Transient Kinetics of Redox Reactions of Flavodoxin: Effects of Chemical Modification of the Flavin Mononucleotide Prosthetic Group on the Dynamics of Intermediate Complex Formation and Electron Transfer†

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ABSTRACT: The effects of structual modifications of the flavin mononucleotide (FMN) prosthetic group of *Clostridium pasteurianum* flavodoxin on the kinetics of electron transfer to the oxidized form (from 5-deazariboflavin semiquinone produced by laser flash photolysis) and from the semiquinone form (to horse heart cytochrome *c* by using stopped-flow spectrophotometry) have been investigated. The analogues used were 7,8-dichloro-FMN, 8-chloro-FMN, 7-chloro-FMN, and 5,6,7,8-tetrahydro-FMN. The ionic strength dependence of cytochrome *c* reduction was not affected by chlorine substitution, although the specific rate constants for complex formation and decay were appreciably smaller. On the other hand, all of the chlorine analogues had the same rate constant for deazariboflavin semiquinone oxidation. The rate constants for tetrahydro-FMN flavodoxin semiquinone reduction of cytochrome *c* were considerably smaller than those for the native protein. The implications of these results for the electron-transfer mechanism of flavodoxin are discussed.

The use of flavin analogues to obtain information concerning the importance of coenzyme structural features in binding and redox processes in flavoproteins has received previous attention in this laboratory (Edmondson et al., 1972; Shiga & Tollin, 1976; Jung & Tollin, 1981) as well as in others [for an excellent review, see Massey & Hemmerich (1980); see also Claiborne et al. (1982)]. The availability of variously substituted and modified flavins offers possibilities for studying virtually every aspect of flavin functionality. X-ray crystal structure analysis (Mayhew & Ludwig, 1975), earlier stopped-flow studies (Shiga & Tollin, 1976; Jung & Tollin, 1981), and a computer graphics investigation of the structure of a putative flavodoxin–cytochrome *c* electron-transfer complex (Simondsen et al., 1982) have suggested the involvement of the partially exposed dimethylbenzene moiety of the flavin ring system in electron-transfer reactions of flavodoxin. Laser flash photolysis observations (Ahmad et al., 1981) of electron transfer from free flavin semiquinone to cytochrome *c* also have implicated this region of the flavin in interactions with the bound heme prosthetic group of the cytochrome. It is to this aspect of flavin structure/function relationships that the present studies are directed.

Electron paramagnetic resonance (EPR) and electron nuclear double resonance (ENDOR) spectroscopies have indicated that the 8-position in the dimethylbenzene ring carries substantial unpaired spin density in the semiquinone forms of both free flavin and flavodoxin (Guzzo & Tollin, 1964; Eriksson & Ehrenberg, 1973) whereas the 7-position does not. This would be consistent with the participation of the 8-position in electron transfer at the semiquinone level. The validity of this possibility may be investigated by individually substituting the methyl groups at these positions. An ideal substituent would be chlorine since its replacement of the similarly sized methyl group probably would not lead to any large changes in the conformation of the protein caused by steric effects, whereas its high electronegativity should significantly perturb the flavin electronic structure (Jung & Tollin, 1981). Both crystallographic (Mayhew & Ludwig, 1975) and computer (Shrake & Rupley, 1973) analyses indicate that the 7- and 8-methyl groups of flavin mononucleotide (FMN) are well exposed to solvent in flavodoxin. The present work utilizes such chlorinated flavins bound to flavodoxin in a kinetic study of electron-transfer reactions from free flavin semiquinone to the oxidized analogue protein and from the analogue protein semiquinone to oxidized cytochrome *c*. We have also investigated an FMN analogue in which the dimethylbenzene ring is partially reduced (5,6,7,8-tetrahydro-FMN). Previous work (Harzer & Ghisla, 1979) has shown that this analogue possesses 90% of the catalytic activity of FMN when bound to the apoprotein of *Micrococcus elsdenii* flavodoxin, as measured by the chloroplast photoreduction of nicotinamide adenine dinucleotide phosphate (NADP). Inasmuch as this is a complex assay system whose kinetics are not well understood, we felt it would be of use to examine this FMN derivative in a simpler system. In this case, however, it is not at all certain that the protein conformation would remain unchanged upon analogue binding, inasmuch as the steric properties of a cyclohexene-type ring are considerably different from those of a planar aromatic ring. Thus, results obtained with the use of this analogue bound to apoflavodoxin must be interpreted with some caution. Furthermore, tetrahydro-FMN has properties which are quite different from those of ordinary flavins with regard to photoreduction and reduction by dithionite (see below).

In a previous study (Simondsen et al., 1982), the ionic strength dependence of the kinetics of the flavodoxin semiquinone–cytochrome *c* redox reaction provided evidence that electrostatic interactions involving negatively charged protein carboxyl groups located close to the dimethylbenzene end of the FMN and positively charged lysine residues near the heme edge in cytochrome *c* were important in orienting the two proteins prior to electron transfer. Although these proteins are not normal physiological partners, they are both well characterized structurally and thus provide a useful model for investigating protein–protein interactions during electron transfer. It is possible to extend these findings by carrying out chemical modification studies [for example, as in Ng et al. (1977)] which change the charge character of the protein or by chemically modifying the bound flavin. In the latter case, any change in the rate of the reaction would be due to the

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nature of the flavin, whereas the electrostatic interactions involving the protein moieties should remain unchanged in the absence of conformational alterations. As will be described below, replacement of the FMN methyl groups by chlorine leads to modifications in the kinetic properties of the flavodoxin–cytochrome *c* electron-transfer reaction which are consistent with our earlier suggestion (Simondsen et al., 1982) of a close juxtaposition between the heme edge and the flavin dimethylbenzene ring in the intermediate complex and which provide some useful and interesting insights into the factors which control complex formation and stability in this system.

Experimental Procedures

Materials. Clostridium pasteurianum (ATCC 6013) flavodoxin was isolated and purified according to published procedures (Mayhew, 1971). Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. and used without further purification. 7,8-Dichlororiboflavin was synthesized as previously described (Shiga & Tollin, 1976). 7-Chlororiboflavin and 8-chlororiboflavin were the generous gifts of Dr. J. P. Lambooy, and tetrahydro-FMN was the gift of Dr. S. Ghisla. The analogue riboflavins were phosphorylated according to the procedure of Scola-Nagelschneider & Hemmerich (1976) to yield the corresponding FMN derivatives.

The apoprotein of flavodoxin was formed by dialysis of the holoprotein against 2.0 M KBr in 0.1 M acetate buffer, pH 3.9 (Mayhew, 1971). The reconstituted protein was prepared in two ways, either by addition of excess analogue followed by dialysis or by addition of excess analogue and removal of unbound material by gel filtration using Sephadex G-25.

Procedures. The stopped-flow experiments were carried out in 0.005 M potassium phosphate buffer, pH 7.2. The temperature was 23.5 °C. The concentration of analogue-containing flavodoxin was approximately 6×10^{-6} M, and ethylenediaminetetraacetic acid (EDTA) concentration was 1.7×10^{-3} M. The neutral semiquinone form of the flavodoxin was produced by anaerobic photoreduction; the EDTA present functioned as the hydrogen donor. Samples were degassed for 1 h and then transferred to the stopped-flow spectrophotometer where they were irradiated for 10 min. This procedure reduced only a portion of the flavodoxin present in the sample. The exact amount could not be determined since the irradiation was carried out in situ. However, even at concentrations of oxidant as low as 12 μM, pseudo-first-order behavior was observed, indicating that only a small fraction of the flavodoxin was converted to the semiquinone. The reactions were observed at 580 nm, a wavelength which allows the disappearance of flavodoxin semiquinone to be followed. Ionic strength was varied by addition of appropriate amounts of NaCl. The stopped-flow apparatus has been described previously (Jung & Tollin, 1981).

In the case of tetrahydro-FMN flavodoxin, dithionite was used as the reductant to produce the semiquinone. The reaction was performed in a glovebox purged overnight with nitrogen. The semiquinone was separated from excess dithionite by gel filtration in the glovebox using Sephadex G-25 with 0.005 M phosphate buffer containing sufficient NaCl to give the desired ionic strength. The column was first treated with buffer containing dithionite to remove any trapped oxygen, followed by a buffer treatment to remove excess dithionite. The semiquinone was anaerobically transferred to the stopped-flow apparatus by using a syringe. The redox reaction was observed at 565 nm, where the decrease in absorption is due to the disappearance of both flavodoxin semiquinone and oxidized cytochrome *c*.

The flash photolysis experiments utilized a pulsed nitrogen laser (PRA LN100) to pump a dye laser which consisted of a 1.2 mM solution of 1,4-bis(2-methylstyryl)benzene (Bis-MSB) in *p*-dioxane in a 1-cm path length square cuvette. Stimulated emission by Bis-MSB provided an approximately 300-ps pulse (at 421 nm). The monitoring beam consisted of a quartz–iodine projection lamp focused through a monochromator onto the sample and then onto an RCA 4463 photomultiplier tube (S-20 response). The laser excitation beam made an angle of about 10° with the monitoring beam. The measuring system consisted of a Nicolet 1170 signal averager which was interfaced to a Northstar computer. The maximum time resolution was approximately 1 μs, limited mainly by fluorescence and scattering artifacts. Normally, 1000 points along the decay curve were obtained per experiment, and 32 flashes were signal averaged.

The flash photolysis experiments were carried out in 0.025 M phosphate buffer containing 0.015 M EDTA, pH 7.5. Samples contained 3.5×10^{-5} M 5-deazariboflavin, whose photoreduction by EDTA to the semiquinone form by the laser flash provided the source of reducing equivalents for flavodoxin reduction. The samples were prepared in a Thunberg cell, with the protein solution separated from the 5-deazariboflavin solution. Oxygen was repeatedly removed from the solutions under vacuum. The solutions were mixed and then flashed. Observations were made at 600 nm where there is no absorption due to the 5-deazariboflavin or its semiquinone. In this way, the kinetics of formation of flavodoxin semiquinone could be measured.

The redox potential determinations for the one-electron reduction of the monochloro analogues from the oxidized to the semiquinone forms were performed by Douglas Root in the laboratory of Dr. G. S. Wilson, Department of Chemistry, University of Arizona, by using previously published methods (Wilson, 1978).

Results

7,8-Dichloro-FMN Flavodoxin Semiquinone Oxidation by Horse Heart Cytochrome c. The oxidation of 7,8-dichloro-FMN flavodoxin semiquinone by cytochrome *c* was examined over a range of ionic strengths (0.02–0.1 M). Plots of log Δs vs. time were linear over at least three half-times (data not shown). The kinetics are complicated by the fact that two rate processes are observed, a fast and a slow process. This is consistent with data obtained previously for the reaction of the native flavodoxin semiquinone with horse heart cytochrome *c* (Simondsen et al., 1982). Only the fast reaction has been considered here. All rate data were corrected for this phenomenon as described earlier (Simondsen et al., 1982).

As has been observed previously for the dichloro analogue at high ionic strength (Jung & Tollin, 1981) and for native flavodoxin at low ionic strength (Simondsen et al., 1982), k_{obsd} was found to saturate at high cytochrome concentrations for all the ionic strengths examined (Figure 1). This behavior is evidence for complex formation. The simplest kinetic mechanism consistent with this is as follows:[1]

$$\mathrm{FldH}\cdot + \mathrm{cyt}\ c_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{complex} \xrightarrow{k_2} \mathrm{Fld}_{ox} + \mathrm{cyt}\ c_{red} \quad (1)$$

Under a steady-state approximation

$$k_{obsd} = k_1 k_2(\mathrm{cyt}\ c)/[k_1(\mathrm{cyt}\ c) + k_{-1} + k_2]$$

Saturation will be observed when $k_2 < k_1(\mathrm{cyt}\ c) + k_{-1}$. As discussed in Jung & Tollin (1981) and in Simondsen et al.

[1] See Simondsen et al. (1982) for a discussion of alternative mechanisms.

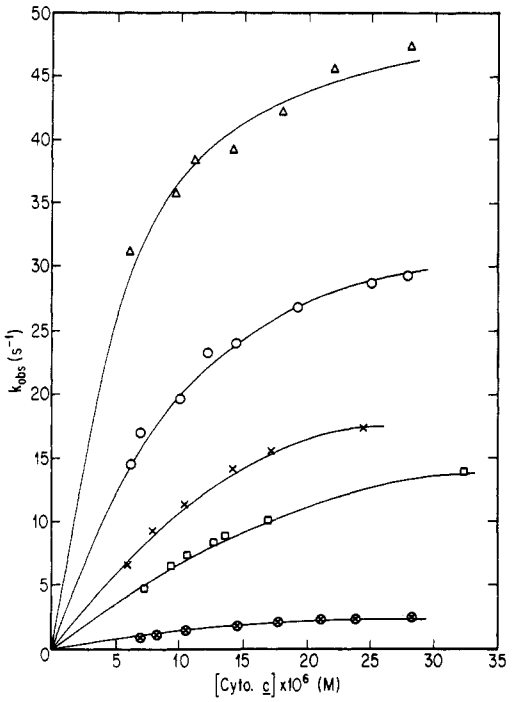

FIGURE 1: Dependence of k_{obsd} on cytochrome *c* concentration. Rate constants obtained from semilog plots of kinetic data obtained at 23.5 °C upon mixing of *C. pasteurianum* 7,8-dichloro-FMN flavodoxin (6×10^{-6} M before mixing) and horse heart cytochrome *c* under anaerobic conditions in 0.005 M phosphate buffer, pH 7.2. Ionic strength changed by the appropriate addition of a sodium chloride solution. (△) $I^{1/2} = 0.141$; (○) $I^{1/2} = 0.200$; (×) $I^{1/2} = 0.245$; (□) $I^{1/2} = 0.265$; (⊗) $I^{1/2} = 0.283$. Curves represent computer fits to data (see text for details).

(1982), under such conditions a minimum value for k_1 is all that can be obtained from the experimental data. Such a value can be calculated in two ways: from the initial slopes of plots of k_{obsd} vs. (cyt *c*) (i.e., Figure 1) or by use of a computer to fit the k_{obsd} vs. (cyt *c*) data according to the mechanism of eq 1 [cf. Ahmad et al. (1982)]. The latter procedure involves treating the mechanism as two consecutive first-order reactions (since all reactions were conducted under pseudo-first-order conditions) and programming an explicit mathematical solution of the rate equations (Frost & Pearson, 1961) into the computer. We restricted possible solutions with the constraint that they be 90% or greater monophasic (which corresponds to our experimental observations). The program used a steepest descents procedure to obtain a minimum least-squares error in fitting the experimental data. It was found that $K_a(k_1/k_{-1})$ and k_2 could be determined directly. However, for k_1 (and k_{-1}), we could only specify a minimum value, with any value larger than this giving identical fits within the experimental error (with K_a constant).

Both the graphical and computer procedures yielded closely equivalent results. We report only the computer-derived values, inasmuch as these are probably more reliable. Table I lists the calculated values for K_a, k_1, k_{-1}, and k_2. As is evident, k_1 increases with decreasing ionic strength. This is indicative of electrostatic interactions between functional groups of opposite charge. Similar results have been observed for the native flavodoxin (Simondsen et al., 1982). Figure 2 shows a plot of these values vs. $I^{1/2}$. Also included for comparison are the values for native flavodoxin obtained previously. Although the rate constants for the dichloro analogue are approximately 10 times smaller than those for the native flavodoxin, the functional dependence on ionic strength remains the same. We may thus conclude that the electrostatic interactions between the protein moieties of flavodoxin semiquinone and cytochrome *c* during complex formation (as measured by ionic strength effects) are essentially unchanged by chlorine substitution of the FMN methyl groups.

Table I: Rate Constants and Association Constants for 7,8-Dichloro-FMN Flavodoxin Semiquinone Oxidation by Cytochrome *c* at Various Ionic Strengths[a]

$I^{1/2}$	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	K_a (M^{-1})
0.141	1.0×10^7	29	52	3.5×10^5
0.200	4.7×10^6	26	37	1.8×10^5
0.245	2.1×10^6	16	26	1.3×10^5
0.265	1.2×10^6	13	21	9.4×10^4
0.283	2.4×10^5	3	4.1	8.3×10^4
0.316	1.1×10^5	1.4	1.4	7.9×10^4

[a] Values obtained by computer analysis as described in the text; k_1 values represent lower limit. Data measured at 23.5 °C in 0.005 M phosphate buffer, pH 7.2. Estimated error is ±10%.

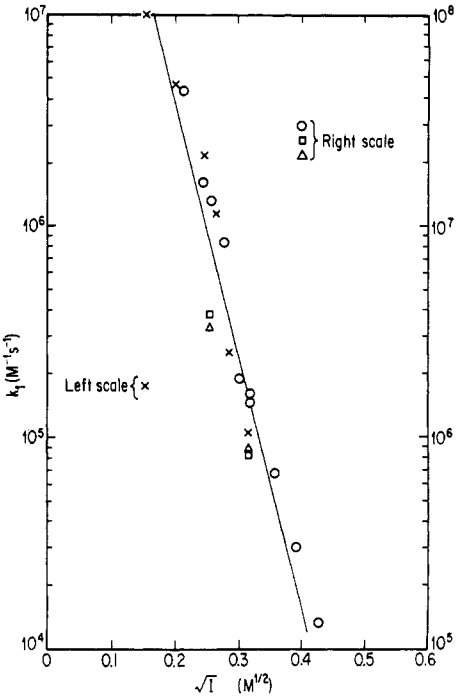

FIGURE 2: Ionic strength dependence of k_1 for oxidation of various flavodoxin semiquinones by cytochrome *c*. (○) *C. pasteurianum* flavodoxin (Simondsen et al., 1982); (×) 7,8-dichloro-FMN flavodoxin; (□) 7-chloro-FMN flavodoxin; (△) 8-chloro-FMN flavodoxin. The estimated error in the rate constants is ±10%.

As we found previously for the native flavodoxin, the association constant, K_a, decreases with increasing ionic strength. This is undoubtedly due to the fact that unlike charges are being screened from each other because of the presence of counterions, thus decreasing the magnitude of the electrostatic interactions. Matthew et al. (1983) were able to account quantitatively for the K_a values as a function of ionic strength for the native flavodoxin–cytochrome *c* complex by an electrostatic mechanism. The K_a values for the dichloro analogue protein are approximately one-half those obtained previously for native flavodoxin (at all but the lowest value of *I*, which is the least reliable). There is also a marked ionic strength dependence of k_2, the limiting first-order rate constant for product formation, and of k_{-1}, the first-order rate constant for complex dissociation. Comparison of the k_2 values with those obtained previously for native flavodoxin indicates that this rate constant is also considerably decreased upon chlorine substitution (approximately a factor of 3), as is k_{-1} (3–4-fold decrease). The k_{-1} and k_2 values are plotted in Figure 3 as

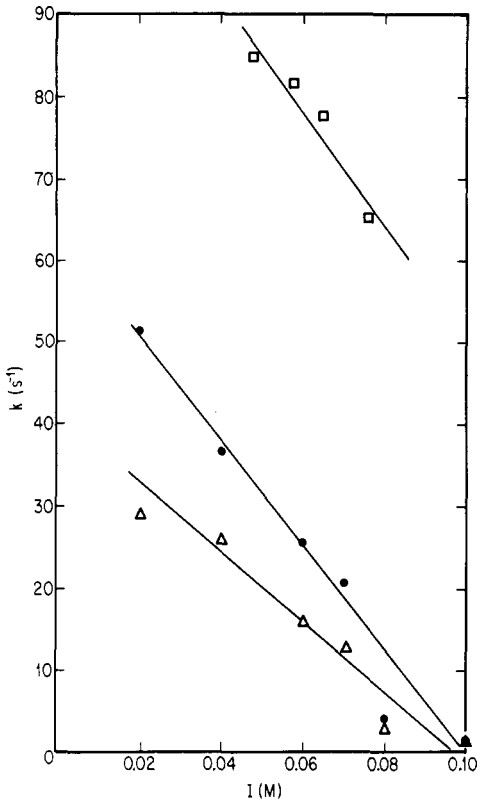

FIGURE 3: Ionic strength dependence of k_2 and k_{-1} for oxidation of native and 7,8-dichloro-FMN flavodoxin semiquinones by cytochrome *c*. (□) k_2 for *C. pasteurianum* flavodoxin (Simondsen et al., 1982); (●) k_2 for 7,8-dichloro-FMN flavodoxin; (△) k_{-1} for 7,8-dichloro-FMN flavodoxin.

Table II: Rate Constants and Association Constants for Oxidation of Monochloro-FMN and Tetrahydro-FMN Flavodoxin Semiquinones by Cytochrome *c*[a]

flavodoxin	$I^{1/2}$	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	K_a (M^{-1})
7-Cl-FMN	0.316	8.2×10^5	*b*	*b*	*b*
	0.255[c]	3.8×10^6	57	91	6.6×10^4
8-Cl-FMN	0.316	8.8×10^5	*b*	*b*	*b*
	0.255[c]	3.3×10^6	51	70	6.6×10^4
tetrahydro-FMN	0.316[c]	3.9×10^5	2.5	3.5	1.5×10^5
	0.255	~1.8×10^6 [d]		~3.0	

[a] Data obtained at 23.5 °C in 0.005 M phosphate buffer, pH 7.2. Estimated error is ±10%. [b] No saturation observed. [c] Constants at this ionic strength obtained by computer analysis as described in the text. [d] Estimated from ionic strength dependence in Figure 3.

a function of ionic strength. Also included for comparison are the k_2 values obtained previously for native flavodoxin.

7-Chloro-FMN Flavodoxin and 8-Chloro-FMN Flavodoxin Semiquinone Oxidation by Cytochrome c. Figure 4a shows plots of k_{obsd} vs. (cytochrome *c*) for the oxidation of the semiquinone forms of the monochloro-FMN analogues of flavodoxin obtained at $I^{1/2} = 0.316$ and $I^{1/2} = 0.255$. No saturation effects were observed at the higher ionic strength. This is similar to the behavior of native flavodoxin and unlike that of the dichloro analogue and thus indicates that the k_2 values for the monochloro derivatives are considerably larger than those for the dichloro-FMN protein under these conditions (cf. Figure 1). Values for k_1 may be calculated from the slopes of these lines and are listed in Table II.[2] The rate constants for the monochloro-FMN derivatives at $I^{1/2} = 0.316$

[2] If a rapid preequilibrium exists between flavodoxin and cytochrome *c*, these values will also be minimal for k_1.

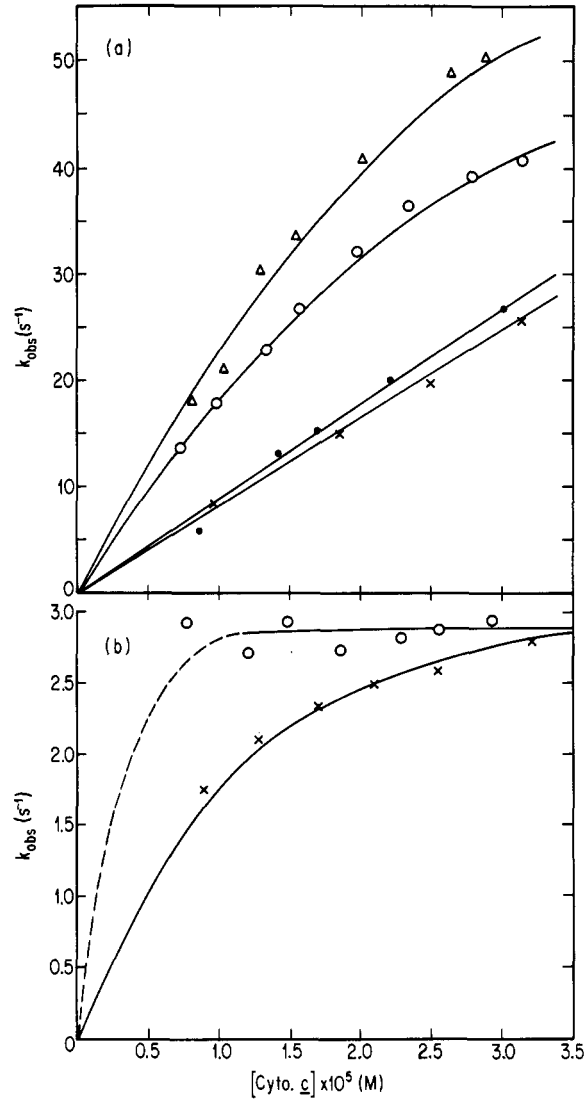

FIGURE 4: (a) Dependence of k_{obsd} on cytochrome *c* concentration. Conditions as in Figure 1. (×) 7-Chloro-FMN flavodoxin, $I^{1/2} = 0.316$; (●) 8-chloro-FMN flavodoxin, $I^{1/2} = 0.316$; (○) 7-chloro-FMN flavodoxin, $I^{1/2} = 0.255$; (△) 8-chloro-FMN flavodoxin, $I^{1/2} = 0.255$. (b) Conditions as in (a). (○) Tetrahydro-FMN flavodoxin, $I^{1/2} = 0.255$; (×) tetrahydro-FMN flavodoxin, $I^{1/2} = 0.316$. Curves represent computer fits to data except for tetrahydro-FMN flavodoxin at $I^{1/2} = 0.255$ (see text for details).

are the same within experimental error and are approximately half of those of the native flavodoxin (cf. Figure 2). This is to be compared with an approximately 13-fold decrease in k_1 upon dichloro substitution at this ionic strength (cf. Figure 2).

At $I^{1/2} = 0.255$, saturation behavior is observed for both of the monochloro-FMN analogue proteins (Figure 4a), again analogous to the native protein. The constants obtained from these data by computer analysis are also given in Table II. As expected, for both analogues, the values for k_1 are larger at the lower ionic strength and parallel those obtained for the native and dichloro-FMN proteins (cf. Figure 2). Thus, although the data are limited, it would appear that chlorine substitution again has not significantly altered the electrostatic interactions between the two proteins (see above). The k_1 values for both analogues are approximately the same and are 3–4 times smaller than that for the native protein (compared to an approximately 10-fold decrease for the dichloro-FMN protein). The K_a values for both monochloro proteins are also the same and are approximately 3 times smaller than the K_a value for the native protein, as well as being smaller than that

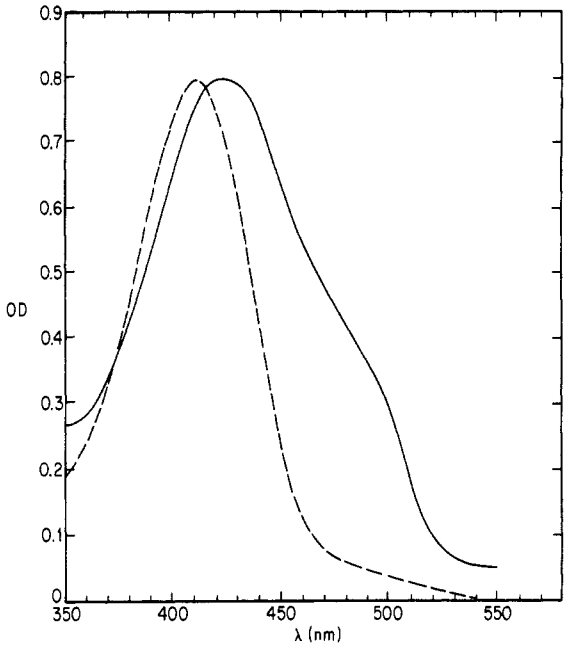

FIGURE 5: Visible absorption spectra for free tetrahydro-FMN (dashed line) in 0.025 M phosphate buffer, pH 7.5, and tetrahydro-FMN flavodoxin (solid line) in 0.005 M phosphate buffer, pH 7.2.

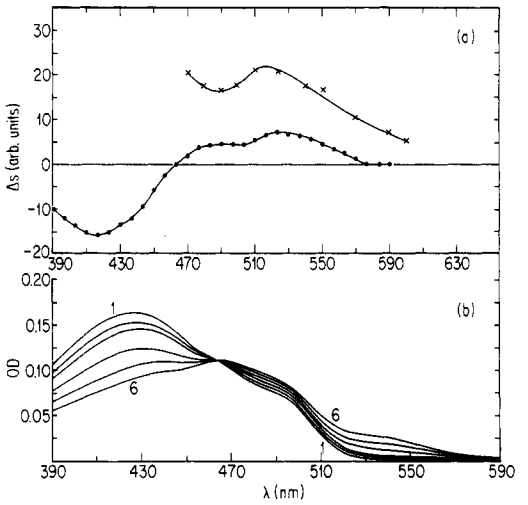

FIGURE 6: (a) (×) Difference spectrum of transient for free tetrahydro-FMN semiquinone generated by laser flash photolysis in 0.025 M phosphate buffer and EDTA (3.0×10^{-2} M), pH 6.4; (●) oxidized minus semiquinone difference spectrum for tetrahydro-FMN flavodoxin in 0.025 M phosphate buffer, pH 7.5. (b) Titration of tetrahydro-FMN flavodoxin in 0.025 M phosphate buffer, pH 7.5, with dithionite. Spectrum 1 is for oxidized protein with subsequent spectra representing various stages of reduction to the semiquinone (spectrum 6).

for the dichloro protein (about a factor of 2, as estimated by interpolation). The latter result is somewhat unexpected and will be discussed below. The k_{-1} values follow the same pattern as do the k_1 values. In fact, the only significantly different constant for the two monochloro analogues at this ionic strength is k_2: in this case, the constant for the 8-chloro-FMN flavodoxin is smaller than that of the 7-chloro derivative. Both constants, however, are larger than those obtained with the dichloro-substituted flavodoxin. It is interesting that the k_2 value for 7-chloro-FMN flavodoxin is slightly larger than that for the native protein. These relationships will be discussed below.

Properties of Free and Flavodoxin-Bound 5,6,7,8-Tetrahydro-FMN. As shown by Harzer & Ghisla (1979), the partial reduction of the dimethylbenzene ring in this analogue causes a substantial change in its spectral properties as compared to ordinary flavins. The oxidized free analogue exhibits a single absorption in the visible region with a λ_{max} at 414 nm and an estimated extinction coefficient of approximately 7800 cm^{-1} M^{-1}. The λ_{max} is red shifted to 425 nm with the appearance of a shoulder at 495 nm on binding to the apoprotein. The corresponding spectra are shown in Figure 5. Although we have not determined an association constant, the tetrahydro-FMN appears to be bound to apoflavodoxin more weakly than is FMN. As with FMN, fluorescence is quenched upon binding. These results are similar to those obtained by Harzer & Ghisla (1979) with *M. elsdenii* flavodoxin.

The reduction of the free analogue was studied by using dithionite as well as illumination in the presence of EDTA. In both instances, full reduction occurred as indicated by the total loss of the absorption maximum at 414 nm with a small amount of absorption remaining in the visible region of the spectrum. However, upon reoxidation of the analogue with air, only a partial return of the absorption peak at 414 nm was observed, suggesting that a loss of a portion of the analogue occurred. An increase in absorption between 350 and 400 nm was observed for the reoxidized sample which indicates the presence of at least one new compound. It should be noted that ordinary flavins diplay fully reversible reduction under these same conditions.

The free analogue was also examind by using flash photolysis in the presence of EDTA. A very weak transient which we attribute to the formation of the semiquinone was obtained. A difference spectrum for this transient is presented in Figure 6a. Under these conditions, ordinary flavins produce much larger semiquinone signals.

The reduction of tetrahydro-FMN flavodoxin was also examined. Behavior similar to that of the free analogue was observed for the photoreduction with EDTA. Again, full reduction occurred upon prolonged exposure to light, whereas upon air reoxidation, only a portion of the original absorption returned. In addition, there was increased absorption between 350 and 400 nm indicative of the appearance of a new species.

As was shown by Harzer & Ghisla (1979) for *M. elsdenii* flavodoxin, the reduction of the tetrahydro-FMN flavodoxin with dithionite yielded the semiquinone as shown in Figure 6b (compare the difference spectrum shown in Figure 6a with that obtained by flash photolysis of the free analogue). The half-time for the reduction was approximately 20 min. The isosbestic point at 463 nm is indicative of the formation of only the semiquinone, and thus, full reduction apparently did not occur. This behavior is analogous to that observed with native flavodoxin, although in the latter case a mixture of semiquinone and fully reduced forms is produced. Upon admission of air, the original oxidized absorption spectrum was quantitatively restored. Although we have not measured the kinetics of air oxidation, it appears to occur considerably more rapidly than for the native flavodoxin semiquinone.

Figure 4b shows plots of k_{obsd} vs. (cytochrome *c*) obtained from stopped-flow studies of the oxidation of the semiquinone which was produced by using dithionite. At both ionic strengths investigated ($I^{1/2} = 0.255$ and $I^{1/2} = 0.316$), saturation behavior was observed. Table II lists values for k_1, k_{-1}, k_2, and K_a. At $I^{1/2} = 0.255$, the rate was completely saturated at all of the concentrations of cytochrome *c* that were used. This situation probably arises because k_1 has increased whereas k_2 has not appreciably changed from the value obtained at $I^{1/2} = 0.316$. It is of course difficult to obtain a value for k_1 in this instance. Our estimate given in Table II for this constant is based on the assumption that the analogue exhibits the same

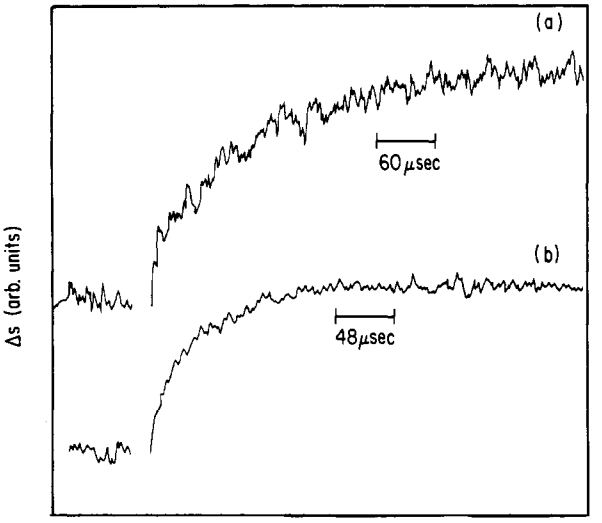

FIGURE 7: (a) Transient obtained at 600 nm for the reduction of *C. pasteurianum* flavodoxin (1.9×10^{-5} M) to the semiquinone by using 5-deazariboflavin (3.5×10^{-5} M) in 0.025 M phosphate buffer with EDTA (1.5×10^{-2} M), pH 7.5, by laser photolysis (excitation λ = 421 nm). The absorbance increase is due to production of the semiquinone. (b) Transient obtained at 600 nm for the reduction of 8-chloro-FMN flavodoxin (5.5×10^{-5} M) to the semiquinone. Conditions as in (a).

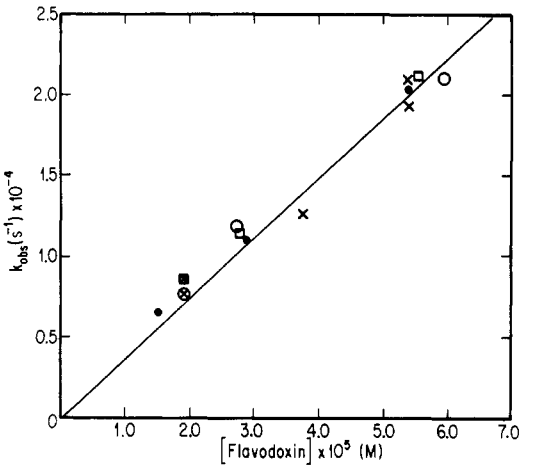

FIGURE 8: Dependence of k_{obsd} upon flavodoxin concentration; conditions as in Figure 7. (×) *C. pasteurianum* flavodoxin; (●) 7-chloro-FMN flavodoxin; (□) 8-chloro-FMN flavodoxin; (○) 7,8-dichloro-FMN flavodoxin.

dependence of k_1 upon ionic strength as does the native protein and the chloro analogues. At $I^{1/2} = 0.316$, k_2 is 2-fold greater for the tetrahydro-FMN flavodoxin than for the 7,8-dichloro-FMN flavodoxin. At $I^{1/2} = 0.255$, k_2 is approximately 30-fold smaller than that for the native protein, as well as being considerably below that for all of the chlorine analogue containing proteins. We will discuss these rate constant comparisons further below.

Reduction of Flavodoxin and Its Analogues by 5-Deazariboflavin Semiquinone. Flash photolysis offers a method by which to examine the kinetics of reduction of flavodoxin from the oxidized to the semiquinone form and thus to determine whether or not chlorine substitution has any effect upon this reaction. The reductant, 5-deazariboflavin semiquinone, was generated in the sample by laser photolysis using EDTA as the hydrogen donor. Figure 7 shows some representative data obtained in this way. Again, first-order behavior over approximately three half-lives was found.

Figure 8 shows a plot of k_{obsd} vs. flavodoxin concentration for the native protein and the various chlorine analogues. No

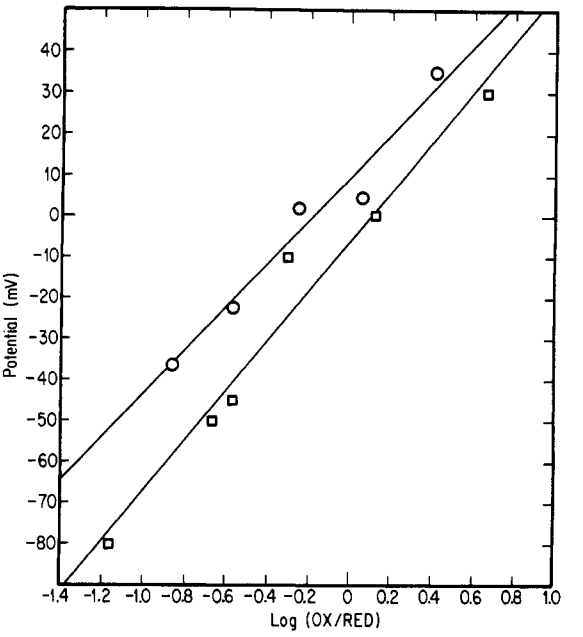

FIGURE 9: Nernst plots for determination of the reduction potentials of the monochloro-FMN flavodoxins in 0.025 M phosphate buffer, pH 7.5. $Fe(CN)_6^{3-}$, methyl viologen, and 1,5-anthraquinone disulfonate were used as mediators under anaerobic conditions. (○) 8-Chloro-FMN flavodoxin; (□) 7-chloro-FMN flavodoxin. Plots for both analogues followed one-electron reductions with least-square slopes of 53.2 and 61.2 and correlation coefficients of 0.982 and 0.983, respectively.

evidence of saturation was found, and thus if a complex exists along the reaction pathway, its rate of decay must be greater than 2×10^4 s^{-1}. Because the protein concentrations were considerably higher than that of the laser-generated deazariboflavin semiquinone, all of the transient curves that were used to calculate the k_{obsd} values followed pseudo-first-order kinetics. It is apparent from Figure 8 that the rate constants for reduction of all of the proteins examined were similar, if not identical within experimental error (< ±20%). The second-order rate constant calculated from the slope of the line in Figure 8 is 3.7×10^8 M^{-1} s^{-1}. Thus, these results demonstrate that chlorine substitution does not affect the rate of reduction of the protein-bound coenzyme by 5-deazariboflavin semiquinone. This conclusion is different from that reported previously (Jung & Tollin, 1981). It is likely that this is due to the greater accuracy and improved sensitivity of the instrument used in this study, as well as to the use of computer analysis in obtaining rate constants. The earlier study also reported a difference in the rate constants for the reduction of the semiquinone forms of native and dichloro-FMN flavodoxins by deazariboflavin semiquinone. In light of the present results, this needs to be reinvestigated.

Determination of Redox Potentials of Monochloro-FMN Flavodoxins. As shown previously (Jung & Tollin, 1981), chlorine substitution at the 7- and 8-positions of FMN substantially shifts the redox potentials of flavodoxin to higher values. It was thus of interest to ascertain the effect of monochloro substitution. Figure 9 shows the results of the redox titrations. The reduction potentials found for the conversion of the oxidized to the semiquinone species were 10 and −6.0 mV for the 8-chloro-FMN and 7-chloro-FMN flavodoxins, respectively. This represents a positive shift of approximately 160 mV as compared to the native flavodoxin and a negative shift of approximately 120 mV with respect to the 7,8-dichloro-FMN flavodoxin (Jung & Tollin, 1981). Thus, the monochloro proteins lie approximately midway between the native and dichloro flavodoxins.

The (two-electron) redox potentials for the free analogues (Walsh et al., 1978) are −128 and −144 mV for 7-chlororiboflavin and 8-chlororiboflavin, respectively. Thus, the potential for the 7-chloro derivative is more positive than for the 8-chloro analogue. However, binding of the monochloro-FMN derivatives by apoflavodoxin shifts the (one-electron) potentials so that the 8-chloro flavodoxin is now more positive than the 7-chloro flavodoxin. Thus, the influence of protein binding is greater at the 8-position than at the 7-position. The possible relationships between these redox potentials and the rate constants for semiquinone oxidation will be discussed below.

Discussion

According to the mechanism of eq 1, the rate constant k_1 reflects complex formation between flavodoxin semiquinone and cytochrome *c*. As was shown in Figure 2, this rate constant depends upon ionic strength in a parallel manner for both the native and the chlorine-substituted flavodoxins, although the values for the chloro analogues are significantly smaller. This indicates that the electrostatic shielding effects of solvent ions are the same for all of the proteins and thus that the replacement of FMN by mono- or dichloro-FMN has not resulted in any conformational change which has affected the topological distribution of negatively charged carboxyl groups in the protein. On the other hand, it is to be expected that the introduction of electronegative chlorine atoms into the coenzyme would alter its dipole moment in such a way as to render the benzene ring region of the molecule more negative. Inasmuch as this portion of the FMN is in van der Waals contact with the nonpolar heme edge of the cytochrome according to the structure of the previously postulated electron-transfer complex (Simondsen et al., 1982), it is not unreasonable to suggest that such an unfavorable interaction between the prosthetic group edges would increase the activation energy and thus result in a decrease in the rate constant for association. The smaller decrease in k_1 for the monochloro analogues (Figure 2) than for the dichloro analogue is consistent with such an interpretation. Furthermore, the fact that all of the protein analogues give the same k_1 values in the laser photolysis experiment shows that the effect of chlorine substitution is specifically related to the nature of the flavin–heme interaction. A factor of 10 in the rate constant (for the dichloro-FMN analogue relative to the native protein) would correspond to an activation energy increase of about 1.4 kcal/mol, which is not unreasonable for a dipole effect. It should be pointed out, however, that his unfavorable interaction could be at least partially compensated by the delocalized positive charge which is due to the ferriheme iron. Perhaps this accounts for an apparently smaller effect of chlorine substitution on K_a for the dichloro analogue compared to the native protein (see above), and for the larger value of K_a obtained for the dichloro-FMN flavodoxin compared to the monochloro analogues at $I^{1/2} = 0.255$.

Other factors may also be involved here. For example, the increased dipole moment of the dichloro-FMN could cause a localized distortion of the electrical potential field at the surface of the protein (Matthew et al., 1983). Localized dielectric constant effects might be important (i.e., an increase in ϵ would decrease the interaction energy) as well as the solvation of the exposed flavin ring which could result in an increased energy requirement for water expulsion during complex formation with the dichloro analogue protein. A quantitative treatment analogous to that of Matthew et al. (1983) might prove enlightening.

It is necessary also to recognize the possibility that an increase in the amount of negative charge localized in the benzene ring portion of the FMN could result in a distortion of the structural arrangement of the immediately adjacent protein backbone and side-chain groups, thus introducing a steric component into the rate and association constants. While we cannot completely eliminate this in the absence of an X-ray structure analysis, it is made less likely by the fact that all of the protein analogues yield the same rate constants for reduction by deazariboflavin semiquinone. This latter result also argues against any influence of the different redox potentials of the analogues on k_1 and K_a.

The limiting first-order rate constant, k_2, can be a measure of the actual electron-transfer step (which should be influenced by redox potential) as well as of any conformational steps which may exist along the reaction pathway. As an example of the latter, if the initial interaction between the two proteins can occur in a variety of ways, not all of which result in proper overlap between the two prosthetic groups, a rearrangement within the complex may be required prior to electron transfer to bring the heme and flavin into a productive alignment. The facts that k_{-1} and k_2 are of similar magnitude for the dichloro-FMN flavodoxin (this is also true for the monochloro analogues) and that both depend upon ionic strength in a roughly parallel manner (Figure 3) suggest that such a conformational step may be an important component of the limiting rate. Thus, k_{-1} must necessarily reflect the ability of the two proteins to move relative to one another inasmuch as this step involves complex dissociation. The ionic strength dependence of these rate constants can be rationalized as follows: At high values of I, salt ions may be tightly bound to the charge-paired groups within the complex, thus making it kinetically more difficult to break these apart than would be the case at low I where solvation largely involves more loosely bound water molecules. The fact that the ionic strength dependence of k_2 for the native protein is parallel to that for the dichloro-FMN protein (Figure 3) is consistent with such an interpretation. An alternative viewpoint would involve the solvation of the entire protein, with the counterion interactions increasing the "rigidity" of the protein, thereby making it more difficult for conformational fluctuations to disrupt charge-pair interactions.

The k_{-1} values for the dichloro analogue are smaller than the corresponding constants for the native protein. This is to be expected inasmuch as the K_a values (and thus the thermodynamic stabilities) for the two complexes are not very different (approximately a factor of 2, which corresponds to a free-energy difference of about 0.4 kcal/mol). For a situation in which K_a is maintained approximately constant, an increase in activation energy for complex formation would lead to a decrease in the relative values of both the forward (k_1) and reverse (k_{-1}) rate constants.

The k_2 values for the chlorine-containing analogues are clearly correlated with the difference in redox potentials for the reactants. The smallest k_2 (estimated to be 23 s^{-1} at $I^{1/2} = 0.255$; cf. Table I) is obtained with 7,8-dichloro-FMN flavodoxin which also has the highest redox potential for the oxidized/semiquinone conversion, whereas the k_2 values (Table II) for the monochloro analogues are larger and in proportion with their respective potentials (Figure 7). Thus, k_2 must also contain a contribution from the rate of electron transfer, rather than solely reflecting the rate of a conformational rearrangement.

The correlation of k_2 with redox potential breaks down, however, for the native flavodoxin, which has a $k_2 = 78$ s^{-1} at $I^{1/2} = 0.255$ as well as the most negative redox potential (Simondsen et al., 1982). This suggests that there may also

be specific substituent effects occurring at the 7- and 8-positions. Since chlorine is an electron-withdrawing substituent, it would be expected to decrease the electron density of the π orbital of the atom to which it is covalently bonded. Inasmuch as the 8-position has a higher unpaired spin density in the semiquinone (see above) than does the 7-position, chlorine substitution at this location should be most effective in retarding electron transfer, as the experimental results indeed suggest. The small observed increase in k_2 for the 7-chloro-FMN analogue over that of the native protein may then be a consequence of two effects, i.e., the electron-withdrawing property of the chlorine which pulls electron density away from the N5 region of the FMN toward the benzene ring and the electron-donating property of the methyl group which specifically increases electron density at the 8-position. While these differences in k_2 do not prove conclusively that electron transfer occurs from the 8-position, they certainly strengthen this supposition.

The behavior of the tetrahydro-FMN flavodoxin provides further insight into the nature of k_2. For this analogue, k_2 does not appear to change with ionic strength, which suggests that the rate of electron transfer is probably rate limiting at both ionic strengths, i.e., that the conformational rearrangement is occurring more rapidly than the heme reduction. That this slow rate of electron transfer is due to structural rather than redox potential[3] considerations may be inferred from the behavior of the analogue protein with dithionite and O_2. Thus, there is no formation of the fully reduced flavodoxin upon dithionite treatment, which suggests that the redox potential for the semiquinone/hydroquinone conversion is more negative than that for the native flavodoxin. Furthermore, the rate of dithionite reduction to the semiquinone is considerably slower than that for the native protein, whereas the rate of O_2 oxidation is appreciably faster. This implies that the redox potential for the oxidized/semiquinone transformation is at least as negative as the redox potential of the native protein and perhaps more so. These properties would be consistent with the decrease in the extent of double-bond conjugation in tetrahydro-FMN and would thus argue for a greater reactivity of the semiquinone relative to native flavodoxin (i.e., a larger k_2 value). The low k_2 value can be rationalized as follows, assuming no significant *protein* structural changes. If electron transfer proceeds by an outer-sphere mechanism, reduction of two of the double bonds in the dimethylbenzene ring would act to decrease the extent of orbital overlap with the heme prosthetic group of cytochrome *c*. If, on the other hand, a tunneling mechanism is operative, the FMN structural modification, by eliminating the unpaired spin density in the exposed region of the flavin, would increase the distance over which the electron must tunnel in order to achieve heme reduction. In either case, a significant decrease in the rate of electron transfer would be expected.

Conclusions

The experiments described above have provided some interesting new insights into the factors which influence complex formation, stability, and dynamics during the interaction between two protein molecules which are involved in an electron-transfer reaction. We have seen that the ionic milieu plays an important role both during and subsequent to complex formation. Thus, in the present case which involves proteins having oppositely charge interaction surfaces, a low salt ion concentration in the solution environment acts to increase the following: the rate of the initial collisional encounter, the rate of any subsequent reorientation process required to bring the prosthetic groups into effective alignment, and the overall thermodynamic stability of the encounter complex. The low free ion content (and lowered dielectric constant) which would exist in the interior regions of a membrane would be expected to have a similar effect.

We have also seen that the polarity of the region of the prosthetic group which is exposed to the solvent can have a significant influence upon these same properties. For this reason, it is probably not coincidental that for both the *c*-type cytochromes and for flavodoxin a completely nonpolar edge of the coenzyme is presented at the site of electron transfer. This would serve to allow the course of the reaction to be controlled effectively by the electrostatic properties of the amino acid side chains adjacent to the active site, as well as to minimize any potentially unfavorable effects caused by dipolar or electrostatic properties of the coenzymes in their interactions with one another, with the electrostatic field surrounding the active site, and with water molecules located in the active site region. Furthermore, it ensures that the actual electron transfer will proceed within a low dielectric environment. Further studies along these lines are important to test the generality of the effects described here, as well as to probe other aspects of the electron-transfer protein interaction.

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Registry No. 8-Chloro-FMN, 73358-82-4; 7-chloro-FMN, 85479-44-3; 5,6,7,8-tetrahydro-FMN, 70841-66-6; 5-deazariboflavin semiquinone, 78548-68-2; cytochrome *c*, 9007-43-6.

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Differential Accessibility of the Amino and Carboxy Termini of Histone H2A in the Nucleosome and Its Histone Subunits†

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ABSTRACT: In our efforts to determine the role of the contact interface of the H2A:H2B dimer and the $(H3:H4)_2$ tetramer as a possible site for the regulation of nucleosome structure and function, we have probed this interface by the use of proteolytic enzymes. The accessibility of the amino and carboxy termini of H2A to cleavage by trypsin was tested in the H2A:H2B dimer, the core histone octamer, chromatin, and nuclei. The H2A-specific protease [Eickbush, T. H., Watson, D. K., & Moudrianakis, E. N. (1976) *Cell* (*Cambridge, Mass.*) *9*, 785–792] was utilized to establish the order of cleavage of the terminal regions of H2A. Three consecutive slab gel electrophoretic systems were used to monitor the histone 2A cleavage products. In the absence of DNA, the amino-terminal region of H2A was observed to be readily cleaved by trypsin when the H2A:H2B dimer is associated with the $(H3:H4)_2$ tetramer in the form of a solubilized octamer. However, in this case, the carboxy terminus of the molecule is quite resistant to cleavage by trypsin. In contrast, when the substrate for trypsinization was the H2A:H2B dimer or several forms of chromatin or nuclei, there was an ordered cleavage of the amino terminus of H2A followed by cleavage of its carboxy-terminal region. It therefore appears then that the carboxy terminus of H2A is somehow involved in the formation of the dimer–tetramer contact interface in the octamer. Furthermore, this interface is accessible to external enzymatic modifications when the octamer is associated with DNA in the form of a nucleosome, while it appears to be much less accessible in free octamers as they exist in high ionic strength solutions. This observation may have important implications in the regulation of chromatin structure through modification of specific regions along this contact interface by either covalent or associative interactions, both of which could alter the compaction state of the nucleosome.

In recent years, a great deal of experimental data has been gathered on the organization of histones and DNA in chromatin. Brief digestion of eukaryotic chromatin with micrococcal nuclease yields particles call nucleosomes which contain about 200 base pairs of DNA associated with two each of the four core histones H2A, H2B, H3, and H4 in the form of histone octamer, and one molecule of H1. Further nuclease digestion brings about the production of the core nucleosome particle which consists of 146 base pairs of DNA wrapped around the core histone octamer [see review by McGhee & Felsenfeld (1980)]. The octamer, which is extractable as a unit from chromatin in 2 M NaCl, is formed by the association of two H2A:H2B dimers with an $(H3:H4)_2$ tetramer (Eickbush & Moudrianakis, 1978). This laboratory has previously presented the results of gel filtration and sedimentation equilibrium experiments which indicate that in solutions of high ionic strength, the core histones behave as a reversibly associating system of histone octamers in equilibrium with $(H3:H4)_2$-H2A:H2B hexamers, $(H3:H4)_2$ tetramers, and H2A:H2B dimers (Godfrey et al., 1980). The balance of this equilibrium is affected by the ionic strength, pH, temperature, and the presence of urea. On the basis of these findings, it has been suggested that the interaction of the dimer and the tetramer involved predominately the formation of a limited number of hydrogen bonds (Eickbush & Moudrianakis, 1978). It is our hypothesis that the metastable dimer–tetramer contact interface may function as a site of regulation of nucleosome function.

A great amount of work has been done to establish the histone–histone contacts within the nucleosome. Much of this has been accomplished through the use of chemical cross-linking reagents. Another approach has involved the use of proteolytic enzymes to determine the accessibility of the histones to their external environment. Weintraub & Van Lente (1974) also examined the digestion of the core histones when they are released from the DNA in 2 M NaCl. Trypsin-modified nucleoprotein complexes were subjected to DNase I digestion (Whitlock & Simpson, 1977) and protein cross-linking with dimethyl suberimidate (Whitlock & Stein, 1978), and it was concluded that the trypsin-limit peptides of the core histones retained both the structural elements necessary for proper protein–protein associations and the ability to organize the DNA into a nucleoprotein complex which resembled the chromatin core particle. A series of recent studies by Bohm and co-workers (Bohm et al., 1980, 1981, 1982) have defined the limit digest products of the core histones when chicken erythrocyte nuclei were extensively digested with trypsin. Of particular interest to this study was the characterization of the major trypsin-limit peptide of H2A which was comprised of residues 12–118 from the original H2A of 128 amino acids in length.

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