# Kinetic Studies of Reduction of a 1:1 Cytochrome *c*–Flavodoxin Complex by Free Flavin Semiquinones and Rubredoxin†

James T. Hazzard,‡ Michael A. Cusanovich,‡ J. A. Tainer,§ E. D. Getzoff,§ and Gordon Tollin*,‡

*Department of Biochemistry, University of Arizona, Tucson, Arizona 85721, and Department of Molecular Biology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

*Received October 22, 1985; Revised Manuscript Received January 14, 1986*

ABSTRACT: The kinetics of reduction by free flavin semiquinones and reduced rubredoxin of the individual components of the 1:1 complex formed between horse heart cytochrome *c* and *Clostridium pasteurianum* flavodoxin have been studied. Complex formation did not affect the rate constant for reduction of flavodoxin by 5-deazariboflavin semiquinone, indicating that the accessibility of the flavin mononucleotide (FMN) of complexed flavodoxin is the same as in the free protein. Reduction of the complexed cytochrome *c* by the neutral flavin semiquinones of lumiflavin and riboflavin was significantly affected by complex formation (2–3-fold rate constant decrease), indicating that there are steric constraints on the accessibility of the cytochrome heme to small exogenous reductants. Reduction of complexed cytochrome *c* by the negatively charged semiquinones of FMN and $Cl_2$FMN was also characterized. A repulsive electrostatic interaction between the reductants and complexed cytochrome was observed, whereas with free cytochrome an attractive interaction had previously been found. This is consistent with the presence of negative electrostatic potential at the protein interface due to uncompensated flavodoxin carboxylates, as predicted by Matthew et al. [Matthew, J. B., Weber, P. C., Salemme, F. R., & Richards, F. M. (1983) *Nature (London) 301*, 169–171]. Further, pseudo-first-order rate constants for the reduction of complexed cytochrome by these flavins had a nonlinear concentration dependence, rather than obeying simple second-order kinetics. This is interpreted by using a mechanism involving a rate-determining structural isomerization of the protein complex prior to the second-order electron-transfer step. The magnitude of the decrease in the rate constant for reduction of complexed cytochrome *c* by the negatively charged reduced rubredoxin was approximately the same as observed for free flavins. Furthermore, simple second-order kinetics were obtained, and the apparent electrostatic interaction between rubredoxin and the complex was attractive. These results suggest that flavodoxin was partially displaced from its complex with cytochrome *c* by a collisional interaction with rubredoxin. The effects of complexation on the kinetics have been correlated with a solvent-accessible surface representation of the computer-generated model of the flavodoxin–cytochrome *c* complex [Simondsen, R. P., Weber, P. C., Salemme, F. R., & Tollin, G. (1982) *Biochemistry 21*, 6366–6375]. The experimental observations are generally consistent with the structural model but clearly require the invocation of dynamic motions at the protein–protein interface.

The nature of the intermediate complex formed by a pair of electron-transfer proteins during their reaction is of interest in understanding the factors that govern the interaction mechanisms of physiological redox couples. Since direct structure determinations of such complexes have not thus far been achieved, computer models of several electron-transfer complexes have been generated on the basis of crystallographic structures of the isolated proteins (Salemme, 1976; Poulos & Kraut, 1980; Simondsen et al., 1982; Poulos & Mauk, 1983). The unifying themes underlying such models of electron-transfer complexes (Salemme, 1978) are that the protein–protein interaction is primarily electrostatic in nature, the prosthetic groups are located at the protein interface in a relatively nonpolar environment (i.e., $H_2O$ is excluded from the interaction domain), and the prosthetic groups are oriented with their planes relatively parallel to one another and are close enough such that direct electron transfer from one macrocycle to another can readily occur or, as in the case of the cytochrome *c*–cytochrome *c* peroxidase complex (Poulos & Kraut, 1980), electron transfer can take place through a hydrogen-bonded network lying between the prosthetic groups.

Recent attempts have made use of kinetic measurements to experimentally test the proposed electron-transfer models. Two complexes, cytochrome *c*–cytochrome *c* peroxidase (Pettigrew & Seilman, 1982; Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985) and *Azotobacter vinelandii* flavodoxin–cytochrome *c* (Dickerson et al., 1985), have been covalently cross-linked at low ionic strengths. It was shown that cross-linking the peroxidase to cytochrome *c* resulted in a 95% decrease in peroxidase activity toward exogenous cytochrome *c*(II).[1] A 16-fold decrease was also observed in the rate constant for ascorbate reduction of cross-linked cytochrome *c*(III) relative to free cytochrome at low ionic strength. No kinetic studies of the flavodoxin–cytochrome *c* complex were reported. One drawback of kinetic experiments involving cross-linked complexes is that, by virtue of the formation of covalent bonds between the components,

† This work was supported in part by research grants from the National Institutes of Health, AM15057 (G.T.) and GM21277 (M.A.C.).

‡ University of Arizona.

§ Scripps Clinic and Research Foundation.

[1] Abbreviations: cytochrome (or Cyt) *c*(II) and *c*(III), ferrous and ferric cytochrome *c*, respectively; FMN, flavin mononucleotide; Fld, flavodoxin; Rd, rubredoxin; LFH·, 5-DRFH·, RFH·, and FMNH·, neutral semiquinones of lumiflavin, 5-deazariboflavin, riboflavin, and FMN, respectively; $Cl_2FMN^{\cdot-}$, anion semiquinone of 7,8-dichloro-FMN; $NADP^+$, oxidized nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; $E_m$, midpoint reduction potential at pH 7.

0006-2960/86/0425-3318$01.50/0 

dynamic motions between the two proteins must certainly be diminished and extrapolation of data interpretation to the non-cross-linked complex can be misleading.

In a recent kinetic study of an electrostatically stabilized cytochrome *c*–cytochrome *c* peroxidase complex, Hoth and Erman (1984) have reported that at low ionic strength (10 mM) the interaction between the proteins decreased the rate of $CN^-$ binding to cytochrome *c* by 90%. This was interpreted in terms of electrostatic repulsion between the incoming cyanide and the negatively charged cytochrome *c*–peroxidase interface region. On the other hand, these workers found that the reactions of the anionic $F^-$ and neutral $H_2O_2$ with the complexed peroxidase were not affected by complex formation. These results are in good agreement with an earlier report by Mochan and Nicholls (1972) that, as a result of complex formation, ascorbate reduction of cytochrome *c* was inhibited by 90% while the reaction between peroxidase and peroxide was not affected. However, in both studies the effects of ionic strength were not investigated so it is difficult to differentiate between the roles of steric and electrostatic factors in influencing the kinetics of these reactions.

Another kinetic method of investigation of preformed protein–protein complexes has been extensively used in our laboratory. This involves rapid formation (<1 $\mu$s) of free flavin semiquinones by laser flash photolysis, which provides in situ generation of small reductants that react with the prosthetic groups of the individual proteins comprising the complexes. The proper choice of flavin analogue permits investigation of the effects of reductant size, electrostatic charge, and reduction potential on the interaction. This technique has been used to study intramolecular electron transfer in flavocytochromes (Cusanovich & Tollin, 1980; Tollin et al., 1984; Cusanovich et al., 1985; Bhattacharyya et al., 1985), the cytochrome *c*–cytochrome *c* oxidase complex (Ahmad et al., 1982), the *Clostridium pasteurianum* rubredoxin–spinach ferredoxin-$NADP^+$ reductase complex (Pryzsiecki et al., 1985a), and the complex between spinach ferredoxin and spinach ferredoxin-$NADP^+$ reductase (Bhattacharyya et al., 1986).

In this work, we report on the effect of complexation between cytochrome *c* and *C. pasteurianum* flavodoxin on the reduction kinetics of both proteins by exogenous one-electron donors. Although this complex is nonphysiological, a large body of information is available on the kinetic characteristics of the electron-transfer reaction between cytochrome *c*(III) and the semiquinone of *C. pasteurianum* flavodoxin (Simondsen et al., 1982; Simondsen & Tollin, 1983; Tollin et al., 1984), on cytochrome *c*(III) reduction kinetics using a wide variety of structural analogues of free flavins, and on flavodoxin reduction kinetics by 5-DRFH· (Ahmad et al., 1981, 1982; Meyer et al., 1983, 1984). On the basis of the kinetics and three-dimensional structural data for the components, a computer-generated model of a putative electron-transfer complex has been proposed (Simondsen et al., 1982; Weber & Tollin, 1985). Theoretical calculations have been performed that demonstrate a correlation between the electrostatic interaction energies and the rate and association constants for complex formation (Matthew et al., 1983; Weber & Tollin, 1985). The complex is characterized by a relatively high association constant at low ionic strength ($\sim 10^7$ $M^{-1}$ at $\mu$ = 20 mM), presumably due to the strong electrostatic interaction between four charge pairs consisting of cytochrome *c* lysines and flavodoxin carboxylates (Simondsen et al., 1982). The model also predicts that the heme *c* and the dimethylbenzene portion of the flavodoxin FMN are in van der Waals contact at the protein–protein interface. At low ionic strength the proton NMR spectrum of cytochrome *c* is significantly affected by complexation with flavodoxin (Hazzard & Tollin, 1985). Several of the resonances from protons of or near the heme *c* are broadened more than expected from the increased molecular weight and rotational correlation time of the complex, relative to the free cytochrome. In addition, several heme resonances in the complex are shifted from their frequencies in the free cytochrome.

In order to gain further insight into the nature of this electron-transfer complex, we have compared the reduction kinetics of cytochrome *c* and flavodoxin in the preformed complex with those of the individual components, utilizing free flavin semiquinones and reduced rubredoxin. The results obtained from these studies are in general agreement with the proposed structural model for this complex but provide some new perspectives concerning dynamic interactions occurring within the cytochrome *c*–flavodoxin structure.

## Experimental Procedures

Flavodoxin was isolated and purified from *C. pasteurianum* (ATCC 6013) by the procedure of Knight and Hardy (1966) to a purity index of $A_{280}/A_{443}$ = 4.28; the concentration was calculated from $\epsilon^{ox}_{443}$ = 10 $mM^{-1}$ $cm^{-1}$ (Mayhew, 1971). *C. pasteurianum* rubredoxin was purified to a purity index of $A_{280}/A_{490}$ = 4.1 (Lovenberg & Sobel, 1965); the concentration of rubredoxin was determined from $\epsilon^{ox}_{530}$ = 3.7 $mM^{-1}$ $cm^{-1}$ (Przysiecki et al., 1985a). Horse heart cytochrome *c* (type VI) was obtained from Sigma and used without further purification. Concentrations of the oxidized and reduced cytochrome were calculated from the following extinction coefficients: $\epsilon^{ox}_{550}$ = 8.16 $mM^{-1}$ $cm^{-1}$ and $\epsilon^{red}_{550}$ = 30.8 $mM^{-1}$ $cm^{-1}$. Preparation of lumiflavin and $Cl_2$FMN and purification of FMN were performed as previously described (Simondsen & Tollin, 1983). 5-Deazariboflavin was generously donated by Drs. William McIntire and Thomas P. Singer. Riboflavin was obtained from Sigma and used without further purification.

Laser flash photolysis experiments were performed as previously described (Ahmad et al., 1982; Simondsen & Tollin, 1983). The flavin concentration for kinetic experiments was 50 $\mu$M in 4 mM phosphate buffer containing 500 $\mu$M EDTA; the ionic strength was adjusted by the addition of the appropriate amount of NaCl. The percentage of dissociated cytochrome *c* at low ionic strengths ($\leq$20 mM) was calculated to be $\leq$9%, on the basis of the dissociation constants given by Simondsen et al. (1982) and Matthew et al. (1983). Cytochrome *c*(III) reduction was followed by the decrease of absorbance at 575 nm as described in Meyer et al. (1984). Flavodoxin photoreduction to the semiquinone by 5-deazariboflavin was followed by the loss of absorbance at 475 nm. Under these experimental conditions, the concentration of free flavin semiquinone generated by each laser flash was $\leq 10^{-7}$ M. The protein concentrations used for the determination of rate constants ($\geq$5 $\mu$M) were large enough so that flavin semiquinone disproportionation did not effectively compete with protein reduction and that pseudo-first-order conditions obtained. Semilog plots of kinetic data were linear over at least 4 half-lives, and the slopes of these plots were used to calculate $k_{obsd}$. The second-order rate constants $k_2$ were calculated from the slopes of linear plots of $k_{obsd}$ vs. protein concentration. The error in the rate constants presented below is estimated to be $\pm$10%.

Stopped-flow data for the reaction between reduced rubredoxin (produced by in situ illumination in the presence of riboflavin) and cytochrome *c*(III) were obtained on a Durrum-Gibson Model D-110 apparatus connected to an Olis Model 3600 interface and a Northstar computer. For each

experiment 200 data points were collected after a 3-ms mixing time. The data represented the increase in absorbance at 547 nm due to the reduction of cytochrome *c* and the oxidation of rubredoxin. As has been observed previously (Simondsen et al., 1982), reduction of cytochrome *c* (both free and in a 1:1 mixture with flavodoxin) was characterized by distinct biphasic kinetics. The slow phase of the reaction amounted to ≤30% of the total absorbance change, and the observed rate constant did not vary significantly with cytochrome concentration. Kinetic data for the stopped-flow experiments were analyzed by subtracting the contribution of the slow phase. Observed rate constants were obtained from semilog plots of the fast-phase absorbance change vs. time; plots of $k_{obsd}$ vs. cytochrome concentration gave the second-order rate constants, $k_2$.

Visible spectra were obtained on a Cary 15 spectrophotometer (with an Olis 3820 modification interfaced to a Northstar computer). Midpoint redox potential ($E_m$) determinations for free cytochrome *c* were performed by two different techniques. In the first method a spectroelectrochemical procedure (Earl, 1981) using an EG&G Model 363 potentiostat with Pt and Ag/AgCl electrodes in a solution containing ferricyanide (1.2 mM), FeEDTA (0.5 mM), and methyl viologen as mediators was used; reductive and oxidative titrations were carried out from +500 to +100 mV (SHE). In the second method oxidized cytochrome *c* in 4 mM phosphate buffer containing 500 μM EDTA ($\mu$ = 10 mM) at pH 7.2 was titrated with ferrocyanide [see Wilson (1978) for a description]. The ionic strength at the end of the titration was 20 mM, which is sufficiently low to minimize protein complex dissociation. In all cases, equilibrium was achieved within several minutes. The two methods gave essentially identical results (±3 mV). Determination of $E_m$ for the cytochrome *c*–flavodoxin complex was accomplished with the ferrocyanide titration procedure. The reduction potential used for $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, corrected for the phosphate buffer and ionic strength (O'Reilly, 1973), was 397 mV.

For computer-graphic analysis atomic coordinates were taken from the Brookhaven Protein Data Bank (Bernstein et al., 1977). Exposed molecular surfaces were calculated with the program MS (Connolly, 1983), which mathematically positions a water-sized molecule wherever it can touch the van der Waals surface of the protein. Molecular surface calculations used individual van der Waals radii, including implicit hydrogen atoms (Getzoff et al., 1986). Computer-graphic docking and analysis of the complexes was done by using the interactive graphics language Gramps (O'Donnell & Olson, 1981) and the molecular modeling program Granny (Connolly & Olson, 1985). Accessibility of the heme and FMN in the cytochrome *c*–flavodoxin complex and chemical and shape complementarity were judged by using color graphics to define shape, stereochemical interactions, and degree of fit, as described elsewhere (Tainer et al., 1985).

## Results and Discussion

*Reduction Potential Measurements for Free and Complexed Cytochrome c.* Reduction potential measurements for free cytochrome *c*(III) at low ionic strengths (10 mM ≤ $\mu$ ≤20 mM) gave an $E_m$ of +275 ± 3 mV ($n$ = 0.88 ± 0.06; data not shown). The $E_m$ value for the complexed cytochrome *c* was determined to be +248 ± 3 mV ($n$ = 0.86 ± 0.05; data not shown). We estimate from the complex association equilibrium constants over the ionic strength range of this measurement (Matthew et al., 1983; Weber & Tollin, 1985) that approximately 95% of the cytochrome *c* was complexed at the end of the titration. Thus, complex formation with flavodoxin resulted in a 27-mV decrease in the $E_m$ of cytochrome *c*. Similar decreases in redox potential for cytochrome *c* have been reported for complexation with liposomes, submitochondrial particles, mitochondria, and cytochrome *c* oxidase [cf. Nicholls (1974)]. This is consistent with a recent proposal by Rees (1985) that the reduction potential of cytochrome *c* should decrease as a result of complex formation with the negatively charged flavodoxin because of the neutralization of positively charged lysines near the heme crevice and the resultant stabilization of the Fe(III) heme relative to the Fe(II) heme. It is also consistent with cytochrome *c*(III) being bound more tightly to flavodoxin than cytochrome *c*(II). On the basis of the results of Meyer et al. (1983, 1984), a redox potential change in this magnitude would be expected to result in a decrease in the rate constant for flavin semiquinone reduction of cytochrome *c*(III) of only 10–20%.

*Laser Flash Photolysis Difference Spectra.* In order to determine whether it was possible to reduce each protein individually within the 1:1 complex, we performed photochemical titrations using free flavin semiquinones as reductants. Figure 1a shows reduced minus oxidized difference spectra of 1:1 mixtures of cytochrome *c* and oxidized flavodoxin at low ionic strength (20 mM) over a wavelength range of 500–650 nm obtained in both equilibrium and time-resolved laser flash experiments using lumiflavin semiquinone ($E_m$ = −231 mV). The positive maxima at 520 and 550 nm and the isosbestic points at 504, 526, 540.5, and 557 nm are the same as obtained in the difference spectrum of free cytochrome *c*. There is no detectable flavodoxin semiquinone in these spectra. This is expected, based on reduction potential difference for the cytochrome *c*(III)/lumiflavin couple ($\Delta E$ = 508 mV) relative to the flavodoxin/lumiflavin couple ($\Delta E$ = 99 mV). The smaller intensity of the 550-nm band in the time-resolved spectrum, relative to that of the equilibrium spectrum, can be attributed to the greater monochromator slit width of the monitoring beam in the kinetic flash experiment. The satisfactory agreement between the time-resolved difference spectrum and the equilibrium spectrum illustrates that in the 1:1 complex only the cytochrome has undergone reduction on the time scale used in the kinetic studies to be discussed below; i.e., flavodoxin reduction followed by intracomplex electron transfer was not observed under these experimental conditions.

In order to obtain similar difference spectra for flavodoxin in the 1:1 complex, we used 5-DRF ($E_m$ = −650 mV) and formed the complex using cytochrome *c*(II). Figure 1b presents the visible difference spectrum (solid line) of a solution of flavodoxin, in the presence of 5-DRF but with no cytochrome *c* present, that had been exposed to a high-intensity light source for approximately 60 s. The maximum at 578 nm and the isosbestic point at 492 nm are consistent with the difference spectrum of semiquinone minus oxidized flavodoxin (Mayhew, 1971). The time-resolved difference spectra obtained from laser flash experiments in the absence and presence of equimolar cytochrome *c*(II) at low ionic strength are shown as the data points in Figure 1b. Again, within experimental error, there is good agreement between the equilibrium and laser flash induced difference spectra, with no indication of a cytochrome contribution. These results clearly establish that individual reduction of either of the components within the complex can be accomplished under the appropriate conditions.

*Kinetic Titration of Cytochrome c with Flavodoxin: Stoichiometry of Flavodoxin Binding to Cytochrome c.* In order to investigate the stoichiometry of complex formation and the effect of flavodoxin binding on the rate constant for heme *c* reduction by free flavin semiquinones, we titrated

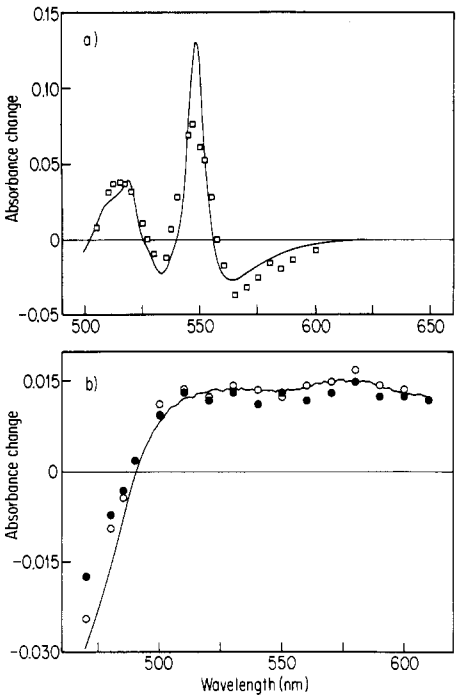


FIGURE 1: Equilibrium and transient-reduced minus oxidized difference spectra of flavodoxin and cytochrome *c*, both free and in 1:1 mixtures. (a) Data points (□) represent the laser flash induced difference spectrum obtained from a 1:1 mixture of cytochrome *c*(III) and flavodoxin at low ionic strength ($\mu$ = 20 mM) in the presence of lumiflavin (average of five flashes). The amplitude of each data point was calculated by subtracting the intensity of the signal at zero time from the signal at 25 ms. The equilibrium difference spectrum (solid line) was measured on the same sample after irradiation with ~100 laser flashes. The units for the data points are arbitrary; the points were normalized with respect to the absorption spectrum by using the 520-nm band. The buffer was 4 mM phosphate (pH 7.1) containing 500 $\mu$M EDTA and the appropriate amount of NaCl to bring the ionic strength to 20 mM. (b) The laser flash induced difference spectrum (○) obtained from a solution containing 14.3 $\mu$M flavodoxin in the presence of 5-DRF on a 25-ms time scale; the difference spectrum (●) obtained from a 1:1 mixture of flavodoxin and cytochrome *c*(II) (13.7 $\mu$M each) at 20 mM ionic strength. The solid line represents the equilibrium difference spectrum of the flavodoxin solution. The units for the data points are arbitrary; the points were normalized with respect to the absorption spectrum by using the 590-nm region. Data acquisition methods for laser flash and equilibrium spectra and buffer conditions were as in (a). The cuvette path length for all spectra was 1 cm.

cytochrome *c*(III) with oxidized flavodoxin at low ionic strength, measuring the pseudo-first-order rate constant for reduction of the heme by LFH·. The results of this titration are shown in Figure 2. As flavodoxin was added, the rate constant decreased from a maximum value of 3000 $s^{-1}$ to a value of ~800 $s^{-1}$ at a molar ratio of 1:1. Further addition of flavodoxin produced only very small changes in the $k_{obsd}$ value.

The transient decay curves from which these data were collected were monophasic within experimental error over 4 half-lives. Since for concentration ratios of less than unity both free and complexed cytochrome were simultaneously present in solution, it might be expected that biphasic kinetic traces would have been observed. However, since the rate constant difference is only ~3.5-fold, it is unlikely that distinct biphasicity would have been resolvable. These results are consistent with the formation of a 1:1 complex between oxidized flavodoxin and cytochrome *c*(III) and an effect of such complex formation on the kinetics of heme *c* reduction by LFH·.

*Direct Determination of Rate Constants for Reduction of the 1:1 Complex between Flavodoxin and Cytochrome c.* As

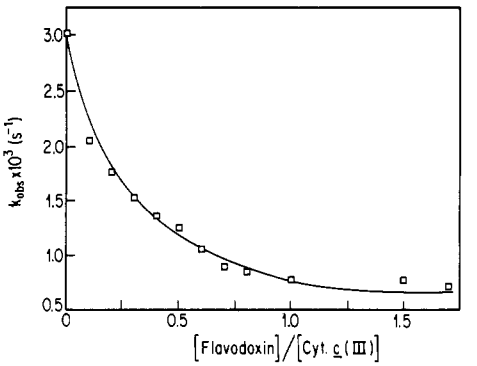


FIGURE 2: Dependence of $k_{obsd}$ for cytochrome *c*(III) reduction by LFH· on [flavodoxin]/[cytochrome *c*(III)]. Cytochrome *c* (40 $\mu$M) was titrated with flavodoxin in phosphate buffer (pH 7.1, $\mu$ = 20 mM) containing 50 $\mu$M lumiflavin and 500 $\mu$M EDTA. The monitoring wavelength was 575 nm.

Table I: Rate Constants for Reduction of Free and Complexed Flavodoxin and Cytochrome *c*(III) by 5-DRFH·, LFH·, and RFH·

| protein | flavin | $\mu$ (mM) | $k_2 \times 10^7$ ($M^{-1}$ $s^{-1}$)[a] free | complex |
|---|---|---|---|---|
| Fld | 5-DRFH· | 20 | 6.9 ± 0.5 | 6.9 ± 0.5 |
| Cyt *c* | LFH· | 20 | 7.1 ± 0.5 | 2.9 ± 0.3 |
| Cyt *c* | LFH· | 100 | 7.0 ± 0.5 | 5.9 ± 0.4 |
| Cyt *c* | RFH· | 20 | 5.4 ± 0.3 | 1.7 ± 0.2 |
| Cyt *c* | RFH· | 100 | 5.4 ± 0.2 | 4.6 ± 0.2 |

[a] Rate constants were calculated from the slopes of the plots of Figure 3.

a means of obtaining further insight into the nature of the cytochrome *c*–flavodoxin complex, we measured the kinetics of reduction of the two proteins within the complex using free flavin semiquinones that vary with respect to reduction potential, size, and electrostatic charge. 5-DRFH·, generated by laser flash photolysis, was used to determine the rate constant for flavodoxin reduction in the absence and presence of equimolar cytochrome *c*(II) at low ionic strength ($\mu$ = 10 mM). The plots of $k_{obsd}$ vs. concentration obtained from these experiments are shown in Figure 3a, and the calculated rate constants are given in Table I. The presence of cytochrome *c*(II) had no measurable effect on the flavodoxin reduction rate constant.

The second-order rate constant for reduction of free flavodoxin given in Table I ($7 \times 10^7$ $M^{-1}$ $s^{-1}$) is significantly smaller than that reported previously ($4 \times 10^8$ $M^{-1}$ $s^{-1}$; Simondsen & Tollin, 1983). However, the earlier experimental conditions were different (pH 7.5 and $\mu$ = 145 mM, as opposed to the present conditions of pH 7.0 and $\mu$ = 10 mM), and improvement has since been made in the methodology of data collection and in the laser apparatus.

Previous studies have shown that the exposed dimethylbenzene ring portion of flavodoxin FMN is the most probable site of electron transfer (Simondsen et al., 1982; Simondsen & Tollin, 1983). As noted above, computer modeling of the cytochrome *c*–flavodoxin complex has indicated that this exposed edge is located at the interface of the protein complex and is in direct contact with the heme (see Figure 5 below). The present kinetic results indicate that the free 5-DRFH· encountered no steric barrier during its interaction with the flavodoxin prosthetic group within the protein complex.[2] We

[2] The reduction potential for flavodoxin, both free and in the complex, was not measured in these studies. We assume, however, that any alteration of the flavodoxin redox potential within the complex has a negligible effect on the reaction rate constant.

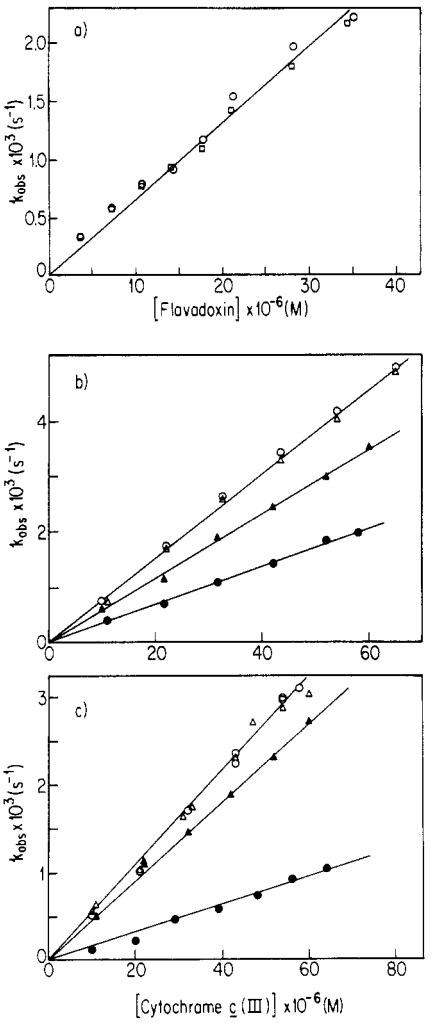


FIGURE 3: Plots of $k_{obsd}$ vs. concentration for reduction of flavodoxin and cytochrome $c$(III), both free and in 1:1 mixtures. (a) Flavodoxin reduction by 5-DRFH·, both free and in a 1:1 mixture with cytochrome $c$(II), at low ionic strength ($\mu$ = 10 mM): (○) free flavodoxin reduction; (□) flavodoxin reduction in a 1:1 mixture with cytochrome $c$(II). The monitoring wavelength was 475 nm. (b) LFH· reduction of cytochrome $c$(III), both free and in a 1:1 mixture with flavodoxin at low and high ionic strengths: (○ and △) free cytochrome $c$ at $\mu$ = 20 and 100 mM, respectively; (● and ▲) cytochrome $c$ and flavodoxin mixture (1:1) at $\mu$ = 20 and 100 mM, respectively. (c) RFH· reduction of cytochrome $c$(III), both free and in 1:1 mixtures, at low and high ionic strengths: (○ and △) free cytochrome at $\mu$ = 20 and 100 mM, respectively; (● and ▲) cytochrome $c$ and flavodoxin mixture at $\mu$ = 20 and 100 mM, respectively. The monitoring wavelength was 575 nm. Reaction conditions were as in Figure 1, except for ionic strength. Second-order rate constants calculated from these plots are listed in Table I.

shall return to this point below.

Investigation of the kinetics of cytochrome $c$(III) reduction within the 1:1 complex produced rather different results than were found for flavodoxin. Figure 3b shows the $k_{obsd}$ vs. concentration plots for reduction of free and complexed cytochrome $c$ by LFH· (cf. Table I). Reduction of the free cytochrome by LFH· showed no ionic strength dependency. The rate constant at both the ionic strengths studied was determined to be 7 × 10$^7$ M$^{-1}$ s$^{-1}$, which agrees well with previous reports (Meyer et al., 1983). The lack of ionic strength dependency is expected since LFH· is electrically neutral at pH 7.0 (Przysiecki et al., 1985b). Reduction kinetics of the cytochrome in 1:1 mixtures with flavodoxin yielded significantly smaller values for the rate constant and showed a distinct ionic strength dependence. The rate constant increased with ionic strength; at $\mu$ = 20 mM, $k_2$ = 2.9 × 10$^7$ M$^{-1}$ s$^{-1}$, and at $\mu$ = 100 mM, a $k_2$ value of 5.9 × 10$^7$ M$^{-1}$ s$^{-1}$ was obtained.

Utilization of riboflavin semiquinone as a reductant allows an assessment of the effect of increased steric bulk on the reduction of cytochrome $c$(III) both free and in the complex, due to the ribityl group that replaces the methyl group of lumiflavin at the $N_{10}$ position. The plots of $k_{obsd}$ vs. concentration for these experiments are shown in Figure 3c, and the rate constants are given in Table I. The rate constant for reduction of free cytochrome $c$(III) by RFH· is significantly smaller than that obtained with LFH· (Table I). This rate constant is also ionic strength independent, a value of 5.4 × 10$^7$ M$^{-1}$ s$^{-1}$ being found at $\mu$ = 20 and 100 mM. These results, again, are in good agreement with previously reported values (Meyer et al., 1984). At low ionic strength, the second-order rate constant for reduction of cytochrome $c$ in the 1:1 complex was 1.7 × 10$^7$ M$^{-1}$ s$^{-1}$, which corresponds to a 3.2-fold decrease relative to the $k_2$ value for free cytochrome $c$ reduction. In the case of LFH·, the decrease in $k_2$ was 2.4-fold at this ionic strength. At the higher ionic strength, $\mu$ = 100 mM, the rate constant for RFH· reduction of cytochrome $c$ in the presence of flavodoxin was 4.6 × 10$^7$ M$^{-1}$ s$^{-1}$, which is only 1.2-fold less than that for free cytochrome reduction. In the lumiflavin case, the decrease at high ionic strength was also 1.2-fold.

Unlike the results obtained for reduction of complexed flavodoxin, a comparison of the rate constants for the reduction of complexed cytochrome $c$ by these small neutral flavin semiquinones indicates that the heme edge is significantly less accessible in the protein complex than in the free cytochrome. Thus, at low ionic strength, the rate constants for lumiflavin and riboflavin reduction of complexed cytochrome were both substantially less than those found for the free protein. The fact that the effect on the riboflavin rate constant was greater than that for lumiflavin is consistent with the greater degree of steric constraint imposed by the larger size of the ribityl side chain. As $\mu$ was increased, the rate constants for both flavin semiquinone reactions were closer to the values obtained with free cytochrome $c$, which is consistent with the electrostatic nature of the interaction between cytochrome $c$ and flavodoxin (Simondsen et al., 1982; Matthew et al., 1983; Weber & Tollin, 1985); i.e., the complex was more highly dissociated. At 100 mM ionic strength, the rate constants were significantly smaller than those for the free cytochrome, indicating that there was an appreciable fraction of the total cytochrome that was complexed. We have found that even at $\mu$ = 200 mM, the interaction between the two proteins is sufficient to cause a small effect on the NMR spectrum of cytochrome $c$ (Hazzard & Tollin, 1985).

Electrostatic potential calculations on the cytochrome $c$–flavodoxin complex (Matthew et al., 1983) have predicted that there should be a net negative electrostatic potential at the protein interface due to total discharge of the cytochrome positive charge by uncompensated negative charge on the flavodoxin. In order to kinetically probe this feature of the complex, we employed the negatively charged FMN semiquinone as a reductant. Plots of $k_{obsd}$ vs. concentration for FMNH· reduction of free cytochrome $c$(III) over a range of ionic strengths are shown in Figure 4a; the rate constants derived from these plots are listed in Table II. In Figure 4a the plots of $k_{obsd}$ vs. [cytochrome] are all linear for ionic strengths from 10 to 100 mM. The decrease in $k_2$ as $\mu$ increased demonstrates that there was an attractive electrostatic interaction component (of a minus–plus type) in agreement with the data and conclusions reported previously (Meyer et al., 1984; Simondson et al., 1982).

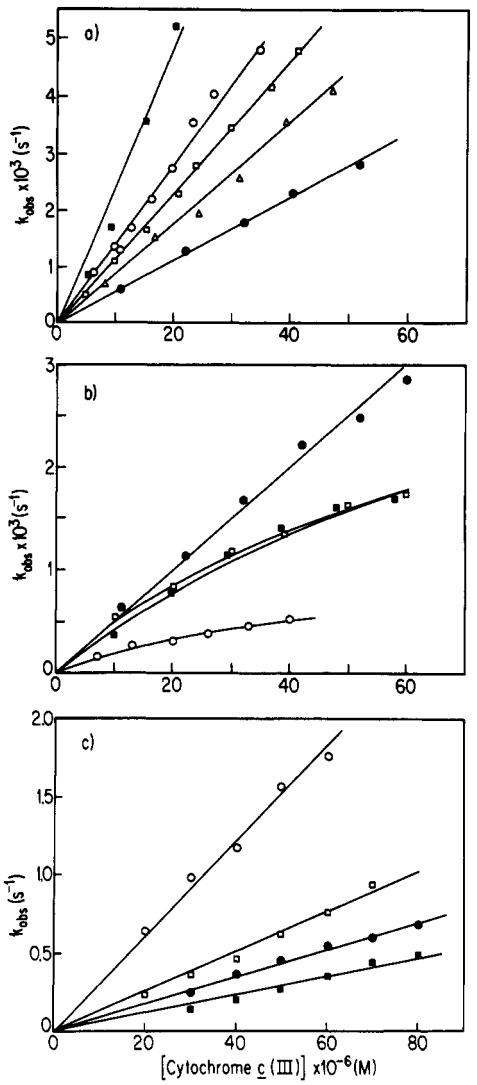


FIGURE 4: Dependence of $k_{obsd}$ on ionic strength for reduction of cytochrome $c$, both free and in a 1:1 mixture with flavodoxin, by FMNH·, $Cl_2FMN^{\bullet-}$, and reduced rubredoxin. (a) Free cytochrome $c$ reduction by FMNH· at $\mu$ = 10 (○), 20 (□), 40 (△), and 100 mM (●); $Cl_2FMN^{\bullet-}$ reduction of free cytochrome at $\mu$ = 20 mM (■); (b) FMNH· reduction of cytochrome $c$ in 1:1 mixtures with oxidized flavodoxin at $\mu$ = 10 (○), 20 (□), and 100 mM (●); $Cl_2FMN^{\bullet-}$ reduction at $\mu$ = 20 mM (■). Solid curves represent theoretical values for $k_{obsd}$ obtained by least-squares analysis according to the mechanism given under Results and Discussion. The monitoring wavelength was 575 nm. Rate and equilibrium constants derived from these plots are given in Table II. (c) Reduced rubredoxin reduction of cytochrome $c$(III), both free and in a 1:1 complex with flavodoxin: (○ and □) reduction of free cytochrome at $\mu$ = 15 and 25 mM, respectively; (● and ■) reduction of cytochrome $c$ in the complex at $\mu$ = 15 and 25 mM, respectively. Rubredoxin (10 $\mu$M before mixing) was reduced by illumination in the presence of riboflavin and EDTA, as described under Experimental Procedures, prior to mixing with the cytochrome solutions. The monitoring wavelength was 547 nm. Buffer conditions were as in Figure 1, except for ionic strength.

The kinetics of reduction of cytochrome $c$ by FMNH· in the presence of flavodoxin are more complicated than observed for the free cytochrome. Plots of $k_{obsd}$ vs. protein concentration in Figure 4b for $\mu$ = 10 and 20 mM are nonlinear, with $k_{obsd}$ becoming independent of changes in protein concentration. At $\mu$ = 100 mM, the nonlinear concentration dependence seen at the lower ionic strengths was no longer observed and the plot of $k_{obsd}$ vs. protein concentration is again linear over the range of concentrations studied.

The kinetic behavior at low $\mu$ ($\leq$20 mM) indicates that the reaction pathway must involve a rate-limiting first-order process that becomes apparent at high protein concentrations. Two mechanisms[3] can be used to account for these results (Strickland et al., 1975). Equations 1 and 2 describe a two-step mechanism that involves a structural isomerization of the protein complex prior to the second-order reduction reaction (see below for further discussion). Equation 2 requires that

$$\text{Cyt } c\text{(III)–Fld} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Cyt } c\text{(III)–Fld}]^* \quad (1)$$

$$[\text{Cyt } c\text{(III)–Fld}]^* + \text{FMNH}\cdot \xrightarrow{k_2} \text{Cyt } c\text{(II)–Fld} + \text{FMN} \quad (2)$$

FMNH· react only with one of the structural forms of the protein complex, denoted as [Cyt $c$(III)–Fld]*. Note that in this mechanism the limiting first-order process must be independent of the chemical nature of the reductant. A second mechanism that can explain the nonlinear kinetic plots is given by eq 3 and 4, in which the limiting first-order process involves a reaction occurring within a ternary electron-transfer complex.

$$\text{Cyt } c\text{(III)–Fld} + \text{FMNH}\cdot \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Cyt } c\text{(III)–Fld–FMNH}\cdot] \quad (3)$$

$$[\text{Cyt } c\text{(III)–Fld–FMNH}\cdot] \xrightarrow{k_2} \text{Cyt } c\text{(II)–Fld} + \text{FMN} \quad (4)$$

In this mechanism, the first-order step, eq 4, would be expected to depend on the chemical nature of the reductant. Both mechanisms assume that the reverse reactions for eq 2 and 4 do not occur, which is consistent with the overall thermodynamics of the electron-transfer reaction ($\Delta E_m \cong +500$ mV for reduction of cytochrome $c$ by FMNH·).

The nonlinear plots of $k_{obsd}$ vs. concentration, shown in Figure 4b, were modeled by least-squares regression of the data to fit the analytical solution to the equations for a two-step mechanism. A further constraint imposed in the modeling was that the data fit a single exponential equation over the first 4 half-lives of the reaction. Since the two mechanisms shown above give mathematically identical solutions [see Strickland et al. (1975)], computer modeling alone cannot distinguish between them. In order to differentiate between the two possible mechanisms, we employed another free flavin, $Cl_2FMN$. The p$K_a$ of the $N_5$ proton of FMN semiquinone is 8.5 (Draper & Ingraham, 1968; Vaish & Tollin, 1971), and under these experimental conditions the electrostatic charge of FMN is due solely to the phosphate group. $Cl_2FMN$ semiquinone, on the other hand, has a p$K_a$ for the $N_5$ proton of 6.5, and thus at neutral pH the predominate one-electron reduced species is the anion radical (Przysiecki et al., 1985b), which has an additional negative charge relative to FMNH·. Furthermore, flavin anion radicals have been shown by Prysziecki et al., (1985b) to be more reactive than neutral radicals. The data obtained for reduction of cytochrome $c$, both free and in the complex, by $Cl_2FMN^{\bullet-}$ at $\mu$ = 20 mM are also shown in Figure 4. For the free cytochrome, the $k_2$ value was larger for $Cl_2FMN^{\bullet-}$ than for FMNH·, as expected (cf. Table II and Figure 4a). In the complex, the values of $k_{obsd}$ for FMNH· and $Cl_2FMN^{\bullet-}$ reduction of cytochrome $c$

[3] An alternative interpretation of the nonlinear concentration dependence is that the limiting rate constant corresponds to a simple dissociation of the cytochrome $c$–flavodoxin complex. We consider this to be unlikely since the dissociation rate constant at $\mu$ = 20 mM can be estimated from the data of Simondsen et al. (1982) to be $\sim$60 s$^{-1}$, which is significantly smaller than the value of 2600 s$^{-1}$ observed in this study.

Table II: Rate Constants for Reduction of Free and Complexed Cytochrome *c*(III) by FMNH· and $Cl_2FMN^{\cdot-}$

| | | free | complex | | |
|---|---|---|---|---|---|
| flavin | $\mu$ (mM) | $k_2 \times 10^7$ ($M^{-1}$ $s^{-1}$)[a] | $k_1$ ($s^{-1}$)[b] | $k_2 \times 10^7$ ($M^{-1}$ $s^{-1}$)[b] | $K$ ($k_1/k_{-1}$)[b] |
| FMNH· | 10 | 14 ± 1.2 | 810 ± 30 | 7 ± 0.4 | 0.5 ± 0.05 |
| FMNH· | 20 | 12 ± 1.1 | 2620 ± 100 | 10 ± 0.5 | 0.9 ± 0.1 |
| FMNH· | 100 | 5.6 ± 0.3 | | 5.4 ± 0.4 | |
| $Cl_2FMN^{\cdot-}$ | 20 | 25 ± 2.3 | 2720 ± 175 | 9.4 ± 0.5 | 0.95 ± 0.1 |

[a] Determined from the slopes of the plots of Figure 4a. [b] Values were determined from rate constants of Figure 4b and computer modeling based on the mechanism discussed under Results and Discussion. $k_2$ and $K$ represent minimum values.

were very similar over the entire concentration range. Importantly, at higher protein concentrations the two plots approach the same limiting value of $k_{obsd}$ (Figure 4b). This result indicates that the limiting first-order process does not depend on the flavin species employed, thus providing support for the mechanism given by eq 1 and 2.

Values for the individual rate constants and the isomerization equilibrium constants, derived from least-squares fits to the data on the basis of the mechanism given by eq 1 and 2 (shown as the solid lines in Figure 4b), are listed in Table II. The effect of complex formation on the second-order reduction rate constant for $Cl_2FMN^{\cdot-}$ was significantly greater than for FMNH·. For the dichloro derivative, a 2.5-fold decrease in $k_2$ was observed, while for FMNH· the decrease was 1.2-fold (cf. Table II). The larger decrease in $k_2$ for the reduction of free vs. complexed cytochrome *c* by the dichloro derivative and the close similarity between the results obtained for the reduction of complexed cytochrome *c* by these two analogues can be explained if, in the case of $Cl_2FMN^{\cdot-}$, two opposing factors influence the kinetics. On the basis of the results with the free protein, the rate constant for the reaction of $Cl_2FMN^{\cdot-}$ with the cytochrome *c* component of the complex should be greater than that of FMNH· (cf. Figure 4a and Table II); however, this effect is counterbalanced by a larger electrostatic repulsion between $Cl_2FMN^{\cdot-}$ and a negative electrostatic field (see below) at the protein complex interface. The data of Figure 4b indicate that these two factors effectively cancel each other out.

The results show that as $\mu$ increased, the equilibrium constant for the protein isomerization step, $K$, also increased. The rate constant for the second-order electron-transfer step, $k_2$, also increased with increasing ionic strength (for $\mu \leq 20$ mM). The value at $\mu = 10$ mM was significantly smaller than that found for the free cytochrome *c* reaction (Table II), while at $\mu = 20$ mM the rate constants for free and complexed cytochrome were approximately the same. The changes in $k_2$ between $\mu = 10$ mM and $\mu = 20$ mM for these two forms of the cytochrome (Table II) were opposite in sign, demonstrating that the electrostatic interaction between free FMNH· and the complex, at low ionic strengths, was of a repulsive (or minus–minus) type, which is opposite in sign to that found for free cytochrome. Therefore, at low ionic strengths a negative electrostatic potential due to the flavodoxin must be close enough to the cytochrome heme that the incoming FMNH· experiences a net electrostatic repulsion. This is consistent with the $Cl_2FMN^{\cdot-}$ results and with the structural and electrostatic modeling of the complex (Simondsen et al., 1982; Matthew et al., 1983; Weber & Tollin, 1985). As $\mu$ increases, a number of factors come into play. Most importantly, the strength of the complex, measured by the electrostatic free energy and the association constant, decreases (Weber & Tollin, 1985), resulting in a greater fraction of free cytochrome. Secondly, the negative electrostatic potential of flavodoxin near the protein interface is diminished, decreasing the repulsive interaction with FMNH·. The net result of these ionic strength effects on the kinetics of reduction of cytochrome *c* in the presence of flavodoxin is an approach of $k_2$ for the complex to that of the free cytochrome.

Table III: Rate Constants for Reduction of Free and Complexed Cytochrome *c* by Rubredoxin

| | $\mu$ (mM) | $k_2 \times 10^3$ ($M^{-1}$ $s^{-1}$)[a] |
|---|---|---|
| Cyt *c* | 15 | 30 ± 3.5 |
| Cyt *c* | 25 | 13 ± 1.0 |
| Cyt *c*–Fld | 15 | 8.6 ± 0.7 |
| Cyt *c*–Fld | 25 | 6.0 ± 0.8 |

[a] Second-order rate constants were calculated from the slopes of the plots of Figure 4c.

Because of the dependence of $k_2$ on ionic strength with FMN and the difference in the nature of the electrostatic interactions between free and complexed cytochrome, it is not possible to quantitate the change in the second-order rate constant for cytochrome reduction caused by steric factors resulting from complexation. However, the reaction mechanism has clearly changed from a simple second-order to a two-step process. The implications of this will be considered further below.

*Reduction of Cytochrome c–Flavodoxin Complex by Reduced Rubredoxin.* In order to further probe the degree to which flavodoxin sterically and electrostatically modulates the reduction of the cytochrome within the complex, we employed a significantly bulkier and more highly charged cytochrome reductant, *C. pasteurianum* rubredoxin. Rubredoxin is a small Fe–$S_4$ protein with a molecular weight of ~6000 (Lovenberg & Sobel, 1965) that undergoes one-electron redox transitions with an $E_m$ of −57 mV. The redox center is located at one end of the long molecular axis (~26 Å) and is buried approximately 2.3 Å from the protein surface [Jensen (1978) and references cited therein]. This small redox protein also has a net charge of −12 (Herriott et al., 1973).

Plots of $k_{obsd}$ vs. concentration for the reduction of cytochrome *c*, free and in a 1:1 complex with flavodoxin, at $\mu$ = 15 and 25 mM are shown in Figure 4c. The plots for both forms of the cytochrome are linear over a 4-fold concentration range, unlike the results obtained with FMNH· reduction of complexed cytochrome *c*. Note, however, that the $k_{obsd}$ values are $10^3$-fold smaller than the values obtained for FMNH·. The second-order rate constants calculated from these plots are given in Table III. For the free cytochrome, an increase in ionic strength from 15 to 25 mM resulted in a 2.3-fold decrease in $k_2$, which is the direction expected from an attractive electrostatic interaction between rubredoxin and cytochrome *c*. In the complex, increasing the ionic strength also resulted in a decrease, albeit considerably smaller, in $k_2$. At both ionic strengths, the rate constants for reduction of complexed cytochrome were less than those found for the free cytochrome. However, the magnitudes of the changes in rate constants are surprisingly small. Thus, at $\mu$ = 15 mM, there was only a 3.5-fold decrease in $k_2$ due to complex formation, while at $\mu$ = 25 mM, the decrease was ~2-fold. The decrease in rate constant for reduction of complexed cytochrome *c* by Rd at the lowest ionic strength is only slightly greater than was found

when small free flavins were used as reductants. However, electrostatic factors again complicate the quantitative comparison.

The ionic strength dependence for complexed cytochrome reduction by Rd was opposite in sign to that found when FMNH· was used as the reductant. The results demonstrate that the net electrostatic interaction between Rd and the complexed cytochrome was of an attractive type, whereas there was a repulsive interaction between FMNH· and the complex.

Considering that Rd is significantly larger than either lumiflavin or riboflavin, and has a greater negative charge than FMN, one might have expected much larger effects of complex formation on the rate constants for the Rd reaction. Our results, in fact, indicate that complex formation had relatively small effects on the kinetics and ionic strength dependency of the Rd reduction of cytochrome *c*. Two possible explanations for this apparent inconsistency can be offered. First, the site of interaction between cytochrome *c* and Rd might not be the same in the free and complexed cytochrome. This would require that electron transfer occur at some region on the surface of the cytochrome away from the cytochrome *c*–flavodoxin interface, where most of the positive charge cancellation would be expected to occur. This would also necessitate that electron transfer occur through the protein to the heme, which would increase the distance between the $Fe–S_4$ cluster of Rd and the heme prosthetic group. Because of the exponential dependence of electron-transfer rate constants on distance (Sutin, 1983), such an increase would be expected to have a greater effect on $k_2$ at $\mu = 15$ mM than was observed. Second, a more likely explanation is as follows. Whereas FMNH· is a relatively small and weakly charged reductant, Rd is much larger and has a net charge of −12 at neutral pH. The combination of these factors could have resulted in such strong repulsive interactions between Rd and flavodoxin that the complex structure was significantly disrupted by a partial displacement of the flavoprotein. This would lead to an increased accessibility of the heme and at least a partial restoration of the positive electrostatic potential near the heme edge and would account in a simple way for our experimental results. The implications of this mechanism will be considered further below.

## Summary and Conclusions

Our studies on the kinetics of reduction of the individual proteins in the cytochrome *c*–flavodoxin complex by free flavin semiquinones and reduced rubredoxin can be summarized as follows. First, complex formation had no significant effect on the rate constant for reduction of the FMN of flavodoxin by 5-DRFH·. Second, the reduction rate constants of complexed cytochrome *c* by the neutral free flavins, LFH· and RFH·, were significantly smaller than those found for the free protein. The magnitude of the decrease was greater for riboflavin than for lumiflavin, as expected from steric considerations. Third, the kinetics of reduction of complexed cytochrome *c* by FMNH· was characterized by nonlinear concentration dependencies that were not seen in any of the other reactions studied. Both the second-order and the limiting first-order rate constants increased with increasing $\mu$. Reduction of free cytochrome *c* by FMNH·, on the other hand, was a simple second-order process with a decrease in the rate constant with increasing $\mu$. This demonstrates that the electrostatic interaction between FMNH· and the protein complex was of a repulsive type, whereas the FMNH–free cytochrome interaction was attractive. Fourth, reduction of complexed cytochrome *c* by rubredoxin was found to be affected by steric factors due to complexation to a similar extent as for the free flavins, despite the significantly greater size of rubredoxin. Furthermore, the decrease in the rate constant for reduction of complexed cytochrome with an increase in $\mu$ indicates an attractive interaction, despite the fact that rubredoxin has a greater net negative charge than FMNH· and therefore should be expected to be more strongly repulsed by the net negative electrostatic potential of the protein cytochrome *c*–flavodoxin complex.

In order to better understand the steric and electrostatic factors giving rise to these kinetic results, accessible surface representations of the protein complex were constructed by using the descriptions and parameters given by Simondsen et al. (1982) and Weber and Tollin (1985). Figure 5a,c shows the complex in the absence of the free flavin reductant; the two views are related by a 180° rotation. For purposes of clarity, amino acid side chains are only shown in the immediate vicinity of the interface and the bridging lysine and carboxylate side chains of the cytochrome and flavodoxin have been left in their free crystallographic protein positions rather than in the extended positions shown previously (Simondsen et al., 1982; Weber & Tollin, 1985).

The model clearly shows several features relevant to our kinetic studies. The extension of the dimethylbenzene ring of flavodoxin FMN into the interprotein crevice results in van der Waals contact with the most exposed region of the cytochrome *c* heme edge, i.e., near the terminal methyl of the thioether bridge of Cys-17. This markedly diminishes the solvent exposure of the heme. The FMN is partially occluded due to two regions of protein–protein contact. In Figure 5a it can be seen that, to the left of the FMN, close protein contact is made between residues 12–17 of the cytochrome and residues 7–10 of the flavodoxin. Figure 5c shows that access to the solvent-exposed surface of the FMN is restricted by the contact between flavodoxin Trp-90, which lies above the flavin ring, and cytochrome residues 79–81. Despite this partial steric hindrance, it is quite evident that within the complex the solvent exposure of the flavodoxin FMN is greater than that of the cytochrome heme.

There are two pockets in the protein interface region that can accommodate the placement of small reductants at the FMN–heme interaction site. In Figure 5b,d, the space-filling structure of lumiflavin has been inserted into these two regions of the complex. Again, it is evident that contact with the FMN is more extensive than with the heme. The riboflavin molecule can also be inserted into these crevices, providing that the ribityl side chain extends away from the isoalloxazine ring. In the case of FMN, however, there is evidence that the ribityl phosphate side chain is constrained to lie over the isoalloxazine ring because of interaction between the phosphate group and the positive charge at the protonated $N_5$ position in the semiquinone species [cf. Meyer et al. (1984) for discussion]. In that conformation, FMN would be unable to enter either of the pockets. Finally, rubredoxin, being much larger than FMN, would also be unable to contact the prosthetic groups within the complex.

As complex formation reduces access of free flavins to the prosthetic groups, one would anticipate a decrease in the rate constants for reduction of both the flavodoxin and cytochrome components relative to the free proteins. Because of the greater degree of the solvent exposure of the flavodoxin FMN prosthetic group relative to that of the heme prosthetic group of the cytochrome *c*, one would expect that a magnitude of the decrease in the rate constant for reduction of this moiety by 5-DRFH· should be smaller than that for the cytochrome reduction by RFH·. Furthermore, because of the constraint

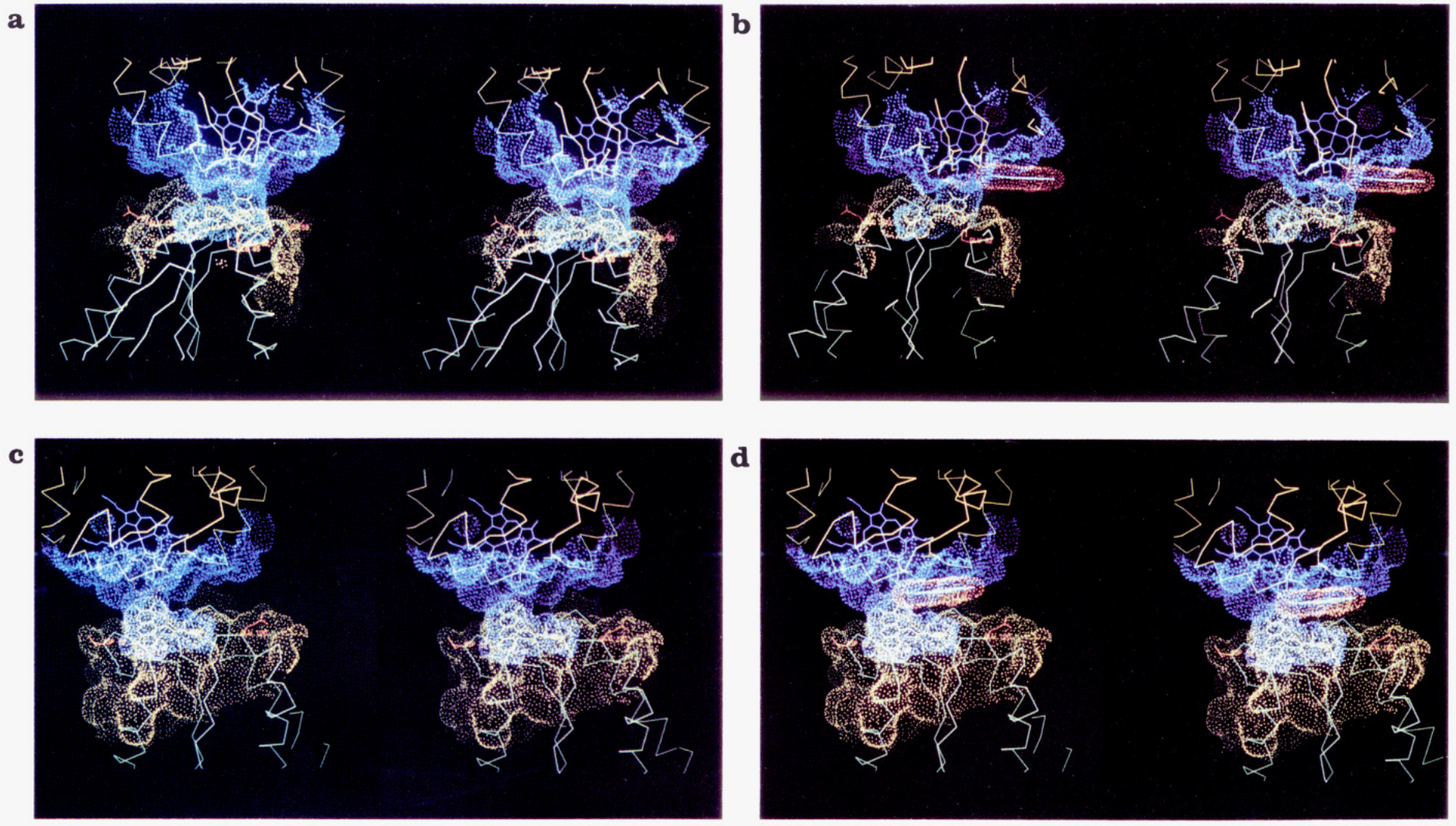


FIGURE 5: Computer-graphic stereoviews of the cytochrome *c*–flavodoxin complex. Cytochrome *c* (top) and flavodoxin (bottom) are displayed as $\alpha$-carbon chains (yellow lines for cytochrome *c* and green lines for flavodoxin) with side-chain bonds for important negative residues (red lines), positive residues (light blue lines), the heme (purple lines), and FMN (yellow lines), and the molecular surface for all atoms is shown by dots (blue for cytochrome *c*, red for heme, yellow for flavodoxin, and cyan for FMN). The lysines and carboxylates postulated to be responsible for salt-bridge formation (Simondsen et al., 1982) have been left in their free protein positions. (a) The complex is oriented so the dimethylbenzene portion of the flavodoxin FMN is pointing toward the viewer. (b) The same view as (a) except lumiflavin has been inserted into the protein crevice within van der Waals contact distance of the FMN. (c) This view is related to (a) by rotation of 180° around a vertical axis, with the solvent-exposed surface of the flavodoxin FMN and the surface of the protein from Trp-90 pointing toward the viewer. (d) The same view as (c) except lumiflavin has been inserted within van der Waals distance of the FMN in an orientation unique from that of (b). In both orientations, it should be noticed that the $\epsilon$-methyl of the cytochrome thioether bridge of Cys-17 is effectively blocked by the 7- and 8-Me of the flavodoxin FMN ring.

on the riboflavin conformation that must occur in order to allow it to enter the interface crevice, the magnitude of the decrease in the reduction rate constant should be greater for this reductant than for the sterically less bulky lumiflavin.

The steric factors for lumiflavin and riboflavin predicted by the structure of the model complex are seen to be in qualitative agreement with the kinetic data for the cytochrome *c*–flavodoxin complex with one exception. As noted above, no measurable effect on complex formation on the kinetics of reduction of flavodoxin by 5-DRFH· was observed. This apparent inconsistency can be rationalized by realizing that the cytochrome *c*–flavodoxin complex is not a static structure and that there must be dynamic motions of the components relative to one another. If such motions acted to increase the accessibility of the prosthetic groups by widening the crevices, the exposure of the flavodoxin FMN could be increased to the point where a kinetic effect would become unobservable. These dynamic movements at the interface would also increase the heme exposure and thus decrease the magnitude of the kinetic effect observed for this prosthetic group as well.

As stated above, the nonlinear concentration dependencies observed for reduction of complexed cytochrome by FMNH· have been interpreted in terms of a rate-limiting process involving an equilibrium between two forms of the protein complex. This can easily be equated with the above concept of a dynamic process that results in a widening of the complex interface crevices. Since the steric bulk of FMNH· is significantly greater than that of lumiflavin or riboflavin, the degree of opening up of the structure would have to be larger for this analogue. Thus, the kinetics of opening of such an enlarged channel could become rate limiting at higher protein concentrations. The fact that the rate-limiting first-order rate constant $k_1$, and the isomerization equilibrium constant, $K$ ($k_1/k_{-1}$), increase with ionic strength is consistent with this concept, since the increased screening of the charged groups would lead to a weakening of the electrostatic interactions that hold the complex together.

As noted previously, the larger reductant rubredoxin cannot be brought within van der Waals distance of the heme in the complex with flavodoxin. Therefore, the attractive electrostatic interaction and the relatively small effect of complex formation on the rate constant can only be explained by a mechanism that involves at least a partial displacement of the flavodoxin relative to the cytochrome within the complex. Such movement could result from the electrostatic repulsion between rubredoxin and flavodoxin, since both are negatively charged, which would facilitate displacement of the flavodoxin from the dynamic complex. Such a process, since it requires considerable movement of the protein molecules, would be expected to be somewhat slower than the rate-limiting step of channel opening observed with FMNH·. However, this opening up of the complex apparently never became rate limiting because the largest pseudo-first-order rate constant obtained with rubredoxin was $\sim 0.7\ s^{-1}$, which is $10^3$-fold less than the limiting first-order rate constant for the FMNH· reaction.

In conclusion, our kinetic results are consistent with and thus provide direct experimental support for the previously proposed structural model for the cytochrome *c*–flavodoxin complex (Simondsen et al., 1982; Weber & Tollin, 1985). The results also suggest that the complex does not have a static structure but rather has characteristic dynamic motions that can be important on the time scale of the laser flash photolysis experiments (milliseconds). These dynamics can affect the ability of reductants, depending on their steric and charge properties, to gain access to the prosthetic groups that are buried at the protein–protein interface in order for electron transfer to take place. Such a mechanism could have important biological implications and thus warrants further investigation.

## ACKNOWLEDGMENTS

The helpful comments and criticisms of Dr. Terrance E. Meyer and Anjan Bhattacharyya during the course of this investigation are greatly appreciated.

**Registry No.** Cyt *c*, 9007-43-6; 5-DRFH·, 101759-37-9; LFH·, 80700-47-6; RFH·, 80700-50-1; FMNH·, 80700-51-2; $Cl_2$FMN·, 101653-97-8.

## REFERENCES

Ahmad, I., Cusanovich, M. A., & Tollin, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6724–6728.

Ahmad, I., Cusanovoch, M. A., & Tollin, G. (1982) *Biochemistry 21*, 3122–3128.

Bechtold, R., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5191–5220.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rogers, J. R., Kennard, O., Shimanouchi, T., & Tasumi, M. (1977) *J. Mol. Biol. 112*, 535–542.

Bhattacharyya, A., Tollin, G., McIntire, W., & Singer, T. P. (1985) *Biochem. J. 228*, 337–345.

Bhattacharyya, A., Meyer, T. E., & Tollin, G. (1986) *Biochemistry* (in press).

Connolly, M. L. (1983) *Science (Washington, D.C.) 221*, 709–713.

Connolly, M. L., & Olson, A. J. (1985) *Comput. Chem. 9*, 1–6.

Cusanovich, M. A., & Tollin, G. (1980) *Biochemistry 19*, 3343–3347.

Cusanovich, M. A., Meyer, T. E., & Tollin, G. (1985) *Biochemistry 24*, 1281–1287.

Dickerson, J. L., Kornuc, J. J., & Rees, D. C. (1985) *J. Biol. Chem. 260*, 5175–5178.

Draper, R. D., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802–808.

Earl, R. A. (1981) Ph.D. Dissertation, University of Arizona, Tucson, AZ.

Getzoff, E. D., Tainer, J. A., & Olson, A. J. (1986) *Biophys. J. 49*, 191–204.

Hazzard, J. T., & Tollin, G. (1985) *Biochem. Biophys. Res. Commun. 130*, 1281–1286.

Herriot, J. R., Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) *J. Mol. Biol. 80*, 423–432.

Hoth, L. E., & Erman, J. E. (1984) *Biochim. Biophys. Acta 788*, 151–153.

Jensen, J. E. (1978) *Colloq. Ges. Biol. Chem. 29*, 74–83.

Jung, J., & Tollin, G. (1981) *Biochemistry 20*, 5124–5131.

Knight, E., Jr., & Hardy, R. W. F. (1966) *J. Biol. Chem. 241*, 2752–2756.

Lovenberg, W., & Sobel, B. E. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 193–199.

Matthew, J. B., Weber, P. C., Salemme, F. R., & Richards, F. M. (1983) *Nature (London) 301*, 169–171.

Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 276–288.

Meyer, T. E., Przysiecki, C. T., Watkins, J. A., Bhattacharyya, A., Simondsen, R. P., Cusanovich, M. A., & Tollin, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6740–6744.

Meyer, T. E., Watkins, J. A., Przysiecki, C. T., Tollin, G., & Cusanovich, M. A. (1984) *Biochemistry 23*, 4761–4767.

Mochan, E., & Nicholls, P. (1972) *Biochim. Biophys. Acta 267*, 309–319.

Nicholls, P. (1974) *Biochim. Biophys. Acta 346*, 261-310.
O'Donnell, T. J., & Olson, A. J. (1981) *Comput. Graphics 15*, 133-142.
O'Reilly, J. E. (1973) *Biochim. Biophys. Acta 292*, 509-515.
Pettigrew, G. W., & Seilman, S. (1982) *Biochem. J. 201*, 9-18.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322-10330.
Poulos, T. L., & Mauk, A. G. (1983) *J. Biol. Chem. 258*, 7369-7373.
Przysiecki, C. T., Bhattacharyya, A., Tollin, G., & Cusanovich, M. A. (1985a) *J. Biol. Chem. 260*, 1452-1458.
Przysiecki, C. T., Tollin, G., Meyer, T. E., Staggers, J. E., & Cusanovich, M. A. (1985b) *Arch. Biochem. Biophys. 238*, 334-343.
Rees, D. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3082-3085.
Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563-568.
Salemme, F. R. (1978) *Annu. Rev. Biochem. 46*, 299-329.
Simondsen, R. P., & Tollin, G. (1983) *Biochemistry 22*, 3008-3016.
Simondsen, R. P., Weber, P. C., Salemme, F. R., & Tollin, G. (1982) *Biochemistry 21*, 6366-6375.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048-4052.
Sutin, N. (1983) *Prog. Inorg. Chem. 30*, 441-498.
Tainer, J. A., Getzoff, E. D., Sayre, J., & Olson, A. J. (1985) *J. Mol. Graphics 3*, 103-105.
Tollin, G., Cheddar, G., Watkins, J. A., Meyer, T. E., & Cusanovich, M. A. (1984) *Biochemistry 23*, 6345-6349.
Vaish, S. P., & Tollin, G. (1971) *Bioenergetics 2*, 61-72.
Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184-5190.
Waldmeyer, B., Bechtold, R., Bosshard, H. R., & Poulos, T. L. (1982) *J. Biol. Chem. 257*, 6073-6076.
Weber, P. C., & Tollin, G. (1985) *J. Biol. Chem. 260*, 5568-5573.
Wilson, G. S. (1978) *Methods Enzymol. 54E*, 396-410.

# Spectroscopic Properties of the Cobalt(II)-Substituted $\alpha$-Fragment of Rabbit Liver Metallothionein†

Meinrad Good and Milan Vašák*
*Biochemisches Institut der Universität Zürich, CH-8057 Zürich, Switzerland*
*Received November 19, 1985; Revised Manuscript Received January 28, 1986*

ABSTRACT: The C-terminal segment of rabbit liver metallothionein 1 ($\alpha$-fragment) containing four paramagnetic Co(II) ions was obtained by stoichiometric replacement of the originally bound diamagnetic Cd(II) ions. The latter form was prepared by limited proteolysis with subtilisin as described previously [Winge, D. R., & Miklossy, K. A. (1982) *J. Biol. Chem. 257*, 3471-3476]. Electronic absorption, magnetic circular dichroism (MCD), and electron paramagnetic resonance (EPR) measurements were employed to monitor the stepwise incorporation of Co(II) ions into the metal-free fragment. Absorption and MCD spectra of the apofragment containing the first 3 Co(II) equiv show the typical features of tetrahedral tetrathiolate Co(II) coordination. However, in the d-d region only small changes in the visible and no apparent change in the near-infrared region are discernible when the fourth Co(II) is bound. This unusual spectral behavior was not seen in Co(II) substitution of native metallothionein [Vašák, M., & Kägi, J. H. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6709-6713] and may indicate a different cluster geometry. In the charge-transfer region, the binding of all 4 Co(II) equiv is accompanied by characteristic increments of the thiolate S→Co(II) bands. As in the formation of $Co(II)_7$-metallothionein, the development of the the charge-transfer and EPR spectral properties upon binding of the first 2 Co(II) equiv to the apofragment is indicative of isolated, noninteracting tetrahedral tetrathiolate Co(II) complexes. The binding of the additional Co(II) ion is accompanied by a red shift in the charge-transfer region and by the dramatic loss of paramagnetism in the EPR spectra, both diagnostic of the formation of metal-thiolate cluster structures. Thus, these data suggest that the four-metal cluster in the isolated $\alpha$-fragment and in $Co(II)_7$-metallothionein is similar but not identical.

Metallothioneins represent a class of low molecular weight metal and sulfur-rich proteins, which are widely distributed in nature (Kägi et al., 1984). An interesting feature of all these proteins is that their biosynthesis can be induced by the administration a variety of agents, among them heavy metals. It is therefore believed that metallothioneins are involved in trace metal metabolism and storage and detoxification of essential and nonessential $d^{10}$ metal ions (Nordberg & Kojima, 1979). All mammalian forms examined to date consist of a single polypeptide chain with 61 amino acids of which 20 residues are cysteine and contain seven diamagnetic metal ions such as Zn(II) and/or Cd(II). There are two major electrophoretically separable isoforms present in most mammals, metallothioneins 1 and -2, that have closely related but distinct amino acid sequences (Kägi et al., 1984).

Spectroscopic studies have established that metallothioneins have a well-defined tertiary structure in which each metal ion is tetrahedrally coordinated by four thiolate ligands (Vašák et al., 1981) and that these metals are arranged in two separate clusters of three and four metals, respectively (Otvos & Ar-

† This work was supported by Swiss National Science Foundation Grant 3.146-085.
* Address correspondence to this author.

0006-2960/86/0425-3328$01.50/0 