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Transient Kinetics of Electron-Transfer Reactions of Flavodoxins[†]

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ABSTRACT: Stopped-flow and laser photolysis methods have been used to investigate the rates of electron-transfer reactions of fully reduced riboflavin and the three oxidation states of *Clostridium pasteurianum* flavodoxin. Both normal and 7,8-dichloroflavin analogues were studied. Redox reagents included oxygen, ferricyanide, ferric EDTA, and several *c*-type cytochromes as oxidants and the semiquinone of 5-deazariboflavin as a reductant. The dependence of the rate of oxidation of the semiquinone form of the dichloro analogue flavodoxin upon oxidant concentration has provided clear evidence for the existence of a complex in the reaction pathway. Rate constant comparisons demonstrate that dichloro substitution decreases the rate of flavodoxin semiquinone oxidation by at least 1–2 orders of magnitude. The limiting first-order rate constants were found to be dependent on the redox potential of the oxidant, as would be predicted by theory if these were reflecting the actual electron-transfer reaction. Rate constant decreases upon chlorine substitution were also observed for the reduction of both oxidized and semiquinone forms of flavodoxin by deazariboflavin semiquinone. These results, considered in conjunction with the redox potential shift of the flavodoxin produced by the chlorine substitution, provide support for the hypothesis that electron transfer to and from the semiquinone form of the flavodoxin involves direct participation of the dimethylbenzene ring of the flavin. A comparison of oxidation rate constants for free and protein-bound fully reduced flavin suggests that the protein environment does not markedly influence coenzyme reactivity in this oxidation state.

The high-resolution X-ray crystal structure determinations which have been carried out on the flavodoxins (Burnett et al., 1974; Mayhew & Ludwig, 1975; Smith et al., 1977) provide an excellent opportunity to investigate the relationships which exist between flavin properties and the protein environment of the bound coenzyme. Among the structural features which have been elucidated by the X-ray analysis are the following: (a) the dimethylbenzene ring of the FMN cofactor is partially exposed to the solvent; (b) the N5–C4a–C10a–N1 region[1] of the flavin, i.e., the most obviously redox-active part of the molecule, is shielded by the protein structure; (c) the isoalloxazine ring of the flavin is constrained to be planar in all three redox states, oxidized (PF), half-reduced (PFH·), and fully reduced (PFH^-).

In the work to be described below, we have addressed the following questions:

(1) Does the planarity of the flavin ring in PFH^-, which is a thermodynamically unfavorable conformation and which has been suggested (Mayhew & Ludwig, 1975) to result in a markedly lowered redox potential for the PFH· to PFH^- conversion, also increase the reactivity of the bound fully reduced cofactor? Since FH· is normally planar, it is conceivable that forcing FH^- to also be planar could allow easier access to the transition state and thus lower the activation energy. The experimental approach used to investigate this question was to determine the rate constant for reaction between FH^-, both free and bound to *Clostridium* flavodoxin, and a variety of one-electron oxidants. Although, as will be shown below, these reactions are too rapid to separate the rate constant for electron transfer from that due to binding of oxidant, the observed second-order rate constants do not indicate any systematic increase in reactivity for the bound coenzyme.

(2) Does one-electron transfer involving interconversion between the PFH·, PF, and PFH^- states of flavodoxin utilize the exposed dimethylbenzene ring of the coenzyme? In the case of FH·, it is well-known that unpaired spin density resides in this region of the isoalloxazine structure (Müller et al., 1970). The experimental approach to this question involved the use of a protein-bound 7,8-dichloro analogue of flavin. By comparing the rate constants for its one-electron conversion between the FH· and FH^- forms to those of the normal coenzyme, the effect of a structural and electronic modification of the exposed ring could be determined. This approach was used in a previous study (Shiga & Tollin, 1976) of the oxidation of PFH· to PF which indicated that the chlorine substitution markedly decreased the rates of reaction with iron-containing oxidants (cytochrome *c*, ferricyanide) for the bound coenzyme only, thereby implicating the participation of the dimethylbenzene ring in the reaction pathway. Although some data were obtained in the earlier investigation on the dependence of reaction rate on oxidant concentration, instrumental limitations did not allow this parameter to be varied over a wide range. With the present apparatus, a better optical and light measurement system has enabled us to measure rates at higher concentrations of oxidant and thus to demonstrate the existence of intermediates in the reaction and, in the case of the dichloro analogue, to directly measure the rate constant for electron transfer. The earlier results obtained with oxidation of the PFH· form showing a decrease in reactivity upon chlorine substitution have been substantiated and extended to include reduction of PF and PFH·.

(3) Is there a relationship between reaction rates and redox potential differences, as would be expected on theoretical grounds (Marcus, 1964; Marcus & Sutin, 1975)? As will be described below, such a relationship is indeed observed.

Flavodoxin, in its function in nitrogen fixation, transfers an electron from its hydroquinone form to the iron-containing subunit of the nitrogenase complex (Orme-Johnson et al.,

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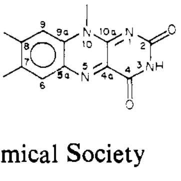
[1] The numbering system of the flavin ring is



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1977). Thus, in the present exploration of flavodoxin electron-transfer mechanisms, we have limited our attention mainly to iron-based oxidizing agents.

Experimental Procedures

Materials. Riboflavin was purchased from Calbiochem and was used without purification. 7,8-Dichlororiboflavin and 7,8-dichloro-FMN were synthesized and purified as previously described (Shiga & Tollin, 1976). Massey et al. (1980) have shown that monochloroflavin analogues substituted in either the 7 or 8 positions undergo photochemical loss of chlorine from the fully reduced state and that this occurs for both the free and the protein-bound analogues. No loss of chlorine was found to occur from the semiquinone state. We have confirmed these results (R. P. Simondsen, unpublished work) with the 7,8-dichloro analogues (both free and bound to flavodoxin). This phenomenon precluded the use of the dichloroflavins in the present study to investigate the reactivity of the fully reduced form of flavodoxin inasmuch as we have used photochemical methods to generate the reduced protein.

The flavodoxin from *Clostridium pasteurianum* was obtained by published procedures (Mayhew, 1971). The 7,8-dichloro-FMN analogue of flavodoxin (Cl_2PF) was prepared by mixing the flavin and the apoflavodoxin, the latter obtained by the KBr dialysis method of Mayhew (1971), in a 1:1.5 mole ratio to assure complete binding of the dichloro-FMN. We have previously shown (Shiga & Tollin, 1976) that the properties of Cl_2PF are closely analogous to those of the normal flavodoxin. In the interpretation of some of the results to be described below, we have assumed that the major features of the X-ray crystallographic results obtained with the *Clostridium* MP flavodoxin also apply to the related *C. pasteurianum* protein.

Semiquinone forms of the flavodoxins were produced by visible light irradiation in the presence of 0.03 M EDTA under anaerobic conditions (Massey & Palmer, 1966). The fully reduced form of riboflavin was also prepared in this manner. For the preparation of fully reduced flavodoxin, however, catalytic amounts of deazariboflavin (Massey & Hemmerich, 1977) were incorporated into the irradiation mixture.

Horse heart cytochrome *c* was obtained from Sigma Chemical Co. and used without further purification. Cytochrome *c*-555 (from *Chlorobium thiosulphatophilum*), *Chromatium vinosum* cytochrome *c′*, *Rhodopseudomonas capsulata* cytochrome *c′*, and *Rhodopseudomonas palustris* cytochrome *c′* were generous gifts from Dr. Michael A. Cusanovich, Department of Biochemistry, University of Arizona.

Ferric EDTA was prepared by dissolving $FeCl_3$ (0.01 M) in an EDTA solution (0.03 M) in 0.025 M phosphate buffer, pH 7.5. AR grade potassium ferricyanide was obtained from Mallinckrodt Chemical Co. and used without purification.

Methods. All reactions were carried out anaerobically (N_2 bubbling) and at constant temperature (usually 23 or 2.5 °C) in 0.025 M phosphate buffer, pH 7.5. The stopped-flow apparatus was constructed in this laboratory by using a mixer, observation cell, and driving piston obtained from a Durrum-Gibson instrument. The driving syringe compartment was made of Plexiglass and utilized Teflon plungers. Although Plexiglass has the disadvantage of low thermal conductivity, its transparency allows samples to be irradiated within the driving syringe compartment. The monitoring light source was a 250-W tungsten–iodine projection lamp powered by a Sorensen DCR 40-10A supply. This passed through a Jarrell-Ash 82–410 monochromator and was focused onto the observation cell (2-cm path length) with microscope optics. The transmitted light was detected by an RCA 4463 photomultiplier tube (S-20 response), the output of which was fed into a Biomation Model 805 transient recorder and then into either a Tektronix oscilloscope for observation purposes or into a strip-chart recorder for a permanent record.

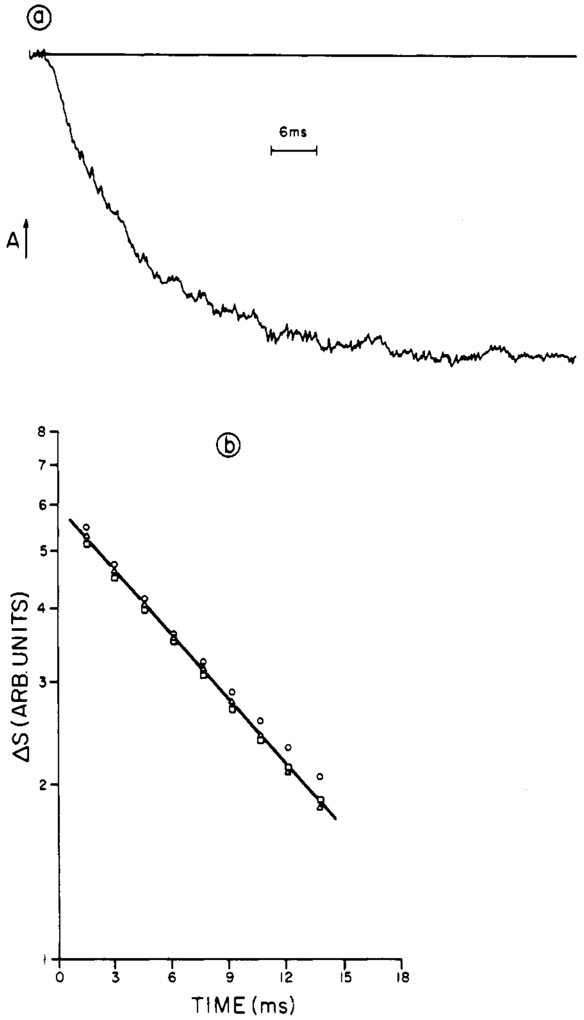

FIGURE 1: (a) Transient absorbance change at 425 nm obtained at 2.5 °C upon stopped-flow mixing of fully reduced riboflavin (7×10^{-7} M) and *Rps. capsulata* cytochrome *c′* (1.9×10^{-6} M) under anaerobic conditions in 0.025 M phosphate buffer, pH 7.5. The decrease in absorbance is due to reduction of cytochrome. (b) Semilog plots of transient absorbance changes obtained from three experiments with the system described in (a).

Although the sensitivity of the apparatus varied with wavelength and type of sample, under most conditions we were able to resolve an absorbance change of 0.001 unit. The mixing dead time also varied with conditions (temperature, viscosity of solutions) but usually was between 5 and 8 ms. A typical reaction trace obtained with one of the most rapid reactions which we have studied is shown in Figure 1a. It is apparent that the signal-to-noise ratio is reasonably good. In Figure 1b is shown a series of semilog plots obtained from several runs with this system. As is evident, reproducibility was also good, with an overall precision of ±10%. We estimate that this is the maximum error range for all of our stopped-flow measurements.

Special care was taken to achieve anaerobic conditions, particularly with the fully reduced flavin species. Small amounts of oxygen which entered the sample during transfer procedures could be eliminated by illumination while the sample was within the driving syringe compartment. This also served to regenerate any fully reduced flavin which was lost by oxidation.

Laser photolysis was used to generate the 5-deazariboflavin semiquinone via triplet-state reduction by EDTA. When this was carried out in the presence of the oxidized or semiquinone forms of flavodoxin, the reduction of the latter species by the deazaflavin radical could be monitored. The photolysis apparatus has been previously described (Tollin et al., 1979). The exciting source was a N_2-laser pumped dye [2-(1-naphthyl)-5-phenyloxazole] which produced a 10-ns pulse of light at 393 nm. The time resolution of the apparatus was approximately 0.3 μs (limited mainly by scattering artifacts). We estimate that rate constant values obtained by laser photolysis with the present systems are accurate to ±20%.

The determination of the redox potentials for the reduction of oxidized dichloro-FMN flavodoxin to the semiquinone and the hydroquinone was carried out in the laboratory of Dr. G. S. Wilson, Department of Chemistry, University of Arizona, by Douglas Root using previously published procedures (Wilson, 1978).

Results and Discussion

Mechanistic Considerations. Since, as will be documented below, PFH· oxidation proceeds via at least a two-step mechanism, it is necessary to consider the consequences of this for data analysis and interpretation. The simplest kinetic mechanism that can be written is as follows (we will assume that this mechanism is applicable to all of the reactions we have investigated, leaving open the possibility that more than one intermediate is actually on the reaction pathway):

$$\mathrm{PFH\cdot\ (PFH^-) + ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \xrightarrow{k_2} \mathrm{PF\ (PFH\cdot) + red} \tag{1}$$

Because of the large redox potential differences which exist in the present systems (see Table I), we can neglect any contribution from the reverse reaction (i.e., $k_{-2} \sim 0$).

If one applies a steady-state approximation to this mechanism, the following relationship can be derived between the observed pseudo-first-order rate constant (k_{obsd}) and the specific rate constants as defined by eq 1:

$$k_{\mathrm{obsd}} = \frac{k_1 k_2(\mathrm{ox})}{k_1(\mathrm{ox}) + k_{-1} + k_2} \tag{2}$$

At low (ox)

$$k_{\mathrm{obsd}} = \frac{k_1 k_2(\mathrm{ox})}{k_{-1} + k_2} \tag{3}$$

and

$$k_{\mathrm{obsd}}/(\mathrm{ox}) = \frac{k_1 k_2}{k_{-1} + k_2} \leq k_1 \tag{4}$$

(depending upon the relative magnitude of k_2 and k_{-1}). Two limiting cases can be recognized:

case I: $k_2 \gg k_1(\mathrm{ox}) + k_{-1}$

Under these conditions, $k_{\mathrm{obsd}} = k_1(\mathrm{ox})$.

case II: $k_2 \ll k_1(\mathrm{ox}) + k_{-1}$

Under these conditions, $k_{\mathrm{obsd}} = k_1 k_2(\mathrm{ox})/[k_1(\mathrm{ox}) + k_{-1}]$, and saturation behavior will be observed as high (ox). Then

$$\frac{1}{k_{\mathrm{obsd}}} = \frac{k_{-1}}{k_1 k_2}\left[\frac{1}{(\mathrm{ox})}\right] + \frac{1}{k_2} \tag{5}$$

and a plot of $1/k_{\mathrm{obsd}}$ vs. $1/(\mathrm{ox})$ will yield a straight line whose slope is $k_{-1}/(k_1 k_2)$ and whose intercept[2] is $1/k_2$. We will define the reciprocal of the slope as k'. It is readily seen that $k'/k_2 = k_1/k_{-1} = K_a$, the association constant for complex formation.

Table I: Redox and Charge Properties of Flavodoxin and Iron-Containing Oxidants

compound	$E_{m,7}$ (mV)	charge at pH 7.5
ferric EDTA	96	−
ferricyanide	410	−
cytochrome *c* (horse heart)	260	+
cytochrome *c*-555 (*Chlorobium thiosulphatophilum*)	150	+
cytochrome *c'* (*Chromatium vinosum*)	0	−
cytochrome *c'* (*Rhodopseudomonas capsulata*)	50	−
cytochrome *c'* (*Rhodopseudomonas palustris*)	100	+
PF (*C. pasteurianum flavodoxin*)	−132	−
PFH· (*C. pasteurianum flavodoxin*)	−419	−

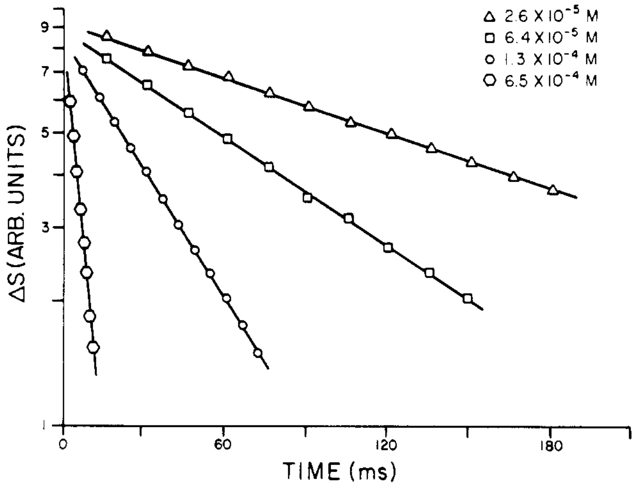

FIGURE 2: Semilog plots of absorbance changes at 580 nm obtained at 23 °C upon stopped-flow mixing of *C. pasteurianum* flavodoxin semiquinone (2×10^{-6} M) and various concentrations of cytochrome *c* (horse heart) under anaerobic conditions in 0.025 M phosphate buffer, pH 7.5. Absorbance decrease due to oxidation of semiquinone.

Oxidation of PFH. As an extension of our previous study (Shiga & Tollin, 1976) of the oxidation of PFH· and its dichloro analogue (Cl_2PFH·), we have determined the dependence of k_{obsd} on (ox) for three reactants: horse heart cytochrome *c*, ferricyanide, and ferric EDTA. A typical set of results is shown in Figure 2. In Figures 3–5, plots of k_{obsd} vs. (ox) are presented (part a). It is apparent that, for all three oxidants, saturation behavior is observed for Cl_2PFH· but not for PFH·. On the basis of the discussion in the preceding section, we conclude that case II holds for the dichloro analogue. In part b of Figures 3–5, we show the double-reciprocal plots based on eq 5 and its analogue for eq 2. As expected, this linearizes the data for the Cl_2PFH· oxidation, providing support for the mechanism given in eq 1. We assume that the mechanism of eq 1 also holds for the normal flavodoxin, with k_2 being large enough so that we cannot reach a high enough (ox) to observe saturation. The values for the rate and equilibrium constants obtained from the data are given in Table II. For the PFH· reactions, we have assumed that the k_2 values must be a minimum of 2 times greater than the highest value obtained for k_{obsd} in order to explain the lack

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[2] This value of the intercept also holds for the most general situation, i.e., eq 2. The value of the slope will differ, however, because of the inclusion of the k_2 term.

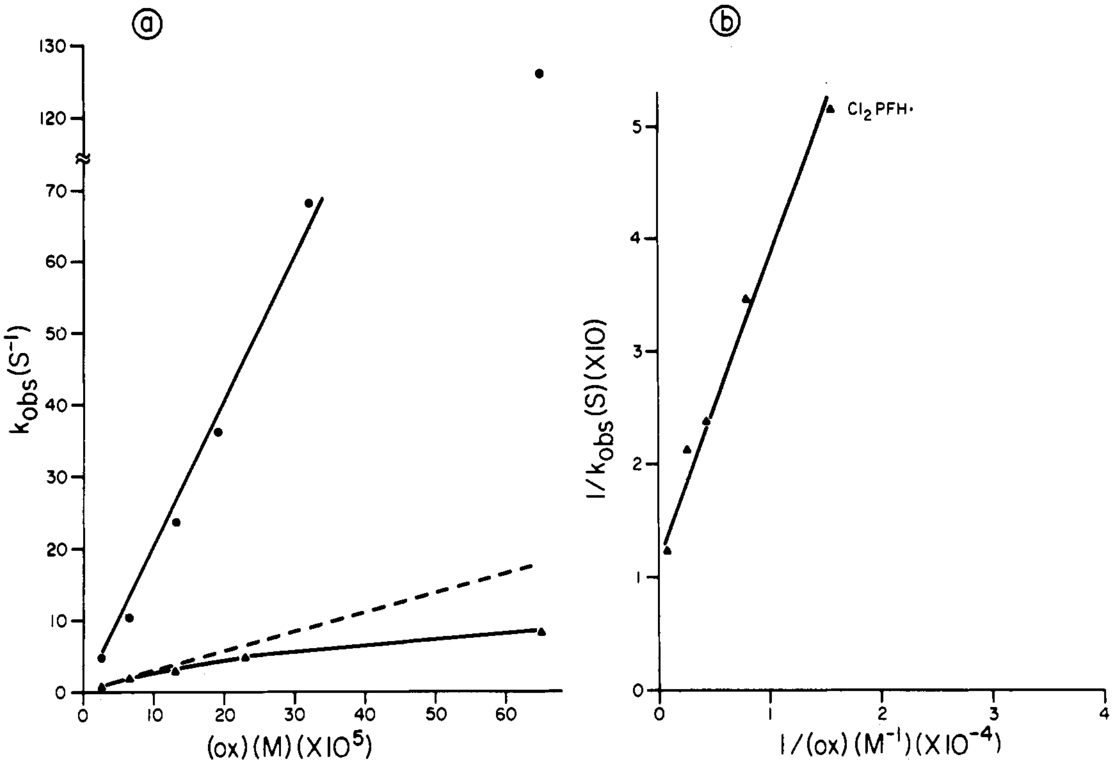

FIGURE 3: (a) Dependence of k_{obsd} on cytochrome *c* (horse heart) concentration. Rate constants obtained from semilog plots such as shown in Figure 2. Conditions as in Figure 2. (●) *C. pasteurianum* flavodoxin semiquinone; (▲) dichloro analogue of *C. pasteurianum* flavodoxin semiquinone. (b) Double-reciprocal plot of data in (a).

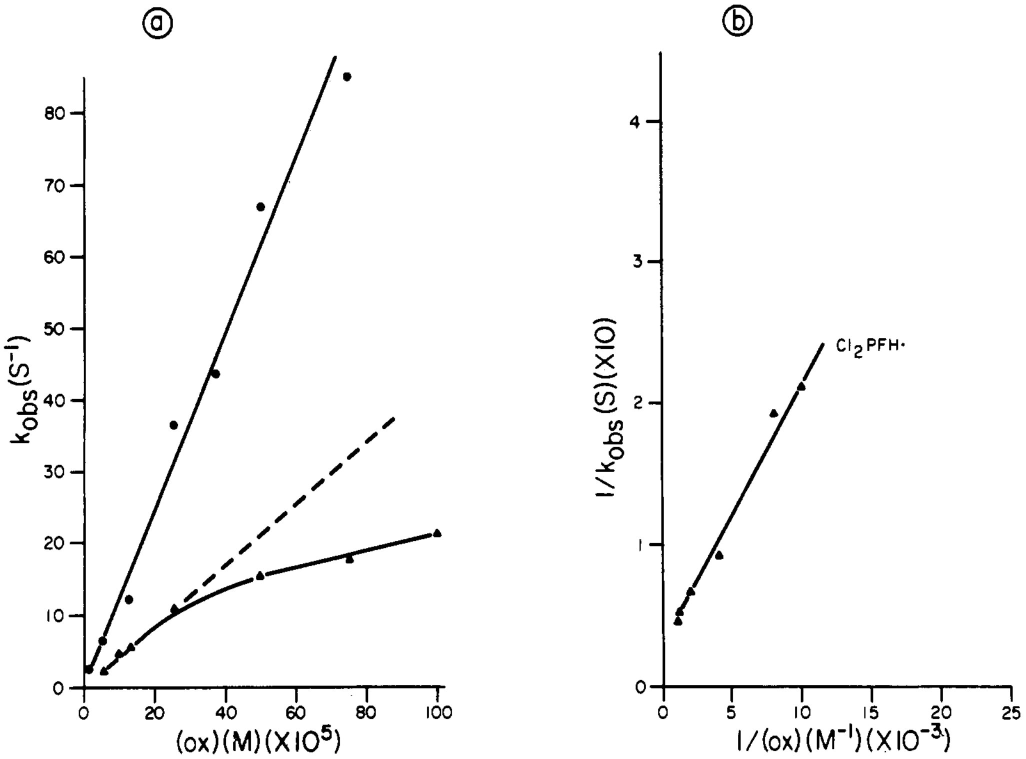

FIGURE 4: (a) Dependence of k_{obsd} on ferricyanide concentration. Conditions as in Figure 2. (●) *C. pasteurianum* flavodoxin semiquinone; (▲) dichloro analogue of *C. pasteurianum* flavodoxin semiquinone. (b) Double-reciprocal plot of data in (a).

of saturation. It is apparent that k_2, the limiting first-order rate constant for appearance of product, which presumably measures electron transfer (see below), is at least 3–30 times larger for the normal flavodoxin than for the dichloro analogue, thus supporting and extending our earlier observations (Shiga & Tollin, 1976). Note also that there is no correlation between K_a, the association constant for complex formation, and k_2. Thus, for example, ferric EDTA accepts an electron from $Cl_2PFH\cdot$ with a rate constant which is 3 orders of magnitude less than that for ferricyanide, yet it is bound almost as tightly. The relative magnitudes of these association constants may be affected by the electrostatic properties of the reactants. Thus, flavodoxin carries a negative charge close to its flavin prosthetic group (Mayhew & Ludwig, 1975) whereas cytochrome *c* is positively charged in the vicinity of its heme prosthetic group (Cusanovich, 1978), ferric EDTA has one

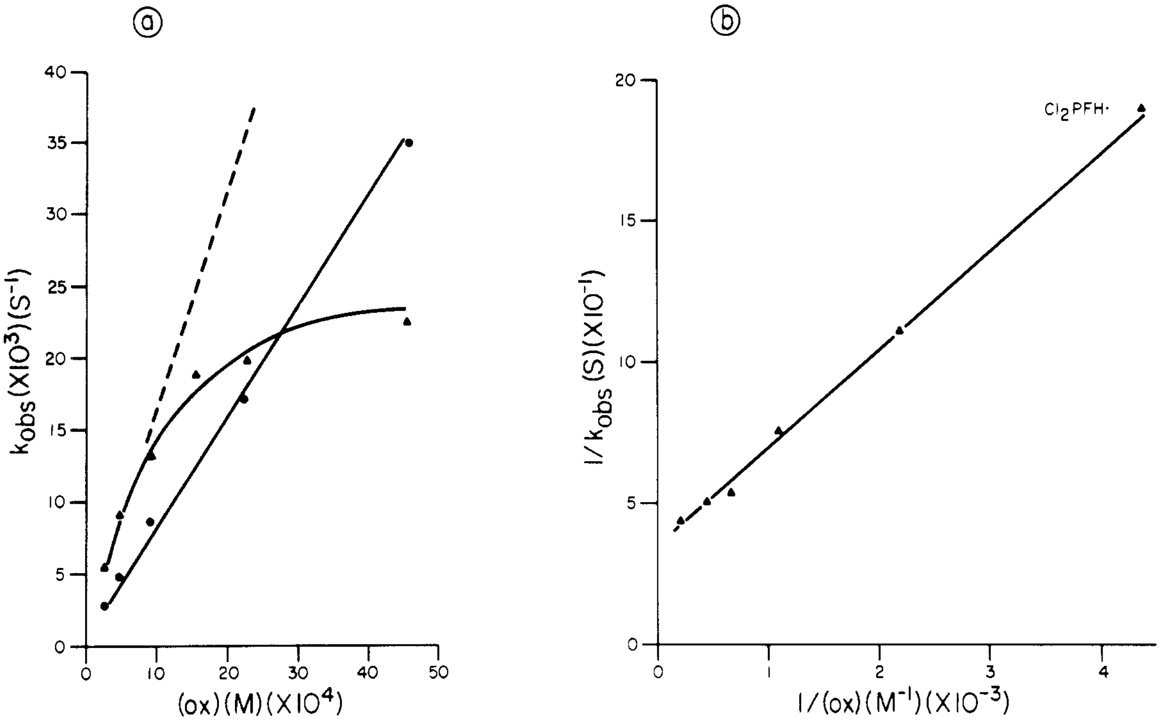

FIGURE 5: (a) Dependence of k_{obsd} on ferric EDTA concentration. Conditions as in Figure 2. (●) *C. pasteurianum* flavodoxin semiquinone; (▲) dichloro analogue of *C. pasteurianum* flavodoxin semiquinone. (b) Double-reciprocal plot of data in (a).

Table II: Rate Constants and Association Constants for Flavodoxin Semiquinone Oxidation[a]

	flavodoxin species					
	PFH·			$Cl_2PFH\cdot$		
oxidant	$k_{obsd}/(ox)$ (M^{-1} s^{-1})	$k_2{}^b$ (s^{-1})	K_a (M^{-1})	k' (M^{-1} s^{-1})	k_2 (s^{-1})	K_a (M^{-1})
horse heart Cyt *c*	1.8×10^5	$\geq 2.5 \times 10^2$		3.8×10^4	8.5	4.5×10^3
$Fe(CN)_6{}^{3-}$	1.2×10^5	$\geq 3 \times 10^2$		5.3×10^4	37	1.4×10^3
FeEDTA	10.5	≥ 0.1		29	0.028	1.0×10^3

[a] Data obtained at 23 °C in 0.025 M phosphate buffer, pH 7.5. Values of k_2 for $Cl_2PFH\cdot$ were obtained by linear regression analyses of double-reciprocal plots. Correlation coefficients were in the range 0.978–0.999. The values of $k_{obsd}/(ox)$ for PFH· oxidation by horse heart cyt *c* and ferricyanide are in good agreement with those obtained earlier (Shiga & Tollin, 1976). [b] These values are estimates (see text for discussion).

negative charge, and ferricyanide has three negative charges. We are presently investigating the ionic strength dependence of these reactions in order to test this possibility.

It can also be seen from Table II that the values of $k_{obsd}/(ox)$ and k' for the normal and analogue proteins differ by factors of from 3 to 5 [k' and $k_{obsd}/(ox)$ are numerically equal for $Cl_2PFH\cdot$, which implies that $k_{-1} > k_2$, i.e., that complex dissociation proceeds more rapidly than electron transfer]. From the definitions of these quantities presented above, this is to be expected if the k_2 values are different. It is also possible, of course, that differing values for k_1 and k_{-1} make a contribution. It is interesting that k' for the ferric EDTA reaction is larger for the dichloro analogue than $k_{obsd}/(ox)$ is for the normal protein. This would argue that either $k_1(Cl_2PFH\cdot) \gg k_1(PFH\cdot)$ or $k_{-1}(Cl_2PFH\cdot) \ll k_{-1}(PFH\cdot)$ or that both inequalities apply. Stated another way, ferric EDTA is bound more tightly to $Cl_2PFH\cdot$ than to PFH·.

Table III: Rate Constants for Reduction of Oxidized and Semiquinone Forms of Flavodoxin by Deazariboflavin Semiquinone[a]

reaction	$k_{obsd}/(ox)$ (M^{-1} s^{-1})
$PF + DRFH\cdot \rightarrow PFH\cdot + DRF$	4×10^9
$Cl_2PF + DRFH\cdot \rightarrow Cl_2PFH\cdot + DRF$	2×10^8
$PFH\cdot + DRFH\cdot \rightarrow PFH^- + DRF$	$2.\times 10^8$
$Cl_2PFH\cdot + DRFH\cdot \rightarrow Cl_2PFH^- + DRF$	4×10^7

[a] Data obtained at 23 °C in 0.025 M phosphate buffer, pH 7.5.

Reduction of PF and PFH·. In order to investigate whether chlorine substitution of the flavin also affects the rate constants for electron transfer to the oxidized flavin and to the flavin semiquinone as it does the removal of an electron from the semiquinone, we have determined rates of reduction by the 5-deazariboflavin semiquinone (Edmondson et al., 1972) using laser photolysis to rapidly generate this species. Separate photolysis experiments were performed starting with either oxidized flavodoxin or flavodoxin semiquinone, the latter produced by prior irradiation of the sample with steady light. Semiquinone formation and decay were monitored at 580 nm, a wavelength at which the deazaflavin semiquinone has negligible absorption (Edmondson et al., 1972). In a separate experiment, the decay of the deazaflavin radical was monitored at 500 nm. Analysis of this latter decay gave results in agreement with those obtained in an earlier investigation (Edmondson et al., 1972). The maximum concentration of the semiquinone generated by the laser in these experiments was approximately 2×10^{-8} M, thus assuring pseudo-first-order conditions with respect to flavodoxin. Typical results

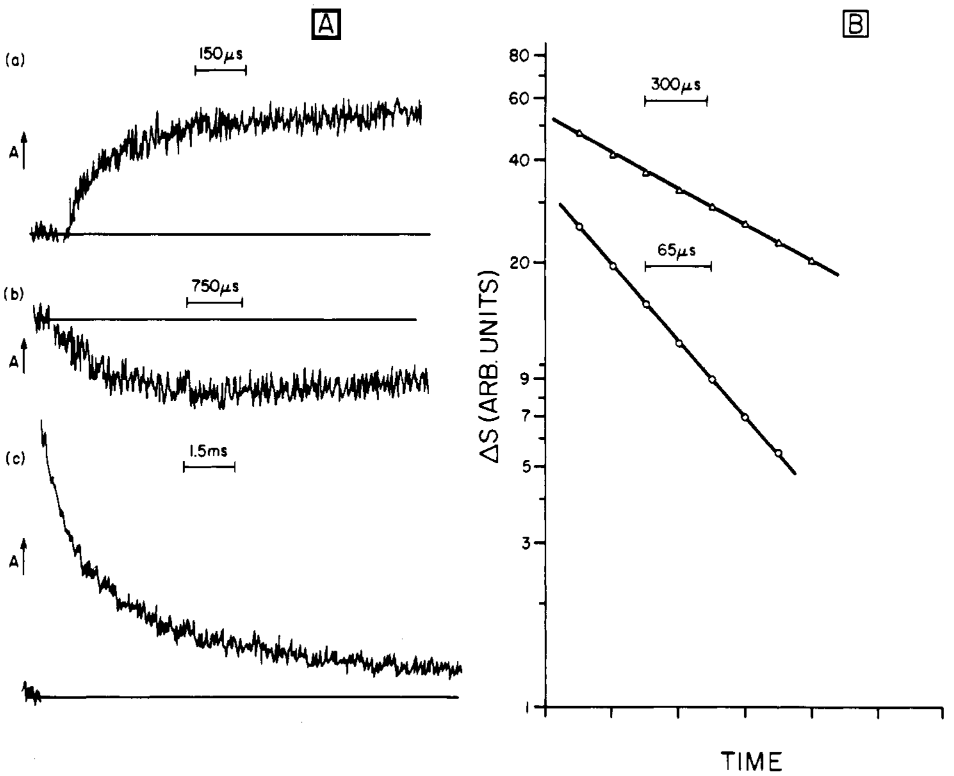

FIGURE 6: (A) (a) Transient obtained at 580 nm upon laser photolysis (excitation λ = 393 nm) at 23 °C of anaerobic solution containing the dichloro analogue of *C. pasteurianum* flavodoxin (3.9 × 10^{-5} M), deazariboflavin (2.1 × 10^{-5} M), and EDTA (0.015 M) in 0.025 M phosphate buffer, pH 7.0. The absorbance increase is due to the reduction of flavodoxin to semiquinone. (b) Transient obtained at 580 nm upon laser photolysis of *C. pasteurianum* flavodoxin semiquinone (1 × 10^{-5} M), deazariboflavin (2.6 × 10^{-5} M), and EDTA (0.015 M). Conditions as in (a). The absorbance decrease is due to reduction of semiquinone to the fully reduced form. (c) Transient obtained at 500 nm upon laser photolysis of deazariboflavin (4.1 × 10^{-5} M) and EDTA (0.015 M) in the absence of flavodoxin. Conditions as in (a). The absorbance changes are due to formation and decay of deazariboflavin semiquinone. Maximum concentration of semiquinone estimated to be 2 × 10^{-8} M. (**B**) Typical semilog plots of data obtained as in (A). (△) Reduction of oxidized form of *C. pasteurianum* flavodoxin; (○) Reduction of dichloro analogue of *C. pasteurianum* flavodoxin semiquinone.

are shown in Figure 6. As can be seen, flavodoxin reduction occurred during the lifetime of the deazaflavin semiquinone, indicating that the latter (as opposed to fully reduced deazaflavin) is the reducing species. The rate constants obtained from these data are presented in Table III. It is evident that, as was the case for oxidation of PFH·, chlorine substitution decreases the rate constant for addition of an electron to both the oxidized and semiquinone forms of flavodoxin. Because of the large values for the observed rates, which precludes study over a wide concentration variation, we cannot be certain that the calculated rate constants are actually those for the electron-transfer step, as opposed to a complex rate constant which includes both binding and electron transfer (i.e., eq 3). Nevertheless, on the basis of the results shown in Table II for PFH· oxidation, it seems reasonable to conclude that the variations in the values of $k_{\mathrm{obsd}}/(\mathrm{ox})$ (5–20-fold) at least partially reflect differences in the k_2 values. It is interesting to note that the reduction of oxidized normal flavodoxin by dRFH· is close to diffusion controlled, whereas the reduction of PFH· is approximately 20 times slower. A similar rate constant decrease is observed with the dichloro analogue, although less marked. This suggests that the reduction rate constants are at least partially reflecting the thermodynamic properties of the oxidants, i.e., the lower redox potential for the PFH· to PFH^- conversion.

Table IV: Rate Constants for the Oxidation Reactions of Fully Reduced Riboflavin and Flavodoxin at 2.5 °C[a]

oxidant	$k_{\mathrm{obsd}}/(\mathrm{ox})$ ($\mathrm{M}^{-1}\,\mathrm{s}^{-1}$) RFH^-	 PFH^-
O_2	2.3 × 10^4	3.1 × 10^4
ferric EDTA	5.6 × 10^6	1.8 × 10^7
$\mathrm{Fe(CN)_6}^{3-}$	>5 × 10^7	>5 × 10^7
horse heart cyt *c*	2.8 × 10^7	>2 × 10^8
cyt *c*-555	5.9 × 10^7	>2 × 10^8
Chromatium cyt *c*′	2.3 × 10^7	3.5 × 10^6
Rps. capsulata cyt *c*′	7.4 × 10^7	7.0 × 10^7
Rps. palustris cyt *c*′	9.7 × 10^7	2.2 × 10^7

[a] Concentrations of oxidants were (2–9) × 10^{-6} M except for the reactions with O_2 whose concentration was controlled by air saturation (2.5 × 10^{-4} M) and by oxygen saturation (1 × 10^{-3} M) under 1-atm pressure. The concentrations of riboflavin and flavodoxin ranged from 0.5 × 10^{-6} to 2 × 10^{-6} M; 0.025 M phosphate buffer, pH 7.5.

Oxidation of PFH^-. We have determined the rate constants for oxidation of both free and bound fully reduced flavin with a number of oxidants, including several different *c*-type cytochromes with varying redox potentials and charge properties (see Table I). In all cases, with the exception of O_2, reaction rates were quite large. This again prevented us from varying the oxidant concentration over a range which was wide enough to allow complex formation to be detected or to obtain a definitive value for k_2. However, proportionality between k_{obsd} and (ox) was observed over the accessible range. In the case of the O_2 reaction, proportionality was also obtained between air-saturation levels (2.5 × 10^{-4} M) and oxygen saturation (1 × 10^{-3} M). Thus, in this case either no complex is formed or the binding constant is quite low. Values for $k_{\mathrm{obsd}}/(\mathrm{ox})$ are presented in Table IV. In some cases, e.g., $\mathrm{Fe(CN)_6}^{3-}$, the rates were too rapid to measure under our conditions. For these reactions, we estimated a minimum value of $k_{\mathrm{obsd}}/(\mathrm{ox})$ from the lowest concentrations which we could use. The major inference from these data is as follows: Since no systematic

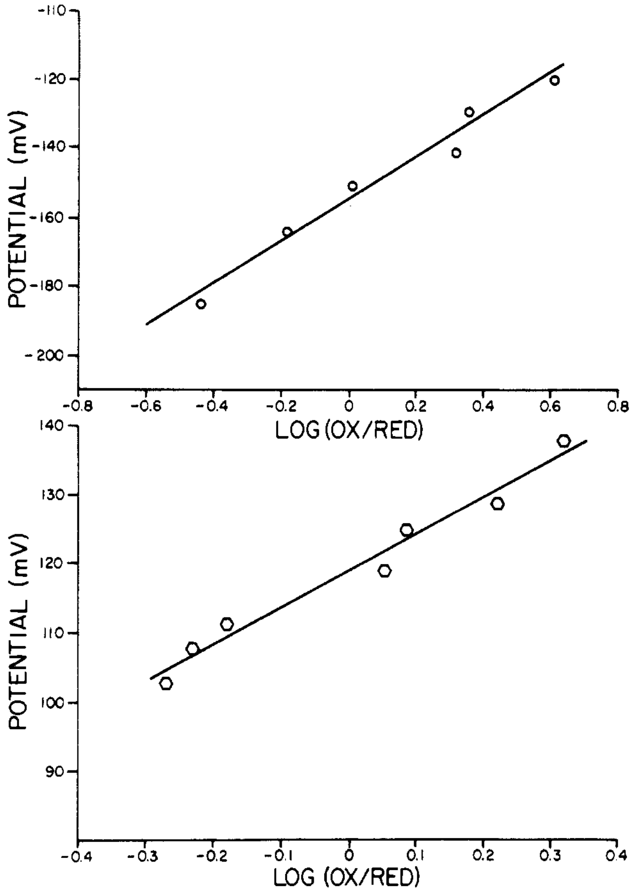

FIGURE 7: Nernst equation plots for the redox titrations of the dichloro analogue of *C. pasteurianum* flavodoxin. (O) Oxidized to semiquinone; anthraquinone disulfonate used as mediator. (O) Semiquinone to fully reduced; indigodisulfonate used as mediator. Anaerobic conditions in 0.025 M phosphate buffer, pH 7.5. E_m values are 119 mV for PF to PFH· and −155 mV for PFH· to PFH$^-$. Plots follow a one-electron fit with a least-square slopes of 55.2 and 61.4, respectively (correlation coefficients = 0.929 and 0.987). Control experiments with the normal flavodoxin gave values for the PF to PFH· conversion which were in agreement with the literature (Mayhew & Ludwig, 1975).

or large differences appear between the values of $k_{obsd}/(ox)$ for free and bound flavins, i.e., in some cases the observed rate constants are greater and in some cases they are smaller, we conclude that the binding of the flavin coenzyme to apoflavodoxin probably does not cause any *major* change in the reactivity of the fully reduced form. The fact that appreciable differences can be found for the $k_{obsd}/(ox)$ values in the reactions of PFH· and $Cl_2PFH·$ (see, for example, Figure 3a and Table III) would lend support to this conclusion, although even in these reactions we do find an inversion in the ferric EDTA case (Figure 5a). It is, of course, possible that changes in the values of k_1 and k_{-1} act to compensate for large changes in the k_2 values. However, this seems improbable for the following reason: While one might anticipate the possibility of such compensation occurring occasionally, it would be entirely unexpected for it to occur for eight oxidants of such widely differing character.

Several other consequences of Table IV are worth noting: (1) No correlations exist between $k_{obsd}/(ox)$ and oxidant properties such as size, charge, or redox potential. (2) For the horse heart cytochrome *c*, cytochrome *c*-555, and ferricyanide oxidations of flavodoxin, the k_2 values must be very large indeed, the k_{-1} step is probably negligible, and the rate is most likely controlled by a diffusion-limited complex formation step. It is interesting to note that these three oxidants have the most positive $E_{m,7}$ values (see Table I) of all the iron-containing oxidants which we have used.

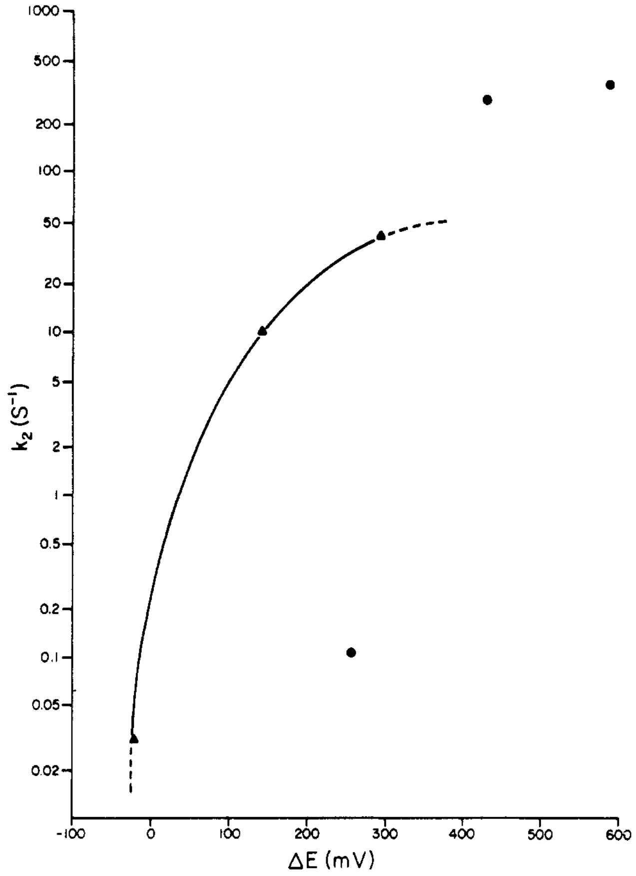

FIGURE 8: Plot of k_2 for oxidation of *C. pasteurianum* flavodoxin semiquinones vs. difference in redox potential values of oxidant and reductant at pH 7.5. (▲) $Cl_2PFH·$; (●) PFH· (minimum values).

According to the Marcus theory of outer-sphere electron-transfer reactions (Marcus, 1964; Marcus & Sutin, 1975), there is a relationship between the log of the rate constant for electron transfer and the redox potential difference between oxidant and reductant. Experimental studies of electron-transfer quenching of excited states (Nagle et al., 1979) have shown that log k increases with ΔE and then levels off as the potential difference becomes very large. Therefore, in order to obtain an understanding of the rate constant differences which we have observed upon chlorine substitution, it is necessary to determine the effect of such substitution upon redox potential. In Figure 7 is shown the results of redox titrations of the one-electron reductions of $Cl_2PF·$. Chlorine substitution causes a shift of the midpoint potential of the oxidized to semiquinone conversion to more positive values by approximately 280 mV. The semiquinone to fully reduced potential is shifted to more positive values by approximately 270 mV. These can be compared with a shift of only 81 mV observed with the free coenzymes (Müller & Massey, 1969). Thus, the flavodoxin is considerably more responsive to the substitution than is free FMN, which implies a very important role for the dimethylbenzene ring in determining the redox properties of the holoprotein.

In Figure 8 we have plotted log k_2 values (from Table II) for the oxidation of PFH· and $Cl_2PFH·$ by various oxidants vs. the difference in redox potential between oxidant and reductant. It is apparent that the expected relationship is approximately obeyed. This supports the contention that the k_2 values do indeed measure the rates of the electron-transfer step rather than some structural change occurring within the intermediate complex. It is also apparent that the two sets of data do not fall on the same curve (this would be true even if one were to shift the values for the PFH· oxidations upward). Thus, if the increase in redox potential were the sole factor determining the change in the rate constants in going from PFH· to $Cl_2PFH·$, the k_2 values for the latter compound should be considerably smaller than they are, especially for the ferric EDTA and cytochrome *c* reactions. Furthermore, we have found decreases in rate constants upon chlorine substitution for the oxidized to semiquinone and semiquinone to fully reduced conversions (Table III), which is in the reverse direction from what would be predicted on the basis of shifts in redox potential; i.e. since the reduction potentials are more positive than for the normal flavodoxin, it should be easier to achieve reduction.

Conclusions

The relationships we have observed between redox potentials and the rate constants for flavodoxin semiquinone oxidation, as measured by stopped flow, and for the reduction of the oxidized and semiquinone flavodoxins, as measured by laser photolysis, lead us to the following conclusion. Replacing the methyl groups in the 7 and 8 positions of the flavin by chlorine exerts an influence on the kinetics of electron transfer to and from the FMN moiety of flavodoxin in a manner which cannot be accounted for solely by the change in the redox potential of the holoenzyme and that this influence serves to facilitate the removal of an electron and hinder the addition of an electron. These effects can be rationalized if the controlling factor is the increase in electron density at position 7 (or 8) caused by the electronegativity of the chlorine substituent and that electrons enter and leave via this region. The kinetic influence of the chlorine atoms could be compensated for in the establishment of the overall redox potential by changes in the binding affinities of the chloro analogue, i.e., $Cl_2FH·$ and Cl_2FH^- bound more tightly than FH· and FH^- and/or Cl_2F bound less tightly than F. This would act to shift the reduction potentials for the dichloro analogue proteins to more positive values without necessarily affecting rate constants. Whether or not this rationalization is correct, it clearly is possible to conclude, with a great deal more certainty than before (Shiga & Tollin, 1976), that the electron-transfer reactions of flavodoxin in its oxidized and semiquinone forms proceed with the direct participation of the dimethylbenzene region of the flavin ring system.

The stopped-flow measurements of the rate constants for oxidation of free and protein-bound fully reduced flavins lead us to the conclusion that the protein environment does not systematically alter the reactivity of the FH^- species, even though its redox potential is made considerably more negative. As in the case of the semiquinone, this could be a consequence of compensating effects due to binding thermodynamics and changes in electron distribution and/or steric accessibility. More work will be required in order to achieve a more complete understanding of this system.

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