Kinetics of Flavin Semiquinone Reduction of the Components of the Cytochrome *c*–Cytochrome b_5 Complex†

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ABSTRACT: The kinetics of flavin semiquinone reduction of the components of the 1:1 complex formed by cytochrome *c* with either cytochrome b_5 or a derivative of cytochrome b_5 in which the heme propionates are esterified (DME-cytochrome b_5) have been studied. The rate constant for the reduction of horse heart cytochrome *c* by the electrostatically neutral lumiflavin semiquinone (LfH·) is unaffected by complexation with native cytochrome b_5 at pH 7. However, complex formation with DME-cytochrome b_5 (pH 7) decreases by 35% the rate constant for cytochrome *c* reduction by LfH·. At pH 8, complex formation with native cytochrome b_5 decreases the rate constant for cytochrome *c* reduction by LfH· markedly, whereas the rate constant for cytochrome *c* reduction, either unbound or in the complex formed with DME-cytochrome b_5, is increased 2-fold relative to pH 7. These results indicate that the accessibility of the cytochrome *c* heme is not the same in the complexes formed with the two cytochrome b_5 derivatives and that the docking geometry of the complex formed by the two native cytochromes is pH dependent. Binding of horse heart and tuna cytochromes *c* to native and DME-cytochromes b_5 decreases the rate constants for reduction of cytochrome *c* by the negatively charged flavin mononucleotide semiquinone (FMNH·) by ~30% and ~40%, respectively. This finding is attributed to substantial neutralization of the positive electrostatic potential surface of cytochrome *c* that occurs when it binds to either form of cytochrome b_5. The rate constants for reduction of both native and DME-cytochromes b_5 by LfH· at pH 7 and 8 increase when these proteins bind to horse heart ferricytochrome *c*. This observation suggests that cytochrome b_5 undergoes either an increase in its reduction potential or a conformational change on binding to cytochrome *c*. At both pH values, DME-cytochrome b_5 reduces more rapidly than the native protein, as expected from its higher reduction potential. FMNH· reduction of ferricytochrome b_5 (pH 7) is appreciably faster in the presence of ferrocytochrome *c*, consistent with a decrease in electrostatic repulsion between the reactants arising from the surface charge neutralization that accompanies cytochrome b_5–cytochrome *c* complex formation. Pseudo-first-order rate constants for the FMNH· reduction of cytochrome b_5 bound to cytochrome *c* display a nonlinear concentration dependence. This effect is more pronounced for tuna cytochrome *c* than for horse cytochrome *c* and is interpreted in terms of either a rate-determining structural isomerization within the complex preceding the second-order electron-transfer event or the formation of a ternary complex followed by first-order intracomplex electron transfer. These kinetic results are consistent with the steric and electrostatic features of the hypothetical models for the complexes formed between native and DME-cytochromes b_5 and cytochrome *c* proposed previously [Salemme, F. R. (1976) *J. Mol. Biol. 102*, 563; Mauk, M. R., Mauk, A. G., Weber, P. C., & Matthew J. B. (1986) *Biochemistry 25*, 7085–7091].

Detailed understanding of the factors that govern interaction and subsequent electron transfer between metallo- and flavoproteins is critical to a rigorous characterization of a wide range of biological oxidation–reduction processes. In recent years, increased emphasis has been placed on analysis of the structural and functional properties of complexes that form between pairs of metallo- and flavoproteins of known three-dimensional structure, in order to eliminate as much uncertainty as possible concerning the nature of the interacting species. In particular, computer graphics modeling has been used to develop working models for the structures of the relatively stable complexes that are now known to form in solution between several pairs of heme proteins [e.g., see Salemme (1976), Poulos and Kraut (1980), Simondsen et al. (1982), and Poulos and Mauk (1983)]. Common characteristics of these model complexes include electrostatic stabilization of protein–protein interactions, minimization of donor–acceptor site separation, and (in most cases) coplanar orientation of prosthetic groups to facilitate π-orbital overlap.

The cytochrome b_5–cytochrome *c* complex is the historical prototype for these computer graphics based modeling studies. Following the original model proposed by Salemme (1976), the interaction between these two proteins has been studied through the use of specifically modified forms of cytochrome *c* (Ng et al., 1977; Stonehuerner et al., 1979), electronic difference spectroscopy (Mauk et al., 1982), NMR spectroscopy (Eley & Moore, 1983; Hartshorn et al., 1987), and fluorescence quenching and energy-transfer measurements (Kornblatt et al., submitted for publication; L. Eltis and A. G. Mauk, unpublished results). Electrostatic calculations combined with equilibrium binding measurements involving a chemically modified derivative of cytochrome b_5 have suggested that the docking geometry for the cytochrome *c*–cytochrome b_5 complex may vary with solution conditions and with the availability of the cytochrome b_5 heme propionate groups (Mauk et al., 1986). Recent molecular dynamics

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calculations indicate that the donor–acceptor site separation distance may be overestimated from static modeling studies and that movement of specific amino acid side chains within the protein–protein interaction domain of this complex may be important in facilitating electron transfer from one heme center to the other (Wendoloski et al., 1987). We note, however, that these calculations were performed on a time scale several orders of magnitude faster than the rate-limiting steps in the electron-transfer reaction.

One experimental approach that has provided useful information about the functional properties of several electron-transfer complexes in solution involves kinetic analysis of the reduction of the individual components of the complexes by exogenous, photolytically generated flavin semiquinones (Bhattacharyya et al., 1986, 1987; Hazzard et al., 1986, 1987). Through judicious selection of the flavins used, it has been possible to obtain information concerning the rate of electron transfer to and within such complexes that have been interpreted in terms of prosthetic group accessibility, electrostatic environments near prosthetic groups, and the occurrence of dynamic motions within electron-transfer complexes. In the present study, we have used this approach to investigate the reduction kinetics of cytochrome b_5 and cytochrome c both individually and together, under conditions that the two proteins are known to form a stable 1:1 complex, in order to determine the effect of complex formation on their electron-transfer properties. We have also investigated the influence of the cytochrome b_5 heme propionate groups on the functional properties of both proteins within their 1:1 complex through parallel studies involving a derivative of cytochrome b_5 in which the heme propionate groups are esterified (DME-cytochrome b_5).[1] The results of these studies are discussed in terms of the hypothetical complexes proposed by Salemme (1976) and Mauk et al. (1986).

Experimental Procedures

The tryptic fragment of bovine cytochrome b_5 and its dimethyl ester heme-substituted derivative were prepared as described previously (Reid & Mauk, 1982; Reid et al., 1984). Horse heart cytochrome c (type VI) and tuna cytochrome c (type XI) were purchased from Sigma and purified by ion-exchange chromatography (Brautigan et al., 1978). Preparation of lumiflavin and purification of FMN were performed as described previously (Simondsen & Tollin, 1983).

Laser flash photolysis reduction studies were carried out according to the procedures given in Hazzard et al. (1986, 1987). Reduction of cytochrome c in the presence of ferricytochrome b_5 was monitored as a decrease in absorption at 565 nm, an isosbestic point in the cytochrome b_5 oxidation–reduction difference spectrum. No evidence for cytochrome b_5 reduction prior to or coincident with cytochrome c reduction was observed; i.e., cytochrome c was preferentially reduced under these conditions. In experiments involving the reduction of ferricytochrome b_5 bound to ferrocytochrome c, the cytochrome c was photoreduced by steady-state illumination of the flavin in the reaction mixture prior to the anaerobic addition of ferricytochrome b_5, and the reaction was monitored as a decrease in absorption at 580 nm. Kinetic experiments were performed in phosphate buffers at pH 7.0 and 8.0 in the presence of 0.5 mM EDTA [ionic strength (I) = 4 mM]. Under these conditions, we estimate the association constant for complexes involving the two cytochromes b_5 and horse cytochrome c to be 10^6 M^{-1} (Mauk et al., 1982, 1986; L. Eltis and A. G. Mauk, unpublished experiments). Reactions were conducted under anaerobic conditions at 24 °C, and kinetic data were analyzed as described previously (Hazzard et al., 1986). We estimate that the uncertainty in the values of the rate constants reported is $<\pm 10\%$.

[1] Abbreviations: native cytochrome b_5 and DME-cytochrome b_5, solubilized tryptic fragment of cytochrome b_5 and the dimethyl-esterified heme derivative of cytochrome b_5, respectively; cyt, cytochrome; LfH· and FMNH·, semiquinone species of lumiflavin and flavin mononucleotide, respectively; $FMNH^-$, fully reduced species of flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid.

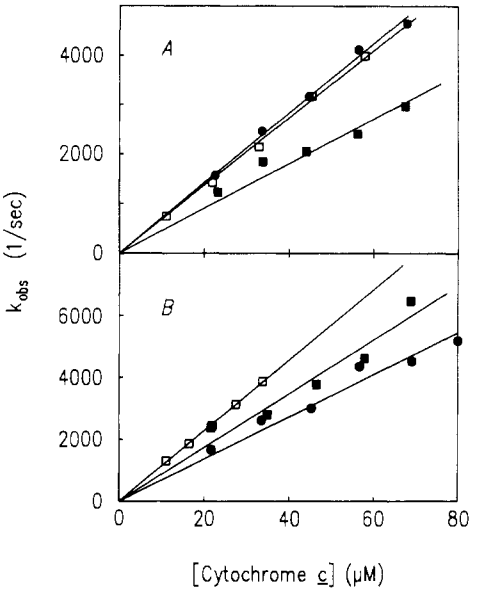

FIGURE 1: Plots of k_{obs} vs cytochrome c concentration for the reduction of horse cytochrome c by LfH· at pH 7 (A) and 8 (B) in the absence (□) and presence of native (●) or DME-cytochrome b_5 (■).

Table I: Second-Order Rate Constants for Flavin Semiquinone Reduction of Cytochrome c in the Presence and Absence of Cytochrome b_5

	Reduction by LfH·	
	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})	
	pH 7	pH 8
horse cytochrome c	6.6	11.0
+cytochrome b_5	7.0	6.7
+DME-cytochrome b_5	4.3	8.4

	Reduction by FMNH· at pH 7
	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})
horse cytochrome c	
free	14.7
+cytochrome b_5	4.4
+DME-cytochrome b_5	5.6
tuna cytochrome c	
free	12.5
+cytochrome b_5	3.2
+DME-cytochrome b_5	5.3

Results and Discussion

Reduction of Cytochrome c by LfH·. Pseudo-first-order rate constants for cytochrome c reduction by LfH· at pH 7 and 8 as a function of protein concentration in the presence and absence of stoichiometric amounts of native and DME-cytochrome b_5 are shown in Figure 1. The second-order rate constants obtained from these plots are given in Table I. At pH 7, the rate constant for cytochrome c reduction was unaffected by the presence of native cytochrome b_5, while it was decreased by 35% in the complex formed with DME-cytochrome b_5. These observations indicate that there is no change in accessibility of the cytochrome c heme when this protein binds to native cytochrome b_5 but that this heme ac-

cessibility is decreased in a kinetically detectable manner when cytochrome *c* binds to DME-cytochrome b_5. In other words, the stereochemical environments of the cytochrome *c* heme edge are not equivalent in the two complexes at pH 7. Presumably, this difference arises either from the increased steric bulk introduced by esterification of the heme propionates or from changes that this modification induces in the geometry of the complex formed by the two proteins. As LfH· is essentially uncharged at pH 7, this kinetic difference cannot be attributed to electrostatic effects.

Increasing the pH to 8 produces two types of kinetic effect. First, the rate constant of cytochrome *c* reduction by LfH· increases substantially. Similar results have been observed previously by Przysiecki et al. (1985) for several small electron-transfer proteins of varying net electrostatic charge and have been attributed to an increase in the inherent reactivity of the lumiflavin radical upon deprotonation to form an anionic species ($pK = 8.27$). Electrostatic effects that result from this deprotonation, if they occur at all, are relatively small (vide infra). Second, binding to native and DME-cytochrome b_5 inhibits the rate constant for horse heart cytochrome *c* reduction by LfH· by 60% and 24%, respectively. Interestingly, the cytochrome *c* reduction rate constant in its complex with native cytochrome b_5 was unaffected by the increase in pH. In this latter case, the increase in LfH· reactivity is perhaps offset by an increase in the steric hindrance of the cytochrome *c* heme edge. In contrast, the rate constant for cytochrome *c* reduction in its complex with DME-cytochrome b_5 undergoes a ~2-fold increase, which is similar to the pH-induced increase observed for free cytochrome *c*.

Overall, these results support the suggestion that esterification of the cytochrome b_5 heme propionate groups induces a change in the docking geometry for the interaction of this protein with cytochrome *c* at pH 7. In addition, the current findings are consistent with a reduction of the kinetic accessibility of the partially exposed cytochrome *c* heme edge in the cytochrome b_5–cytochrome *c* complex at higher pH. The electrostatic environment of the partially exposed native cytochrome b_5 heme edge is highly negatively charged owing to the presence of the two heme propionate groups. This characteristic may inhibit the interaction of the partially anionic lumiflavin semiquinone with the heme of cytochrome *c* in its complex with native cytochrome b_5 at pH 8. Such an electrostatic effect is not expected to contribute to the kinetics of cytochrome *c* reduction in its complex with DME-cytochrome b_5 as the electrostatic environment of the heme edge in this derivative is effectively neutral (Mauk et al., 1986). Analysis of the ionic strength dependence of these reactions is required to clarify this latter point.

Reduction of Cytochrome c by FMNH·. Use of FMN semiquinone (FMNH·) as a reductant permits evaluation of the sign and magnitude of the effective electrostatic charge of the protein near the site of electron transfer (Meyer et al., 1984; Hazzard et al., 1987). In addition, use of two species of cytochrome *c* (horse and tuna) can provide information regarding the effects of amino acid substitution in the region surrounding the cytochrome *c* heme edge [cf. Table VI in Hazzard et al. (1987)] that are thought to be important in the formation of electron-transfer complexes (Salemme, 1976; Mauk et al., 1986).

The dependencies of the rate constants (k_{obs}) for horse heart and tuna cytochrome *c* reduction by FMNH· on cytochrome *c* concentration in the presence and absence of stoichiometric amounts of native or DME-cytochrome b_5 are shown in Figure 2. The second-order rate constants derived from these plots

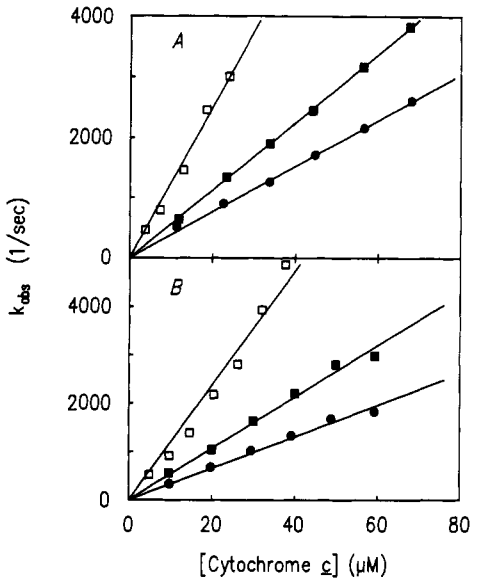

FIGURE 2: Plots of k_{obs} vs cytochrome *c* concentration for the reduction of horse (A) and tuna (B) cytochromes *c* by FMNH· in the absence (□) and presence of native (●) or DME-cytochrome b_5 (■).

are listed in Table I. In the absence of cytochrome b_5, the two species of cytochrome *c* exhibit minimal differences in their reduction behavior, consistent with previous results (Hazzard et al., 1987). The greater reduction rate constants observed with FMNH·, relative to LfH·, can be attributed to the electrostatic attraction of the negatively charged FMNH· to the highly postively charged surface of cytochrome *c* surrounding its partially exposed heme edge at the low ionic strengths used in these experiments (Meyer et al., 1984).

Binding of tuna or horse heart cytochrome *c* to native or DME-cytochrome b_5 significantly decreases the rate constant of cytochrome *c* reduction by FMNH· at pH 7 (by ~73% and ~60%, respectively). This sensitivity of the rate constants observed with the anionic flavin (FMNH·) to the presence of cytochrome b_5 contrasts with the identity of the rate constants observed for cytochrome *c* reduction by the neutral flavin (LfH·) in the presence or absence of native cytochrome b_5. The dependence of kinetic behavior on the electrostatic properties of the flavin employed probably arises from a differential response of the two flavins to the appreciable neutralization of the positive electrostatic potential surface of cytochrome *c* that occurs when it binds to native cytochrome b_5. This conclusion correlates with the smaller effect of cytochrome *c* binding to DME-cytochrome b_5 on the reduction rate constants for FMNH·; i.e., it is consistent with the diminished negative electrostatic potential in the DME analogue near the region of the exposed heme edge (Mauk et al., 1986). Furthermore, this interpretation implies that the heme propionate groups are in sufficiently close proximity to the site of FMNH· approach to cytochrome *c* that they exert a kinetically significant electrostatic influence. Finally, we note the interesting lack of species dependence of cytochrome *c* reactivity within the complexes.

Reduction of Native and DME-Cytochrome b_5 by LfH·. The variations in observed rate constants (k_{obs}) for native and DME-cytochrome b_5 reduction by LfH· in the presence and absence of stoichiometric amounts of ferrocytochrome *c* (pH 7 and 8) are shown in Figure 3. The corresponding second-order rate constants are listed in Table II. At pH 7, the rate constants observed for the free cytochromes vary directly with the thermodynamic driving forces for the reactions (Meyer et al., 1983). For example, the rate constants for reduction of free cytochrome b_5 are 25–45% lower than those obtained

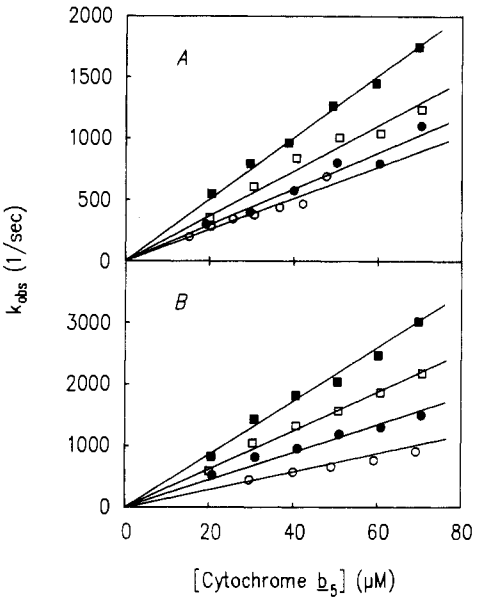

FIGURE 3: Dependence of the rate constant of cytochrome b_5 reduction by LfH· on [cytochrome b_5] at pH 7 (A) and 8 (B). Rates were determined for native cytochrome b_5 in the absence (○) and presence (●) of horse heart cytochrome c and for DME-cytochrome b_5 in the absence (□) and presence (■) of horse heart cytochrome c.

Table II: Second-Order Rate Constants for Reduction of Cytochrome b_5 by LfH· in the Presence and Absence of Ferrous Cytochrome c

	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})	
	pH 7	pH 8
cytochrome b_5		
free	1.3	1.3
+horse cytochrome c	1.5	2.1
DME-cytochrome b_5		
free	1.8	3.0
+horse cytochrome c	2.5	4.2

for free DME-cytochrome b_5, as expected from the finding that heme propionate group esterification increases the reduction potential of this protein by 60 mV (Reid et al., 1982, 1984). Similarly, the rate constants for cytochrome c ($E_{m,7}$ = 260 mV vs SHE) reduction are significantly faster than the rate of reduction of either form of cytochrome b_5. Other, less readily correlated factors, of course, are also expected to contribute to the rate differences between cytochrome c and the cytochrome b_5 derivatives observed here. These factors include the extent of heme exposure to solvent [6% in cytochrome c, 23% in cytochrome b_5; Stellwagen, 1978] [cf. Table I and Figure 1 of Tollin et al. (1986)] and the Franck–Condon activation energies of the two proteins.

With an increase in pH from 7 to 8, the rate constant for native cytochrome b_5 reduction by LfH· is unchanged while the rate constant for DME-cytochrome b_5 reduction is increased to an extent (~1.7-fold) similar to that observed for LfH· reduction of cytochrome c (vide supra). We attribute this differential response of the two cytochrome b_5 derivatives to pH change to the sum of two opposing effects. On one hand, the electrostatic effects of partial deprotonation of LfH· to generate the anionic form of the flavin at alkaline pH should decrease the rate constant for the native cytochrome reduction to a much greater extent than in the case of the modified protein as a consequence of the electrostatic effect of the solvent-exposed heme propionate groups (Reid et al., 1984). On the other hand, the increased reactivity of the anionic form of the flavin (Przysiecki et al., 1985) will tend to increase the rate of reduction of both cytochrome b_5 derivatives. For the native protein, this second effect appears to be exactly offset by the inhibitory electrostatic influence of the heme propionate groups, while for DME-cytochrome b_5 elimination of the electrostatic contribution of the propionate groups permits the increased reactivity of LfH· at alkaline pH to prevail. Clearly, the differential response of the two forms of cytochrome b_5 is not related to changes in thermodynamic driving force as the reduction potentials of native and DME-cytochrome b_5 are largely unaffected by this change in pH (Reid et al., 1982, 1984).

The rate constants for reduction of both native and DME-cytochrome b_5 by LfH· *increase* in the presence of stoichiometric amounts of ferrocytochrome c. For native cytochrome b_5, the increase in the reduction rate constant is more pronounced at pH 8, while for DME-cytochrome b_5 the increase on binding to cytochrome c is significant at both values of pH. While the mechanistic basis for this observation is unknown, it may be related to a decrease in the reorganizational barrier to electron transfer or to an increase in the reduction potentials of both forms of cytochrome b_5 that is induced by binding of either protein to cytochrome c. This latter possibility could be related to the increase in reduction potential observed for native and DME-cytochrome b_5 with increased ionic strength (Reid et al., 1982, 1984), the origin of which is not fully understood.

The increase in reactivity of cytochrome b_5 associated with protein–protein complex formation is not without precedent. For the reduction of bovine cytochrome c oxidase (Ahmad et al., 1982) and of yeast cytochrome c peroxidase compound I (Hazzard et al., 1987) by free flavin semiquinones, electron transfer is not fast enough to compete with semiquinone disproportionation. In the presence of cytochrome c, however, rapid electron transfer from flavin to either the oxidase or the peroxidase does occur, though the mechanism involves direct reduction of the cytochrome c followed by intracomplex electron transfer. In these cases, it has been argued that binding to cytochrome c establishes an effective electron-transfer pathway through structural changes induced in the ultimate electron acceptor protein. Whether a similar mechanism is involved in the present observation requires further study.

Reduction of Native and DME-Cytochrome b_5 by FMNH·. The kinetic results for reduction of native and DME-cytochromes b_5 by FMNH· in the presence and absence of tuna and horse cytochromes c are presented in Figure 4. For both forms of free cytochrome b_5, reduction by FMNH· was slower than semiquinone disproportionation, so cytochrome reduction proceeded primarily via the fully reduced species, $FMNH^-$ (data not shown). This result suggests that $FMNH^-$ reduction kinetics are significantly affected by strong electrostatic repulsion between the negatively charged FMNH· and both free b_5 species, as would be expected from previous electrostatic calculations (Mauk et al., 1986).

The observed rate constants for reduction by $FMNH^-$ are given in Table III. The value for the free native cytochrome b_5 is only slightly smaller than that obtained for the unbound, modified species, as is also the case for LfH· reduction (cf. Table III). The small magnitude of the difference suggests that although the electrostatic repulsion between FMNH and the protein may be affected by the neutralization of the heme propionate charge by esterification, other factors such as heme accessibility and the change in reduction potential also influence the reduction kinetics, resulting in a complex mixture of partially cancelling effects. We note that the second-order rate constants for the reduction of native and DME-cyto-

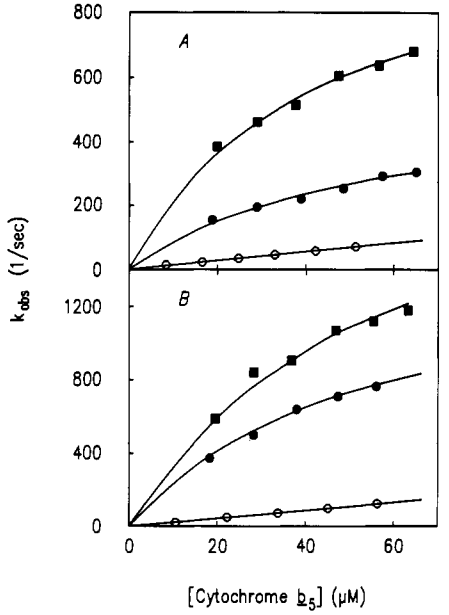

FIGURE 4: Variation of the rate constants for cytochrome b_5 reduction by FMNH· with [cytochrome b_5] in the presence of horse heart (A) and tuna (B) cytochrome *c*. In panel A, the rates of unbound native cytochrome b_5 reduction are shown (○), and in panel B, the rates for unbound DME-cytochrome b_5 reduction are shown (○). In both panels, the effects of binding the respective cytochromes *c* to native cytochrome b_5 (●) and to DME-cytochrome b_5 (■) are indicated. The solid lines represent the theoretical fits to the data (see text for details).

Table III: Reduction of Free and Complexed Cytochrome b_5 by Reduced FMN at pH 7

	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})[a]	k_1[a] (s^{-1})
cytochrome b_5		
free	0.14 (fully reduced)	
+horse cytochrome *c*	2.7 (semiquinone)	460
+tuna cytochrome *c*	5.9 (semiquinone)	1040
DME-cytochrome b_5		
free	0.22 (fully reduced)	
+horse cytochrome *c*	6.4 (semiquinone)	840
+tuna cytochrome *c*	8.1 (semiquinone)	1390

[a] Rate constants for the complexed species were determined from nonlinear least-squares regression analysis of the plots of k_{obs} vs protein concentration in Figure 4 based on the mechanisms given in eq 1 and 2 or eq 3 and 4.

chrome b_5 by $Fe(EDTA)^{2-}$, another small reductant with a net charge of −2, are 112 and 1970 M^{-1} s^{-1}, respectively, at I = 0.1 M and pH 7 (Reid et al., 1984). The difference in the effect of heme propionate esterification on the reaction kinetics of these two reductants [2-fold for $FMNH^-$ vs 10-fold for $Fe(EDTA)^{2-}$] suggests a fundamental difference in the mechanism of reduction of cytochrome b_5 by these reductants, possibly involving requirement for π-orbital overlap between the flavin and heme prosthetic group.

Large effects on the reduction kinetics of native and DME-cytochrome b_5 by reduced FMN occur upon complexation with either horse or tuna cytochrome *c*, as shown in Figure 4. In all cases, reduction of the cytochrome b_5 heme within the complex occurs via the semiquinone species, FMNH·, in marked contrast to the free cytochromes b_5. Contrary to the data obtained for cytochrome *c* reduction in the complex (cf. Figure 2), plots of k_{obs} vs complex concentration for cytochrome b_5 reduction are not linear, suggesting that there is a first-order rate-limiting process in the reaction mechanism. One possible explanation for this is the following:

$$\text{cyt } b_5(\text{III})\text{–cyt } c(\text{II}) \xrightarrow{k_1} \text{cyt } b_5(\text{III})\text{–cyt } c(\text{II})^* \quad (1)$$

$$\text{cyt } b_5(\text{III})\text{–cyt } c(\text{II})^* + \text{FMNH}\cdot \xrightarrow{k_2} \text{cyt } b_5(\text{II})\text{–cyt } c(\text{II}) + \text{FMN} \quad (2)$$

where cytochrome b_5(III)–cytochrome *c*(II)* represents a form of the complex with which FMNH· preferentially reacts, perhaps as the result of increased accessibility of the cytochrome b_5 heme group. Thus, the rate-limiting process at high concentrations is the first-order isomerization of the complex. This mechanism in combination with our current results implies that the active form of the complex must have electrostatic properties that are distinct from those of the inactive form and to which reduction by FMNH· (but not LfH·) is sensitive.

A second plausible mechanism involves the formation of a kinetically detectable precursor complex between the protein–protein complex and FMNH·:

$$\text{cyt } c(\text{II})\text{–cyt } b_5(\text{III}) + \text{FMNH}\cdot \xrightarrow{k_2} \text{cyt } c(\text{II})\text{–cyt } b_5(\text{III})\text{–FMNH}\cdot \quad (3)$$

$$\text{cyt } c(\text{II})\text{–cyt } b_5(\text{III})\text{–FMNH}\cdot \xrightarrow{k_1} \text{cyt } c(\text{II})\text{–cyt } b_5(\text{II}) + \text{FMN} \quad (4)$$

Rate constants obtained from nonlinear least-squares regression analysis of the data according to these two mechanisms are listed in Table III. Inasmuch as these two mechanisms are mathematically equivalent (Strickland et al., 1975), the numerical values for the first- and second-order rate constants are the same. For either mechanism, the second-order rate constant reflects the interaction of the negatively charged FMNH· with the bound cytochrome b_5 and is thus sensitive to the effective electrostatic environment near the interaction site.

The large values for the second-order rate constants for semiquinone reduction compared to those observed with the free proteins indicate that there is a considerable neutralization of the cytochrome b_5 negative charge, and hence a greater reactivity with the reduced FMN, in the complex. The increased rate constant for reduction of DME-cytochrome b_5 in complexes with horse or tuna cytochrome *c*, relative to native b_5, is consistent with the results obtained with the free proteins. Whereas no difference between horse and tuna cytochrome *c* reduction was observed in the complexes with cytochrome b_5, there were marked species-dependent differences in the cytochrome b_5 reduction kinetics. For both native and DME-cytochrome b_5, the values for the second-order rate constants in the complexes with horse cytochrome *c* were significantly smaller than those obtained with tuna cytochrome *c*. This suggests that there is either a less negative or a more positive electrostatic potential near the cytochrome b_5 reduction site in the complex with tuna cytochrome *c* than is the case in the complex with horse cytochrome *c*.

The interpretation of the effects on the rate-limiting first-order rate constant is dependent upon the mechanism. In one previous case, reduction of cytochrome *c* by FMNH· in a 1:1 complex with *Clostridium pasteurianum* flavodoxin, we showed unambiguously that the mechanism given by eq 1 and 2 was correct, based on the independence of the first-order rate-limiting process of the identity of the reductant (Hazzard et al., 1986). In the present case, no such comparison was made. However, some discussion concerning the mechanism can be given. As DME-cytochrome b_5 has a smaller negative electrostatic potential than native cytochrome b_5, it should form a weaker complex with cytochrome *c*. According to the mechanism given by eq 1 and 2, this would predict a larger value for k_1, in agreement with the results. The larger k_1

values obtained with the tuna cytochrome *c* complexes would suggest that a more dynamic complex is formed with this cytochrome than with horse cytochrome *c*. Although it is more difficult to rationalize the results in terms of the mechanism of eq 3 and 4, such arguments are of course not definitive, and further study is required.

Computer graphics modeling of the cytochrome b_5–cytochrome *c* complex suggests that at least two electrostatically isoenergetic docking geometries can exist under certain solution conditions (Mauk et al., 1986). The complex originally proposed by Salemme (1976) is favored by interaction between the two native cytochromes at pH 7 or lower. An alternative orientation in which the two heme groups are further apart and no longer coplanar is electrostatically favored by interaction of DME-cytochrome b_5 with cytochrome *c* at pH 8 and greater. Analysis of these limiting, static models indicates that the exposure of the cytochrome b_5 heme group to solvent is unaffected by binding of cytochrome b_5 to cytochrome *c* in either orientation; the exposure of the cytochrome *c* heme group to solvent is unaffected on binding to cytochrome b_5, but is reduced by ca. 35% on binding to this protein in the alternative orientation. The current results are consistent with these models in that the rate constant for the reduction of cytochrome *c* by $LfH^{\cdot}$ is unaffected by the binding to native cytochrome b_5 at pH 7 but is reduced by 25% upon binding to DME-cytochrome b_5 at pH 8. Nevertheless, the value of estimating the effect of complex formation on heme exposure of the cytochromes on the basis of the modeled complexes is clearly limited in light of recent molecular dynamics calculations of the cytochrome b_5–cytochrome *c* complex, proposed by Salemme, which suggest that there may be some flexibility in the solution conformations (Wendoloski et al., 1987).

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