21*, 5886.

Carney, M. J., Lesniak, J. S., Likar, M. D., & Pladziewicz, J. R. (1984) *J. Am. Chem. Soc. 106*, 2565.

Cassatt, J. C., & Marini, C. P. (1974) *Biochemistry 13*, 5323.

Chapman, S. K., Knox, C. V., & Sykes, A. G. (1984) *J. Chem. Soc., Dalton Trans.*, 2775.

Chen, J., & Kostič, N. M. (1988) *Inorg. Chem. 27*, 2682.

Cheung, E., & English, A. M. (1988) *Inorg. Chem. 27*, 1078.

Conklin, K. T., & McLendon, G. (1988) *J. Am. Chem. Soc. 110*, 3345.

Cusanovich, M. A., Meyer, T. E., & Tollin, G. (1987) *Adv. Inorg. Biochem. 7*, 37.

Eley, C. G. S., & Moore, G. R. (1983) *Biochem. J. 215*, 11.

Erman, J. E., Kim, K. L., Vitello, L. B., Moench, S. J., & Satterlee, J. D. (1987) *Biochim. Biophys. Acta 911*, 1.

Foyt, D. C. (1981) *Comput. Chem. 5*, 49.

Geren, L. M., Stonehuerner, J., Davis, D. J., & Millett, F. (1983) *Biochim. Biophys. Acta 724*, 62.

Gingrich, D. J., Nocek, J. M., Natan, M. J., & Hoffman, B. M. (1987) *J. Am. Chem. Soc. 109*, 7533.

Gupta, R. K., & Yonetani, T. (1973) *Biochim. Biophys. Acta 292*, 502.

Haladjian, J., Bianco, P., & Pilard, R. (1983) *Electrochim. Acta 28*, 1823.

Hazzard, J. T., Cusanovich, M. A., Tainer, J. A., Getzoff, E. D., & Tollin, G. (1986) *Biochemistry 25*, 3318.

Hazzard, J. T., McLendon, G., Cusanovich, M. A., Das, G., Sherman, F., & Tollin, G. (1988a) *Biochemistry 27*, 4445.

Hazzard, J. T., McLendon, G., Cusanovich, M. A., & Tollin, G. (1988b) *Biochem. Biophys. Res. Commun. 151*, 429.

Hazzard, J. T., Moench, S. J., Erman, J. E., Satterlee, J. D., & Tollin, G. (1988c) *Biochemistry 27*, 2002.

Hill, H. A. O., Page, D. J., Walton, N. J., & Whitford, D. (1985) *J. Electroanal. Chem. 187*, 315.

King, G. C., Binstead, R. A., & Wright, P. E. (1985) *Biochim. Biophys. Acta 806*, 262.

Koppenol, W. H., & Butler, J. (1984) *Isr. J. Chem. 24*, 11.

Kostič, N. M. (1988) *Comments Inorg. Chem. 8*, 137.

Liang, N., Kang, C. H., Ho, P. S., Margoliash, E., & Hoffman, B. M. (1986) *J. Am. Chem. Soc. 108*, 4665.

Liang, N., Mauk, A. G., Pielak, G. J., Johnson, J. A., Smith, M., & Hoffman, B. M. (1988) *Science (Washington, D.C.) 240*, 311.

Mauk, M. R., Mauk, A. G., Weber, P. C., & Matthew, J. B. (1986) *Biochemistry 25*, 7085.

McLendon, G. (1988) *Acc. Chem. Res. 21*, 160.

McLendon, G. L., & Miller, J. R. (1985) *J. Am. Chem. Soc. 107*, 7811.

McLendon, G., Miller, J. R., Simolo, K., Taylor, K., Mauk, A. G., & English, A. M. (1986) in *Excited States and Reactive Intermediates: Photochemistry, Photophysics and Electrochemistry* (Lever, A. B. P., Ed.) ACS Symposium Series 307, pp 150–164, American Chemical Society, Washington, DC.
McLendon, G., Pardue, K., & Bak, P. (1987) *J. Am. Chem. Soc. 109*, 7540.
Michel, B., & Bosshard, H. R. (1984) *J. Biol. Chem. 259*, 10085.
Moore, G. R., Eley, C. G. S., & Williams, G. (1984) *Adv. Inorg. Bioinorg. Mech. 3*, 1.
Nishimoto, Y. (1986) *J. Biol. Chem. 261*, 14232.
Northrup, S. H., Boles, J. O., & Reynolds, J. C. L. (1988) *Science (Washington D.C.) 241*, 67.
Osheroff, N., Brautigan, D. L., & Margoliash, E. (1980) *J. Biol. Chem. 255*, 8245.
Peerey, L. M., & Kostić, N. M. (1987) *Inorg. Chem. 26*, 2079.
Peterson-Kennedy, S. E., McGourty, J. L., Ho, P. S., Sutoris, C. J., Liang, N., Zemel, H., Blough, N. V., Margoliash, E., & Hoffman, B. M. (1985) *Coord. Chem. Rev. 64*, 125.
Pladziewicz, J. R., Brenner, M. S., Rodeberg, D. A., & Likar, M. D. (1985) *Inorg. Chem. 24*, 1450.
Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115.
Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563.
Sykes, A. G. (1985) *Chem. Soc. Rev. 14*, 283.
Tam, S. C., & Williams, R. J. P. (1985) *Struct. Bonding (Berlin) 63*, 103.
Taniguchi, V. T., Sailasuta-Scott, N., Anson, F. C., & Gray, H. B. (1980) *Pure Appl. Chem. 52*, 2275.
Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184.
Yang, E. S., Chan, M.-S., & Wahl, A. C. (1975) *J. Phys. Chem. 79*, 2049.

Functional Domain Structure of Calcineurin A: Mapping by Limited Proteolysis

Michael J. Hubbard‡ and Claude B. Klee*
Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892
Received August 2, 1988; Revised Manuscript Received September 29, 1988

ABSTRACT: Limited proteolysis of calcineurin, the Ca^{2+}/calmodulin-stimulated protein phosphatase, with clostripain is sequential and defines four functional domains in calcineurin A (61 kDa). In the presence of calmodulin, an inhibitory domain located at the carboxyl terminus is rapidly degraded, yielding an M_r 57 000 fragment which retains the ability to bind calmodulin but whose *p*-nitrophenylphosphatase is fully active in the absence of Ca^{2+} and no longer stimulated by calmodulin. Subsequent cleavage(s), near the amino terminus, yield(s) an M_r 55 000 fragment which has lost more than 80% of the enzymatic activity. A third, slower, proteolytic cleavage in the carboxyl-terminal half of the protein converts the M_r 55 000 fragment to an M_r 42 000 polypeptide which contains the calcineurin B binding domain and an M_r 14 000 fragment which binds calmodulin in a Ca^{2+}-dependent manner with high affinity. In the absence of calmodulin, clostripain rapidly severs both the calmodulin-binding and the inhibitory domains. The catalytic domain is preserved, and the activity of the proteolyzed 43-kDa enzyme is increased 10-fold in the absence of Ca^{2+} and 40-fold in its presence. The calcineurin B binding domain and calcineurin B appear unaffected by proteolysis both in the presence and in the absence of calmodulin. Thus, calcineurin A is organized into functionally distinct domains connected by proteolytically sensitive hinge regions. The catalytic, inhibitory, and calmodulin-binding domains are readily removed from the protease-resistant core, which contains the calcineurin B binding domain. Calmodulin stimulation of calcineurin is dependent on intact inhibitory and calmodulin-binding domains, but the degraded enzyme lacking these domains is still regulated by Ca^{2+}.

Calcineurin, the major soluble calmodulin-binding protein in brain (Klee et al., 1979), is a Ca^{2+}/calmodulin-stimulated protein phosphatase (Stewart et al., 1982). It appears to be the neural specific isozyme of a widely distributed class of protein phosphatases [as reviewed by Klee et al. (1988) and Klee and Cohen (1988)]. The enzyme is a heterodimer of calcineurin A (61 kDa) and calcineurin B (19 kDa) which remains tightly associated in the presence or absence of Ca^{2+}. Calcineurin A contains the catalytic center (Merat et al., 1985; Gupta et al., 1985) and in the presence of micromolar Ca^{2+} binds calmodulin with high affinity, $K_d = 10^{-10}$ M (Hubbard & Klee, 1987), resulting in a reversible 10–20-fold increase of the phosphatase activity. Calcineurin B binds Ca^{2+} with high affinity and is believed to mediate a small (2–3-fold) Ca^{2+} stimulation of enzyme activity and the Ca^{2+}-dependent association of the two subunits of the enzyme (Merat et al., 1985; Klee et al., 1985). The amino acid sequence of calcineurin B, determined by Aitken et al. (1984), revealed the presence of four "EF-hand" Ca^{2+}-binding domains as defined by Kretsinger (1980). Despite its structural similarity with calmodulin, calcineurin B does not substitute for calmodulin in the stimulation of the phosphatase; conversely, calmodulin cannot replace calcineurin B in the Ca^{2+}-dependent reconstitution of calcineurin (Klee et al., 1983; Merat et al., 1985). Previous studies showed that limited degradation of calcineurin A by trypsin abolishes calmodulin binding and results in the irreversible activation of the phosphatase (Manalan & Klee, 1983; Li & Chan, 1984; Tallant & Cheung, 1984). Activation of calcineurin by limited proteolysis or by calmodulin is not due to a dissociation of calcineurin subunits (Manalan & Klee,

* To whom correspondence should be addressed.
‡ Present address: Department of Biochemistry, University of Dundee, Dundee, Scotland DD1 4HN.