Influence of 8α-Imidazole Substitution of the FMN Cofactor on the Rate of Electron Transfer from the Neutral Semiquinones of Two Flavodoxins to Cytochrome *c*†

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ABSTRACT: The effects of substituting an imidazole ring onto the 8α-position of the FMN cofactor on the kinetics of electron transfer from the neutral semiquinone forms of *Azotobacter* and *Clostridium* flavodoxins to oxidized horse heart cytochrome *c* have been investigated by stopped-flow methods. Although 8α-substitution does not alter the mechanistic pathway of the reaction, the rate constants are decreased by factors of 10–30, without significant changes in the equilibrium association constants of the intermediate electron-transfer complexes. Protonation of the imidazole ring further decreases the observed second-order rate constants for the electron-transfer reaction by factors of 20–50. The pK_a values for the 8α-imidazole ring in both flavodoxin semiquinones were determined to be approximately 7. In contrast, the reactions of the native flavodoxins with cytochrome *c* are pH independent. The results are consistent with a structural model of the intermediate complex [Simondsen, R. P., Weber, P. C., Salemme, F. R., & Tollin, G. (1982) *Biochemistry 21*, 6366–6375], which postulates a close fit between the exposed dimethylbenzene ring of the FMN and the heme edge within a nonpolar interface region. The results further indicate that electron transfer is uncoupled from proton transfer, that it is the rate-limiting step, and that it occurs prior to proton transfer at all pH values. Finally, the results do not provide support for a direct role of the imidazole ring in the facilitation of one-electron transfer in those enzymes containing 8α-*N*-histidylflavin coenzymes.

The kinetics of the electron-transfer reaction between flavodoxin semiquinone and cytochrome *c* have been investigated in considerable detail (Simondsen et al., 1982; Simondsen & Tollin, 1983; Tollin et al., 1984; Cheddar et al., 1986). Although this reaction is nonphysiological, it has a number of features that make it a unique and valuable system for studying protein–protein interactions during electron transfer. These include the following: (1) X-ray crystallographic structures are available for several examples of both components [cf. Mayhew and Ludwig (1975), Meyer and Kamen (1982), and Smith et al. (1983)]; (2) the kinetics of the redox process are rapid and irreversible, and it is thus relatively easy to determine the rate constants that characterize the system; (3) the components form a tight, electrostatically stabilized, complex that can be detected both kinetically (Simondsen et al., 1982) and spectroscopically (Hazzard & Tollin, 1985); (4) the FMN cofactor can be reversibly removed from flavodoxin and replaced with structurally modified flavins (Edmondson & Tollin, 1971).

Previous kinetic studies (Simondsen et al., 1982) have shown that the reaction involves the rapid formation of an equilibrium complex of flavodoxin semiquinone ($FldH^{\bullet}$)[1] and oxidized horse heart cytochrome *c* (cyt *c*) prior to electron transfer from the reduced flavin to the heme.

$$FldH^{\bullet} + \text{cyt } c_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{complex}] \xrightarrow{k_2} Fld_{ox} + \text{cyt } c_{red} \qquad (1)$$

It has also been found (Simondsen & Tollin, 1983) that replacement of the 7- and 8-methyl groups in the isoalloxazine ring of FMN by chlorine atoms results in a 10-fold decrease in k_1, a 3-fold decrease in k_2, and a 2-fold decrease in the kinetically determined value for K_a (see eq 1 above for identification of these constants; $K_a = k_1/k_{-1}$) for the electron-transfer reaction between *Clostridium pasteurianum* flavodoxin and cyt *c*. This was interpreted as providing support for a computer-generated model of the structure of the intermediate complex (Simondsen et al., 1982), in which the exposed dimethylbenzene ring of the flavin cofactor of flavodoxin is in van der Waals contact with the exposed heme edge of the cytochrome during the electron-transfer interaction.

In view of the possible involvement of the benzenoid portion of the flavin ring in this reaction, it is of interest to investigate whether 8α-substitution of the FMN cofactor of flavodoxin might have any influence. A number of naturally occurring flavoenzymes have covalently bound flavin coenzymes in which attachment is via the 8α-position to a histidine, cysteine, or tyrosine residue [see Singer and McIntire (1984) for a recent review]. In several enzyme systems belonging to this class, one-electron-transfer reactions between the flavin and either heme groups or Fe/S clusters are of catalytic significance. Currently, it is not known whether the 8α amino acid substituent plays any direct role in such electron-transfer reactions.

In the present paper, we report the effect of an imidazole ring attached via the nitrogen atom to the 8α-position of FMN (see below for structure) on the kinetics of electron transfer from the neutral flavin semiquinone form of two flavodoxins

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[1] Abbreviations: Fld, flavodoxin; $FldH^{\bullet}$, flavodoxin semiquinone; cyt *c*, horse heart cytochrome *c*; FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid; 8-IMF, 8α-*N*-imidazolyl-FMN; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; TAPS, [[tris(hydroxymethyl)-methyl]amino]propanesulfonic acid; k_{obsd}, observed pseudo-first-order rate constant.

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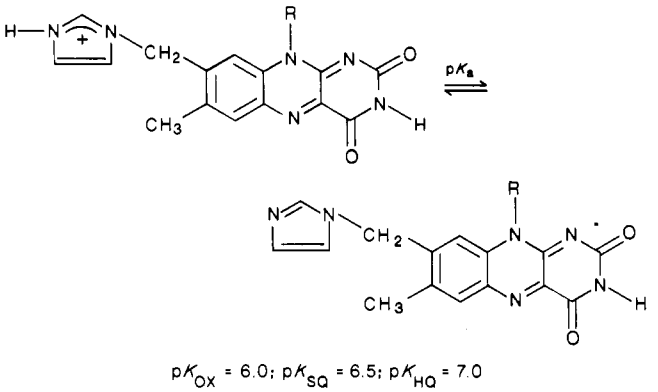
to cyt *c*. Extensive information is available on the redox potential and pK_a values of 8α-*N*-imidazolylflavin (Williamson & Edmondson, 1985a,b) and on the effect of 8α-substitution on the pK_a of the neutral flavin semiquinone (Edmondson et al., 1977). The tight binding of 8α-substituted FMN analogues to apoflavodoxin has been shown (Oestreicher et al., 1976), and the resulting reconstituted 8α-substituted FMN–apoflavodoxin complexes can be readily reduced to form near stoichiometric amounts of neutral semiquinone either by chemical reduction with sodium dithionite or photochemically with the EDTA–deazaflavin system (Massey & Hemmerich, 1978).

Previous electrochemical and NMR data (Williamson & Edmondson, 1985a,b) have shown that the pK_a of the 8α-imidazole moiety is sensitive to the level of flavin reduction, with all pK_a values occurring in the physiological range



$pK_{OX} = 6.0$; $pK_{SQ} = 6.5$; $pK_{HQ} = 7.0$

The functional significance of the state of protonation of the 8α-imidazole group in electron-transfer reactions is presently unknown.

Unpublished ^1H and ^{13}C NMR data from this laboratory suggest that little or no spin density resides in the imidazole ring of the semiquinones of free 8α-*N*-imidazolylflavin analogues. This could be interpreted as evidence against the direct participation of this group in electron transfer from the semiquinone to an acceptor. If this were also the case for an 8α-imidazolylflavin bound to apoflavodoxin, it would be predicted that the presence of such a bulky group attached to the flavin ring would have a large negative effect on the interaction of flavodoxin with cyt *c* in the complex formation step, inasmuch as the computer model for the electron-transfer complex indicates a close fit in the interface between these two redox proteins (Simondsen et al., 1982; Hazzard et al., 1986).

In order to further delineate these effects, we have investigated the interactions of 8α-*N*-imidazolyl-FMN (8-IMF) complexes of the apoflavodoxins from *Clostridium pasteurianum* and from *Azotobacter vinelandii* with cyt *c*. The *Azotobacter* flavodoxin has previously been shown to be less reactive than the *C. pasteurianum* flavodoxin in electron transfer to cyt *c* (Cheddar et al., 1986). This was interpreted as being due to differences in accessibility of the FMN moieties in these two flavodoxins. It is of interest to determine if the presence of an extended 8-position functionality can alter this property. The results of the present study have shown that (a) the protonation state of the 8α-imidazole moiety in the analogue flavodoxin has a large effect on the kinetic properties of the electron-transfer reaction, whereas the pH of the medium has no influence on the rates of electron transfer from the normal FMN flavodoxin semiquinone, and (b) the presence of an 8α-imidazole moiety markedly reduces the reactivity of the flavin semiquinone of both flavodoxins toward cyt *c*. These results will be discussed in terms of our current knowledge of the factors involved in the determination of electron-transfer rates in this system, and from the point of view of the possible role of 8α-imidazole substituents in one-electron-transfer reactions for those redox enzymes containing an 8α-*N*-histidylflavin coenzyme.

Experimental Procedures

Materials. Clostridium pasteurianum cultures (strain W5 ATCC6013) were purchased from the American Type Culture Collection. *Azotobacter vinelandii* (strain OP) was a gift from Dr. D. Yoch, University of California, Berkeley.

Azotobacter and *Clostridium* flavodoxins were purified according to published procedures (Hinkson & Bulen, 1967; Mayhew, 1971b), and the apoproteins were prepared by trichloroacetic acid precipitation as previously described by Edmondson and Tollin (1971). The published extinction coefficients were used in order to calculate the concentration of the two proteins and to judge their purity. Horse heart cytochrome *c* (type VI) was purchased from Sigma and used without any further purification. An extinction coefficient of 30000 M^{-1} cm^{-1} at the reduced α maximum (550 nm) was assumed to calculate the cytochrome concentration.

8α-*N*-Imidazolylriboflavin was synthesized as described by Williamson and Edmondson (1985a). Phosphorylation of this riboflavin analogue to the corresponding FMN level was carried out according to the procedure of Flexser and Farkas (1952). The 5′-phosphate isomers were then purified by preparative HPLC (R. De Francesco and D. E. Edmondson, manuscript in preparation). Commercial FMN (U.S. Biochemical Corp.) was also purified in this manner (Nielsen et al., 1986) for reconstitution experiments. 5-Deazariboflavin was synthesized as described by O'Brien et al. (1970).

Different buffers were used to carry out the kinetic experiments at the various pH values: sodium acetate at pH 5.5; MES/KOH at pH 6.0 and 6.6; HEPES/KOH at pH 7.25 and 8.25; and TAPS/KOH at pH 8.75. The buffer concentration was 5 mM in all cases, and the ionic strength was then adjusted with KCl (45 or 90 mM). EDTA, needed to reduce the photoinduced triplet state of deazariboflavin to the corresponding semiquinone, was included in all buffers at a concentration of 1 mM.

Methods. Samples were made anaerobic by several cycles of alternate degassing under vacuum and flushing with argon that had been previously purged through a BASF catalyst at 140 °C (Williams et al., 1979). Flavodoxin semiquinone was generated by photoreduction in the presence of 5-deazariboflavin and EDTA (Massey & Hemmerich, 1978). Samples were then anaerobically transferred to the stopped-flow spectrophotometer by means of tonometers equipped with three-way stopcocks. The stopped-flow apparatus used was from Kinetic Instruments, Inc., Ann Arbor, MI. Data were collected with a Nicolet Model 4094 digital oscilloscope and analyzed on an IBM XT personal computer system using the MEDAS decay analysis program (EMF Software Inc., Knoxville, TN) by fitting to a single-exponential curve. In some experiments a small amount of a more slowly decaying transient was observed as in previous work (Simondsen et al., 1982). This was not included in the data-fitting procedure.

Electron transfer from flavodoxin semiquinone to oxidized cytochrome *c* can be conveniently monitored at 580 nm, a wavelength at which an absorbance decrease is observed as a consequence of both semiquinone oxidation and cytochrome reduction. Control experiments at the isosbestic wavelengths for the flavodoxin oxidized/semiquinone transformations, which monitor only cytochrome reduction, gave kinetic traces identical with those obtained at 580 nm.

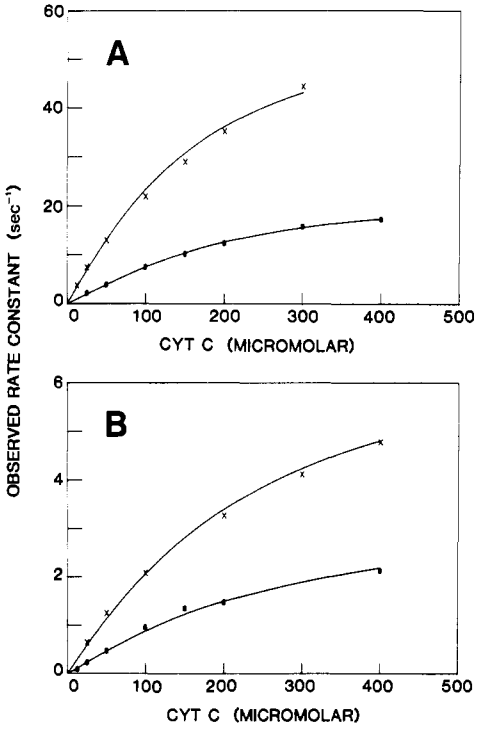


FIGURE 1: Plots of observed pseudo-first-order rate constant for *Azotobacter* flavodoxin semiquinone oxidation vs. cytochrome *c* concentration at pH 8.25 in 5 mM HEPES buffer: (A) native flavodoxin in solutions containing 45 (×) and 90 mM (●) KCl; (B) 8-IMF flavodoxin in solutions containing 45 (×) and 90 mM (●) KCl. Curves are theoretical plots using kinetic constants given in Table I.

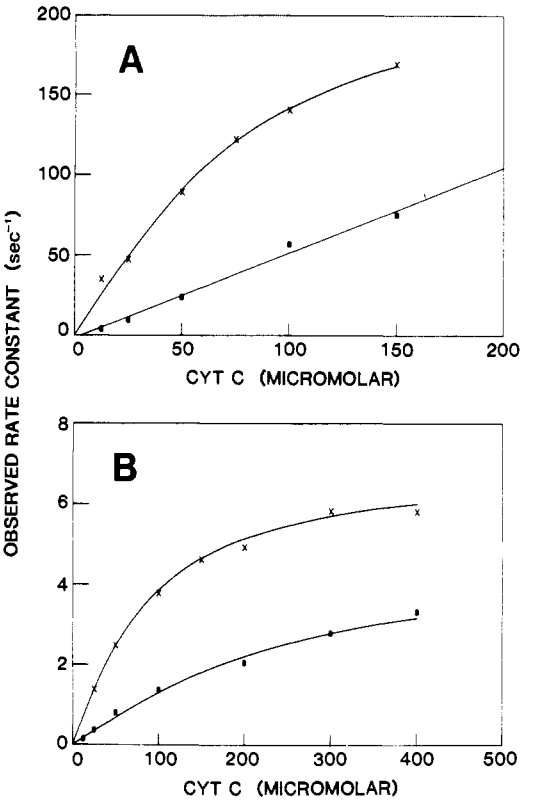


FIGURE 2: Plots of observed pseudo-first-order rate constants for *Clostridium* flavodoxin semiquinone oxidation vs. cytochrome *c* concentration in 5 mM HEPES, pH 8.25: (A) native flavodoxin in solutions containing 45 (×) and 90 mM (●) KCl; (B) 8-IMF flavodoxin in solutions containing 45 (×) and 90 mM (●) KCl. The monitoring wavelength was 580 nm. Curves are theoretical plots using kinetic constants given in Table I.

RESULTS

Factors Influencing the Kinetic Properties of Reconstituted FMN Analogue–Apoflavodoxin Semiquinones. During the course of experiments on reconstitution of *Azotobacter* flavodoxin with 8-IMF, we observed that biphasic kinetics were obtained for cyt *c* oxidation of the semiquinone when apoprotein which had been stored at 4 °C for more than 1 day was used. A similar result was also found with ordinary FMN when it was used for reconstitution. The fast component was completely absent when freshly prepared apoprotein was used for reconstitution. Furthermore, the ratio of fast to slow phases increased with increasing storage time of the apoprotein, with no change in the observed rate constants of the two components. We also found that the pH and cytochrome concentration dependencies observed for this fast component were parallel to those obtained for the slow phase with the freshly reconstituted 8-IMF flavodoxin (see below). These results strongly support the existence of two forms of the reconstituted flavodoxin, both of which generate semiquinones that react with cyt *c* by the same mechanism but which do so with quite different rate constants (the apparent second-order rate constant of the fast component was approximately 50 times larger than that of the slow component for both FMN and 8-IMF reconstituted flavodoxins). It is important to point out that storage of native or reconstituted flavodoxins (prepared from freshly prepared apoflavodoxin) at −20 °C for several weeks resulted in little or no alterations in kinetic properties and that reconstitution or fresh apoprotein with FMN produced holoprotein with kinetic properties identical with those of native flavodoxin. Whatever the changes are that occur upon storage, the process apparently proceeds more readily with the apoprotein than with either the native or reconstituted forms. It is also important to note that *no obvious* differences exist between flavodoxins reconstituted from fresh or aged apoproteins with regard to absorption spectral properties, FMN binding, or the ability to generate stable semiquinone forms upon photoreduction with 5-deazaflavin. No attempts were made to see if a similar behavior is exhibited with *Clostridium* flavodoxin since the apoprotein is inherently less stable. Rates of reaction with FMN-reconstituted *Clostridium* flavodoxin were identical with those observed with the native protein. It is clear from these results that subtle alterations, which are not detected by normal spectral properties but which can produce large changes in kinetic properties, can occur upon storage of apoflavodoxins prior to reconstitution. Thus, careful control of apoprotein preparation and reconstitution with flavin analogues is required, even for relatively stable flavoproteins like the flavodoxins.

Effect of Cytochrome c Concentration on Kinetics of Flavodoxin Semiquinone Oxidation. Plots of k_{obsd} vs. cyt *c* concentration obtained at two ionic strengths at pH 8.25 for the oxidation of native and 8-IMF *Azotobacter* flavodoxins are shown in Figure 1. Similar data for the *Clostridium pasteurianum* flavodoxin systems are shown in Figure 2. Several conclusions can be reached from these data. For both the native and 8-IMF flavodoxins, the k_{obsd} values are smaller at the higher ionic strength. This is consistent with earlier results with the native proteins (Simondsen et al., 1982; Cheddar et al., 1986) and demonstrates that 8α-substitution of the FMN does not substantially alter the electrostatic interaction between flavodoxin and cytochrome *c*. An analogous result was obtained upon chlorine substitution at the 7 and 8 positions of the FMN cofactor (Simondsen & Tollin, 1983). Also consistent with earlier results (Cheddar et al., 1986) is the observation that the rate constants for electron transfer to cyt *c* are faster for *Clostridium* flavodoxin than for *Azo-*

Table I: Values of Kinetic Constants Obtained by Computer Analyses of Stopped-Flow Data for Electron-Transfer Reactions between Native and 8α-Imidazolyl-FMN Flavodoxins and Cytochrome *c* at pH 8.25

flavodoxin	ionic strength (mM)	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	k_2 (s^{-1})	K_a (M^{-1})
Azotobacter, native	45	5.1×10^5	51	62	1×10^4
	90	1.2×10^5	13	24	9.2×10^3
Azotobacter, 8-IMF	45	5.1×10^4	8	7.2	6.4×10^3
	90	1.8×10^4	2.9	3.4	6.2×10^3
Clostridium, native	45	3.3×10^6	145	236	2.3×10^4
	90[a]	5.1×10^5			
Clostridium, 8-IMF	45	1.2×10^5	7	7	1.7×10^4
	90	2.4×10^4	3.2	4.6	7.5×10^3

[a] Nonlinear concentration dependence was not observed for this system.

tobacter flavodoxin. It is interesting, however, that this is less so for the 8-IMF analogues than for the native proteins, which suggests that for these systems the kinetic properties are being dominated by the 8α-substituent rather than by differences in the surface topography of the two flavodoxins.

The data of Figures 1 and 2 show clearly that nonlinear concentration dependences are observed with both the native and 8-IMF flavodoxins. Such behavior was previously (Simondsen et al., 1982) ascribed to intermediate complex formation, according to the mechanism of eq 1 in the introduction. The present results indicate that 8α-substitution probably does not alter the mechanistic pathway along which the electron-transfer reaction proceeds. However, it is immediately apparent from the k_{obsd} values that the 8-IMF flavodoxin semiquinone reactions proceed much more slowly than do those of the native flavodoxins, by factors of 10–30. That this was not due to the reconstitution procedure was shown by control experiments in which ordinary FMN was added to *freshly prepared* apoflavodoxins. The resulting holoproteins gave kinetic results identical with those obtained with the native *Azotobacter* and *Clostridium* flavodoxins. The observed differences in rate also cannot be attributed solely to alterations in redox potential, since the oxidized/semiquinone couple of 8-IMF *Azotobacter* flavodoxin has been determined to be only ~40 mV more positive than that of the native form at pH 8.0 (D. E. Edmondson, unpublished observations). On the basis of previous results (Cheddar et al., 1986) this would be expected to contribute no more than a factor of 2 to the rate-constant decrease.

Further insight into these kinetic effects can be obtained by computer analyses of the data in terms of eq 1 [cf. Ahmad et al. (1982) and Simondsen et al. (1982) for a discussion of the fitting procedure used]. This permits values for k_1, k_{-1}, k_2, and K_a to be estimated. The results of such analyses are given in Table I, and the solid lines in Figures 1 and 2 correspond to curves calculated from these values of the kinetic constants. It is apparent that the agreement between the simulated curves and the experimental data is quite satisfactory.

The results shown in Table I indicate that, for both flavodoxins, the values of k_1 and k_2 are decreased by 1 order of magnitude or more upon 8α-imidazole substitution. The k_1 value reflects the rate of the bimolecular complex formation step, whereas the k_2 value is a composite measure of the rates of processes associated with the electron-transfer step. We can conclude that both of these aspects of the reaction mechanism are slowed down by the structural modification. On the other hand, the K_a values are approximately unaffected by 8α-substitution. This is a consequence of the occurrence

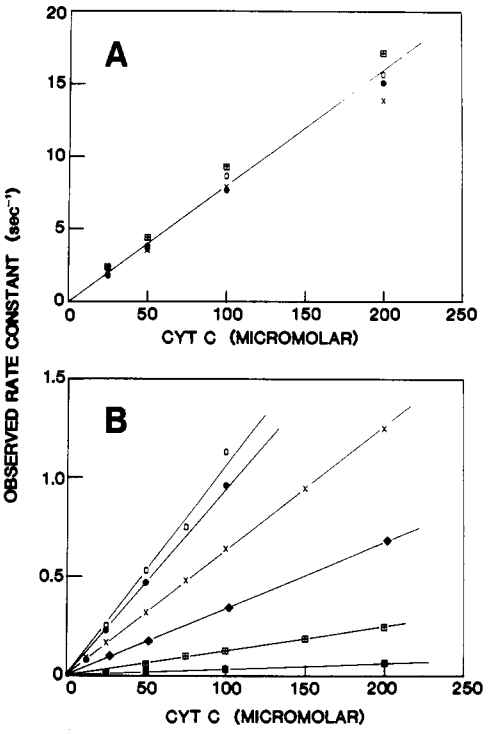


FIGURE 3: Plots of observed pseudo-first-order rate constants for *Azotobacter* flavodoxin semiquinone oxidation vs. cytochrome *c* concentration at various pH values. All solutions contained 90 mM KCl; buffer concentration was 5 mM. (A) Native flavodoxin: (×) pH 5.8, MES buffer; (O) pH 7.3, HEPES buffer; (⊞) pH 7.3, phosphate buffer; (●) pH 8.25, HEPES buffer. (B) 8-IMF flavodoxin: (■) pH 5.5, acetate buffer; (⊞) pH 6.0, MES buffer; (◆) pH 6.6, MES buffer; (×) pH 7.25, HEPES buffer; (●) pH 8.25, HEPES buffer; (O) pH 8.7, TAPS buffer.

of compensating changes in the k_{-1} values. Thus, although the second-order complex formation process is slower for the 8-IMF flavodoxins, once formed, the complexes dissociate more slowly than do the native flavodoxins.

Effect of pH on the Rate of Electron Transfer. Previous electrochemical data on the model compound 8α-*N*-imidazolylriboflavin (Williamson & Edmondson, 1985a) have shown that the pK_a of the 8α-imidazole substituent is dependent on the redox level of the flavin ring. Thus, it was of interest to determine whether the state of protonation of the 8α-imidazole ring would influence the rate of electron transfer to oxidized cytochrome. It is known that the oxidation–reduction potential of cyt *c* is independent of pH in the 5–8 range (Moore et al., 1984), whereas the potential of the oxidized/semiquinone couple of flavodoxin becomes more positive by approximately 60 mV/(pH unit) as the pH decreases (Mayhew, 1971a). Thus, the thermodynamic driving force of the reaction should increase with increasing pH. According to the Marcus theory of electron transfer (Marcus, 1960, 1964) and to our previous results with these and similar systems (Meyer et al., 1983), an increase in rate constant should be observed with increasing pH values if the thermodynamic equilibrium with respect to protons and the kinetics of electron transfer are coupled together.

The results of the pH dependence (at constant ionic strength) for the kinetic rates of native *Clostridium* and *Azotobacter* flavodoxin semiquinone oxidation by cyt *c* are given in Figures 3A and 4A. Also shown is a comparison between phosphate and HEPES buffers at pH 7.3, which suggests that there are no specific buffer ion effects occurring in these reactions. For both flavodoxins, the observed rate constants for electron transfer are independent of pH over the

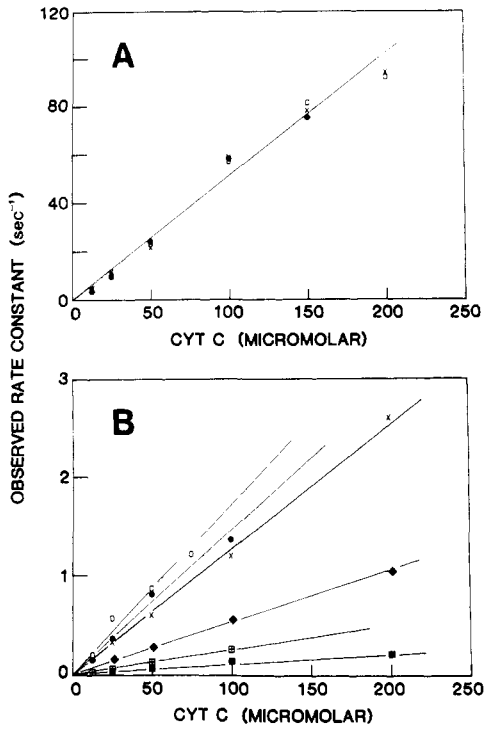


FIGURE 4: Plots of observed pseudo-first-order rate constants for *Clostridium* flavodoxin semiquinone oxidation vs. cytochrome *c* concentration at various pH values. All solutions contained 90 mM KCl; buffer concentration was 5 mM. (A) Native flavodoxin: (O) pH 6.07, MES buffer; (×) pH 7.25, HEPES buffer; (●) pH 8.25, HEPES buffer. (B) 8-IMF flavodoxin: Symbols as in Figure 2B.

range 5.8–8.25. These data constitute strong evidence that the electron-transfer step is not coupled to proton release from the neutral flavin semiquinone during $1e^-$ oxidation; i.e., proton release proceeds much more rapidly than electron transfer (see below for further comment). van Leeuwen et al. (1983) have observed that the rate of *Megasphaera elsdenii* flavodoxin reduction to the semiquinone by the radical form of methylviologen is pH independent between pH 7.0 and 9.2. This observation is also consistent with a kinetic "uncoupling" of electron transfer and protonation of the flavin; i.e., the rate of protonation is much faster than the rate of one-electron reduction.

In contrast to the native flavodoxins, the kinetic rates of cyt *c* reduction by 8-IMF flavodoxin semiquinones show a very strong pH dependence (Figures 3B and 4B). With both substituted *Azotobacter* and *Clostridium* flavodoxins, a substantial increase in the apparent second-order rate was observed as the pH was increased in the range 5.5–8.7 (at constant ionic strength). This could be a consequence of a change in the reaction mechanism with regard to either the proton-release step or the protonation of the imidazole ring, resulting in a less favorable reaction. Unpublished work from this laboratory (R. De Francesco and D. E. Edmondson, manuscript in preparation) has shown that the pK_a of the 8α-imidazole moiety of 8-IMF bound to *Azotobacter* apoflavodoxin (oxidized form) is 6.7, and for the modified *Clostridium* flavodoxin (also in the oxidized form) the pK_a is 6.9. These values are 0.7–0.9 of a unit higher than that of the free oxidized 8α-imidazolylriboflavin ($pK_a = 6.0 \pm 0.1$) (Williamson & Edmondson, 1985a,b). Comparison of the pH dependence for the rate of 8-IMF flavodoxin semiquinone oxidation (Figure 5) with theoretical curves for a single proton ionization shows 8-IMF *Azotobacter* flavodoxin semiquinone to have a pK_a of 6.98 and 8-IMF *Clostridium* flavodoxin semiquinone to have a pK_a of 7.02. These values are slightly higher than

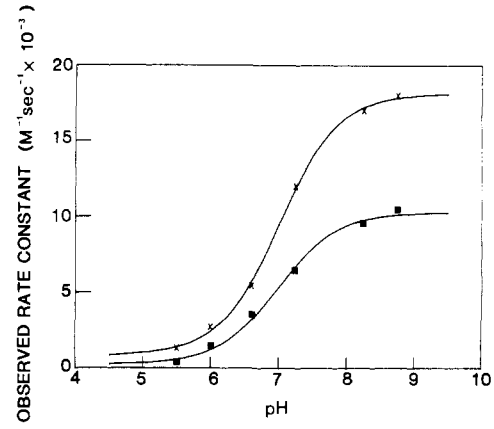


FIGURE 5: Plots of second-order rate constants for 8-IMF flavodoxin semiquinone oxidation vs. pH. (■) *Azotobacter* flavodoxin. Solid line is theoretical curve for a one-proton ionization using the following parameters: $pK_a = 6.98$; k (unprotonated) = 10.27×10^3 M^{-1} s^{-1}; k (protonated) = 0.22×10^3 M^{-1} s^{-1}. (×) *Clostridium* flavodoxin. Solid line is theoretical curve for a one-proton ionization using the following parameters: $pK_a = 7.02$; k (unprotonated) = 18.17×10^3 M^{-1} s^{-1}; k (protonated) = $0.91 \times 10^3 M^{-1}$ s^{-1}.

those found for the imidazole moiety of the oxidized forms (0.1–0.3 pH unit), as is also observed for the free 8α-*N*-imidazolylriboflavin ($pK_a = 6.5$ for the semiquinone form) (Williamson & Edmondson, 1985a). We conclude from this that the pH dependence is most probably due to the protonation of the imidazole substituent in the 8-IMF flavodoxin semiquinone, which results in a less favorable interaction between the flavodoxin and the cytochrome. From the limiting values obtained by the fitting procedure (Figure 5), this amounts to a factor of 47 for *Azotobacter* flavodoxin and to a factor of 20 for *Clostridium* flavodoxin. It is worth pointing out that this conclusion implies that the value of the apparent second-order rate constant (obtained from the slopes of the lines given in Figures 3B and 4B) at a given pH is dependent upon the concentration of the unprotonated imidazole moiety of 8-IMF. This would be the case for the mechanism shown in eq 1 above if the rapid preequilibrium condition applied, i.e., if the intermediate complex formation equilibrium is established rapidly compared with the electron-transfer step. In this situation, the apparent second-order rate constant would be equal to the product of K_a and k_2.

DISCUSSION

The results presented above demonstrate that placing the relatively bulky imidazole ring at the 8α-position of the FMN cofactor of flavodoxin results in large effects on the rate of the electron-transfer reaction from the semiquinone to cyt *c*. This is consistent with the structural model of the electron-transfer complex for this system (Simondsen et al., 1982), which postulates a close steric fit between the exposed dimethylbenzene ring of the FMN and the heme edge of the cytochrome (Hazzard et al., 1986). It is also clear from our results that the imidazole ring is apparently unable to form an effective bridge between the flavin and the heme which can facilitate the electron-transfer event. The fact that the k_2 values are appreciably diminished could be a result of an increase in the effective distance over which electron transfer must occur within the intermediate complex, or of an increase in the number of nonproductive complexes which can occur during collisional interactions between the reactants, or both. Alternatively, the geometry of the 8α-imidazole ring relative to the bound flavin isoalloxazine ring could be constrained on complex formation in such a way that the π-orbitals of the imidazole ring do not have much overlap with those of the

flavin. It remains to be experimentally determined if the binding of 8-IMF to apoflavodoxin confers any restriction of motion upon the 8α-imidazole ring and if complex formation with cyt *c* results in a further restriction. In principle, this could be investigated by NMR methods. Consideration of the complex model based on the X-ray structures of the two proteins (Hazzard et al., 1986) would predict this to be a reasonable possibility.

The lowered reactivity of 8α-imidazolylflavin in this system suggests that the 8α-substituent may not play a direct role in the facilitation of one-electron transfer in those enzymes containing 8α-*N*-histidylflavin coenzymes. It should be kept in mind, however, that the antibonding π-orbital of the imidazole ring lies at a rather high energy level (i.e., the π,π*-absorption of this chromophore lies at a higher energy than does, for example, that of the phenolic ring), and this may be unfavorable to participation in electron transfer. Thus, it would be of interest to carry out experiments similar to those presented above using an 8α-phenolic substitution of the FMN cofactor as a model for the flavocytochrome, *p*-cresol methylhydroxylase, which contains two 8α-*O*-tyrosyl-FAD coenzymes and two *c*-type hemes (McIntire et al., 1981). Furthermore, an 8α-thiol ether substituent on the FMN may be able to utilize sulfur d orbitals for electron transfer, and it would thus be of interest to test this as well as a model for those flavocytochromes containing 8α-*S*-cysteinylflavins (e.g., *Chromatium* cytochrome c_{552} and *Chlorobium* cytochrome c_{553}). In fact, preliminary evidence that the latter is so has already been published (Przysiecki et al., 1985).

The rather large effect of protonation of the imidazole ring on the kinetics of the flavodoxin–cytochrome reaction is also readily interpreted in terms of the structural model of the intermediate complex. Thus, in the model, the 8-methyl group of the FMN points directly toward the rather nonpolar heme edge, and one would anticipate an energetically unfavorable interaction between a positively charged group and this region of the cytochrome. Furthermore, it has been suggested (Salemme, 1978; Matthew et al., 1983) that water is excluded from the interface region in complexes of the kind we are concerned with here. This would also act to disfavor complex formation involving the protonated imidazole ring.

It is interesting that, although the rate constant for the complex formation step is appreciably diminished by 8α-imidazole substitution, the complex stability constant is much less affected. This is most likely a consequence of interactions between the imidazole ring and the cytochrome surface, which occur subsequent to complex formation and which act to slow down the rate of complex dissociation relative to the native protein. The hydration potential for the unprotonated imidazole ring is ~−10.3 kcal/mol (Wolfenden et al., 1981), and thus one might expect to see an appreciable lowering of the K_a of complex formation if total desolvation of the imidazole ring occurred. The observation of no major differences in binding suggests that the imidazole ring is at most only partially desolvated in the complex. These arguments suggest that there may be significant differences between the complexes formed by the native and 8-IMF flavodoxins with cytochrome *c*. Circular dichroism and NMR data (Tollin et al., 1987) on the complexes of cytochrome *c* with native and 8-IMF *Clostridium* flavodoxin are consistent with this interpretation.

Finally, the results support and extend the previous pulse radiolysis work (van Leeuwen et al., 1983) and demonstrate that proton release during the redox reaction of flavodoxin semiquinone and a one-electron acceptor proceeds at a much faster rate than the processes associated with the electron-transfer event (the latter would include any bond distance or bond angle changes or any changes in solvation that occur during the formation of the transition state). The pH independence of the reaction rate constant implies that the difference in flavodoxin semiquinone redox potential between low and high pH does not affect the reaction kinetics, even though it has previously been shown (Meyer et al., 1983; Cheddar et al., 1986; Tollin et al., 1986) that, at a given pH, redox potential differences do exert a measurable influence on the apparent second-order rate constants for electron transfer. This can be readily understood if, during the redox interaction between flavodoxin semiquinone and cytochrome *c*, the electron-transfer event occurs first at all pH values and is followed by rapid proton release to the solvent.

In conclusion, the results presented here demonstrate that it is possible to obtain useful and interesting insights into the electron-transfer mechanisms utilized by redox proteins by investigation of the flavodoxin–cytochrome *c* model system. Furthermore, they also provide the experimental groundwork for more detailed structural and dynamic studies of the 8α-imidazole ring in 8-IMF flavodoxin complexes and for the study of other 8α-substituents, which should provide further insights into the role of these moieties in electron-transfer reactions. Efforts along these lines are currently in progress.

Registry No. cyt *c*, 9007-43-6; FMN, 146-17-8.

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Flavodoxin–Cytochrome *c* Interactions: Circular Dichroism and Nuclear Magnetic Resonance Studies†

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ABSTRACT: Circular dichroism and ^1H and ^{31}P nuclear magnetic resonance spectroscopy have been used to investigate complex formation between cytochrome *c* and the flavodoxins from *Azotobacter vinelandii* and *Clostridium pasteurianum*. Such complexes are known to be involved in the mechanism of electron transfer between these two redox proteins. A large increase in ellipticity in the Soret band of the cytochrome heme was observed upon formation of the *Clostridium* flavodoxin complex, whereas much smaller changes were found for the complexes with either *Azotobacter* flavodoxin or an 8α-imidazolyl-FMN-substituted *Clostridium* flavodoxin analogue. Similarly, the magnitudes of the perturbations of the contact-shifted heme proton resonances obtained upon complexation of cytochrome *c* by *Azotobacter* flavodoxin were much smaller than those previously shown for *Clostridium* flavodoxin [Hazzard, J. T., & Tollin, G. (1985) *Biochem. Biophys. Res. Commun. 130*, 1281-1286]. ^{31}P nuclear magnetic resonance measurements were also consistent with differences in the interactions between the components in the complexes of the two flavodoxins with cytochrome *c*. It is suggested that these spectral changes are due to a loosening or opening of the heme crevice upon *Clostridium* flavodoxin binding, which allows closer contact between the heme and flavin prosthetic groups and results in a faster rate of electron transfer. The implications of these observations for biological oxidation–reduction processes are considered.

The electron-transfer reactions from flavodoxin semiquinones to cytochrome *c* have been extensively studied as a model to probe factors that are important in the determination of electron-transfer rates in redox proteins (Simondsen et al., 1982; Simondsen & Tollin, 1983; Tollin et al., 1984; Cheddar et al., 1986; De Francesco et al., 1987). The observation of saturation kinetics at high cytochrome *c* concentrations and the marked influence of ionic strength on reaction rates have demonstrated the involvement of electrostatistically stabilized complexes of the two proteins in the electron-transfer mechanism. Complex formation between *Clostridium pasteurianum* flavodoxin and horse heart cytochrome *c* has also been observed by NMR spectroscopy (Hazzard & Tollin, 1985). Electrostatic free energy calculations (Weber & Tollin, 1985), based on a computer-generated model of the structure of the complex (Simondsen et al., 1982) in which the exposed redox centers are in van der Waals contact, have shown that the ionic strength dependence of the kinetic rate constants for the electron-transfer reaction is dominated by those charged amino acid side chains which are localized predominantly in the vicinity of the FMN and heme prosthetic groups (lysine residues in the case of cytochrome *c* and aspartate and glutamate residues in the case of flavodoxin).

A previous comparative study (Cheddar et al., 1986) of *Clostridium pasteurianum* and *Azotobacter vinelandii* flavodoxins has shown the latter to be considerably less reactive than the former, despite the significantly lower redox potential for the oxidized/semiquinone couple of the *Azotobacter* protein, which would provide a larger thermodynamic driving force resulting in an increased rate of electron transfer. Cross-linking studies have shown that *Azotobacter* flavodoxin forms a tight 1:1 complex with cytochrome *c*, with an estimated association constant of 4×10^4 M^{-1} at 88 mM ionic strength (Dickerson et al., 1985), which compares favorably with the kinetically determined value for cytochrome *c* and *C. pasteurianum* flavodoxin semiquinone (18×10^4 M^{-1})

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