Flavocytochromes *c*: Transient Kinetics of Photoreduction by Flavin Analogues[†]

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ABSTRACT: Kinetics of reduction of phototrophic bacterial flavocytochromes *c* by exogenous flavin semiquinones and fully reduced flavins generated by laser flash photolysis have been studied. The mechanisms of reduction of *Chromatium* and *Chlorobium* flavocytochromes *c* are more similar to one another than previously thought. Neither protein is very reactive with neutral flavin semiquinones ($k < 10^7$ M^{-1} s^{-1}), and the reactions with fully reduced flavins are slower than expected on the basis of comparison with other electron-transfer proteins of similar redox potentials. Deazaflavin radical is reactive with the flavocytochromes *c* by virtue of its low redox potential, but this reaction is also slower than expected on the basis of comparison with other electron-transfer proteins. These experiments indicate that the active site for reduction of flavocytochrome *c* is relatively buried and probably inaccessible to solvent. Fully reduced FMN does not show an ionic strength effect in its reaction with flavocytochrome *c*, which demonstrates that the active site is uncharged. Sulfite, which forms an adduct with protein-bound FAD, partially blocks heme reduction. This shows that heme is reduced via the FAD. The rate constant for intramolecular electron transfer between FAD and heme must be on the order of 10^4 s^{-1} or larger.

Flavocytochromes *c* from the phototropic bacteria *Chromatium vinosum* and *Chlorobium thiosulfatophilum* offer an opportunity to study electron-transfer processes in a water-soluble multiredox center system involving heme and flavin chromophores. The *Chromatium* protein (also known as cytochrome *c*-552) has two hemes and a flavin per M_r of 72 000 and consists of dissimilar flavin-containing (M_r ~45 000–51 000) and heme-containing subunits (M_r ~15 000–22 000) (Kennel, 1971; Yamanaka & Kusai, 1976; S. L. Brown and M. A. Cusanovich, unpublished observations). In contrast, the *Chlorobium* protein (also known as cytochrome *c*-553) contains one heme and one flavin per M_r ~50 000. The heme subunit has a M_r of 11 000–14 000 and the flavin subunit 47 000 (Yamanaka, 1976; Yamanaka & Kusai, 1976). The midpoint oxidation–reduction potential of the heme moiety of the *Chlorobium* flavocytochrome *c* is approximately 98 mV as compared to 10–35 mV for the heme of *Chromatium* flavocytochrome *c* at pH 7 (Bartsch & Kamen, 1960; Meyer et al., 1968; Vorkink, 1972). It has been established that both the *Chromatium* and *Chlorobium* flavocytochromes can function as sulfide dehydrogenases (Kusai & Yamanaka, 1973a,b).

In previous studies using lumiflavin semiquinone and fully reduced lumiflavin as reductants [produced by laser flash photolysis in the presence of ethylenediaminetetraacetic acid (EDTA)], we characterized some aspects of the electron-transfer properties of the flavocytochromes *c* (henceforth FC) from both *Chromatium* and *Chlorobium* (Cusanovich & Tollin, 1980; Tollin et al., 1982). With both proteins, the evidence suggested that initial reduction occurred at the flavin moiety and was followed by rapid intramolecular electron transfer to the heme center, with the intramolecular rate constants approximately 10^3 s^{-1} and $>10^6$ s^{-1} for the *Chlorobium* and *Chromatium* proteins, respectively.

Chromatium FC was primarily characterized in terms of its reduction by fully reduced lumiflavin, as we were unable to analyze the semiquinone reaction in any detail. Moreover, we observed the transient formation of a protein-bound flavin anion radical at pH 7. *Chlorobium* FC was found to behave in a generally similar manner but with apparently different rate constants.

In an effort to more fully characterize the kinetics of electron transfer by the flavocytochromes *c*, we have investigated their reaction with photoreduced flavins in more detail. The focus of the present study is on the chemistry of the exogenous flavin, with different flavin analogues (lumiflavin, FMN, dichlororiboflavin, riboflavin, and 10-methylisoalloxazine) being used in order to obtain information about the interaction domain on the flavocytochrome. In addition, we have extended our studies of the kinetic influence of pH, ionic strength, and the formation of an endogenous flavin–sulfite adduct. The results reported here allow us to greatly refine our understanding of the mechanism of electron transfer by the flavocytochromes *c*. In addition, we have now established that not only are the two flavocytochromes identical mechanistically but in fact their rate constants are more similar than we had previously indicated.

EXPERIMENTAL PROCEDURES

Chlorobium thiosulfatophilum strain Tassajara flavocytochrome *c* was prepared as described by Meyer et al. (1968). *Chromatium vinosum* strain D flavocytochrome *c* was prepared as described by Bartsch (1971). The laser flash photolysis apparatus, techniques, and methods of data reduction were as previously described (Cusanovich & Tollin, 1980; Ahmad et al., 1981; Simondsen & Tollin, 1983; Meyer et al., 1983). The solid curves in time-resolved difference spectra were subjectively drawn to approximate the data. The monochromator slit widths used for data collection could be expected to introduce a certain amount of error in these spectra. However, all difference spectra, except those for deazariboflavin reduction, have peak ratios that are similar

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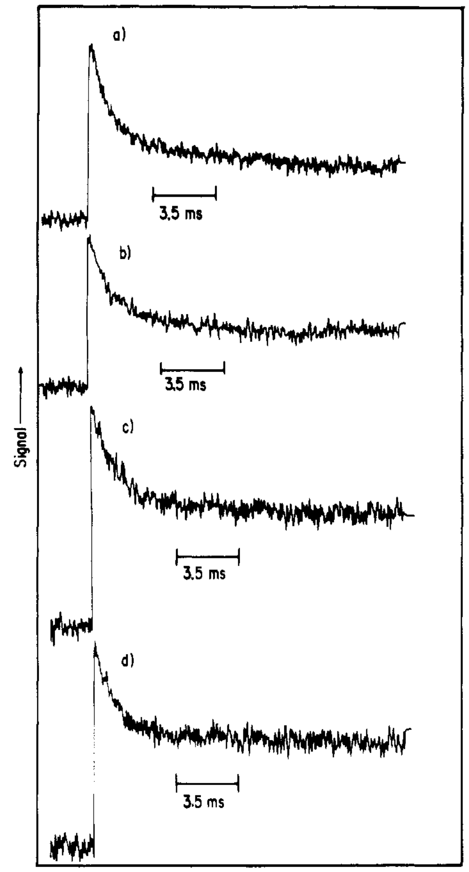
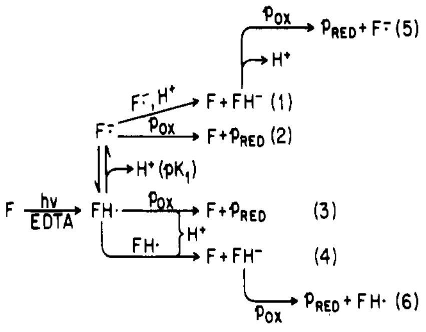


FIGURE 1: Transient kinetics of lumiflavin semiquinone decay in the presence and absence of *Chromatium* FC. Buffer was 20 mM potassium phosphate, 20 μM riboflavin, and 10 mM EDTA, pH 7.0. (a) No added cytochrome, 563 nm; (b) 13.6 μM FC, 560 nm; (c) 27.2 μM FC, 560 nm, (d) 40.7 μM FC, 560 nm. In all cases the total sweep time was 20 ms.

to those obtained from chemical difference spectra recorded on a Cary spectrophotometer, which suggests that the effective band width in our experiment is less than the half-width of the narrowest peak, namely, the α peak (552–553 nm). The difference spectra obtained with deazariboflavin are obviously distorted. This is because the signals were smaller and the slit width had to be increased. The error in relative peak heights in this case is estimated to be less than a factor of 2 as judged from the observed absorbance changes at 552 and 525 nm. All data are presented as change in signal intensity rather than absorbance. Typically, the maximum absorbance changes per point are on the order of 0.01 Δ absorbance units, corresponding to less than 1% reduction of the protein in the observation beam (less than 0.1% of the total protein in the sample). The relevant redox and protonation reactions (Holmstrom, 1964; Vaish and Tollin, 1971) for free flavins are shown in Scheme I, where pK_1 is 8.4, 8.4, 7.7, and 6.4 for lumiflavin (LF), riboflavin (RF), 10-methylisoalloxazine (10MI), and dichlororiboflavin (diClRF), respectively, P_{ox} and P_{red} are oxidized and reduced forms of a redox protein, FH· is the neutral flavin semiquinone, F^-· is the anionic flavin semiquinone, and FH^- represents the predominant ionic form of fully reduced flavin at neutral pH.

Scheme I



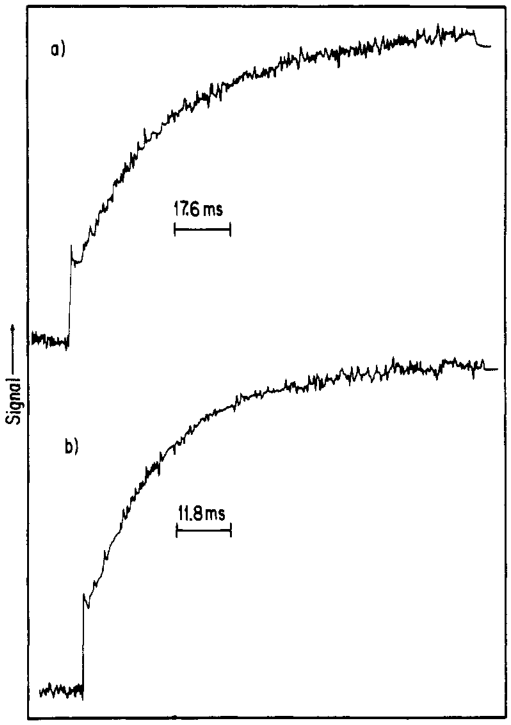


FIGURE 2: Reaction of fully reduced lumiflavin with *Chromatium* FC (slow phase). Buffer and other conditions were as in Figure 1a: 550 nm; 150-ms sweep for upper trace, [heme] = 10.9 μM; 100-ms sweep for lower trace, [heme] = 21.8 μM.

Results and Discussion

Our previous studies of *Chromatium* FC (Cusanovich & Tollin, 1980) focused on its reduction by fully reduced flavins and on intramolecular electron transfer. Although it was possible to establish that some reduction of the flavocytochrome took place during the lifetime of the photoproduced flavin semiquinone radical, we could not determine a rate constant for this reaction. Recent improvements in instrumentation and technique have prompted us to reinvestigate the kinetics of both semiquinone and fully reduced flavin reduction of FC. Two kinetic processes are known to occur following the laser flash. The first of these is a rapid decay of the photoproduced exogenous flavin semiquinone to form, in part, fully reduced flavin via disproportionation (reactions 1 and 4 of Scheme I). The second, slower phase involves reduction of the FC by the fully reduced exogenous flavin (reactions 5 and 6). If FC reduction also occurs during the rapid phase (reactions 2 and 3), the semiquinone decay kinetics should be altered by the presence of the protein. Figure 1 shows the decay kinetics of exogenous LF semiquinone in the presence and absence of several concentrations of added *Chromatium* FC, measured at a heme isosbestic point (560 nm). As can be seen, essentially no kinetic difference is observable; i.e., semiquinone decay is, within our experimental error (±20%), unaffected by the presence of the flavocytochrome. On the other hand, the second, slower kinetic process, reaction with fully reduced exogenous flavin, is pseudo first order at all FC concentrations studied, and the observed first-order rate constant is dependent on the FC concentration. This is consistent with our previous observations (Cusanovich

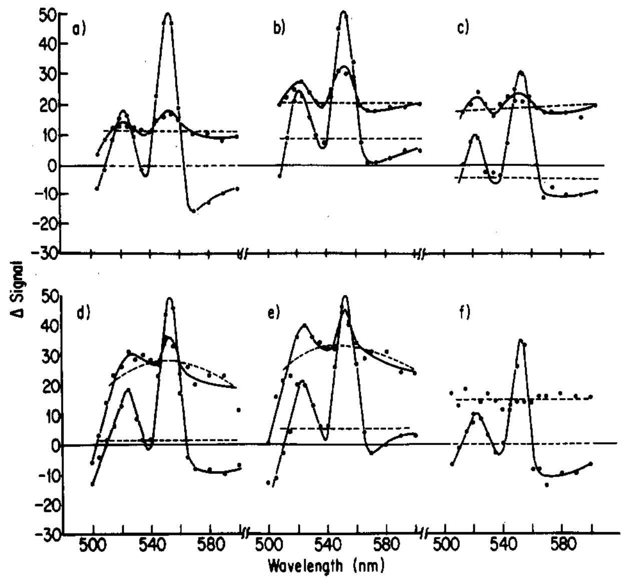


FIGURE 3: Time-resolved reduced minus oxidized difference spectra of *Chromatium* FC in the presence of lumiflavin and riboflavin. Buffer was pH 7.0 20 mM potassium phosphate and 10 mM EDTA or pH 5.0 10 mM sodium acetate and 10 mM EDTA. Flavin analogue concentrations are given in Table I. Data presented in different panels were not necessarily taken with the same samples or instrument settings; hence, absolute values are not comparable. Heme concentration was 11 μM. (a–c) Lumiflavin at pH 7, pH 5, and pH 5 + 10 mM sodium sulfite; (d–f) riboflavin at pH 7, pH 5, and pH 5 + 10 mM sodium sulfite. Open circles are the fast phase, and filled circles are the slow phase.

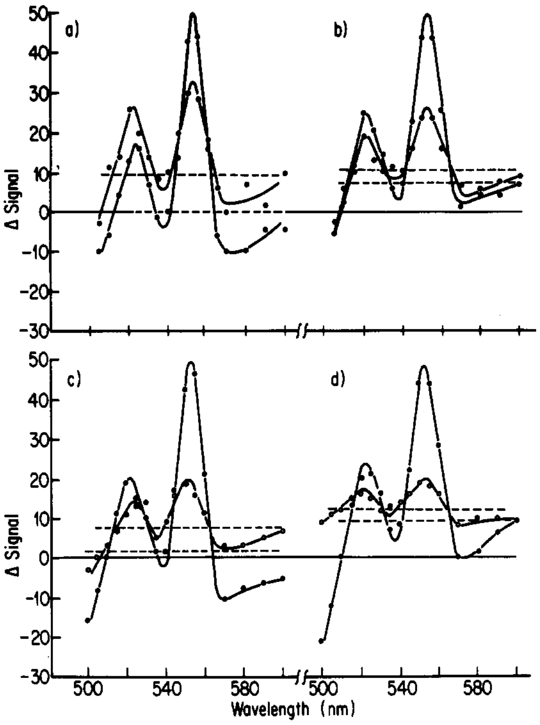


FIGURE 4: Time-resolved reduced minus oxidized difference spectra of *Chromatium* FC in the presence of dichlororiboflavin and 10-methylisoalloxazine. Conditions are as in legend to Figure 3. (a and b) Dichlororiboflavin at pH 7 and pH 5; (c and d) 10-methylisoalloxazine at pH 7 and pH 5.

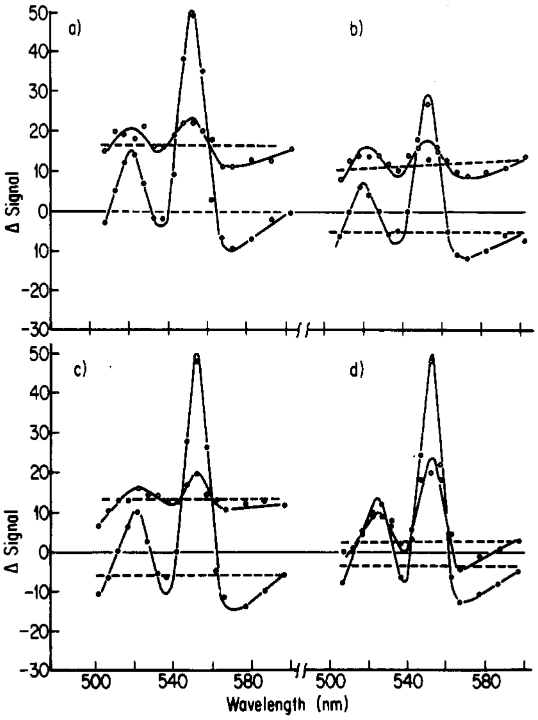


FIGURE 5: Time-resolved reduced minus oxidized difference spectra of *Chlorobium* FC in the presence of different flavin analogues. Buffers and reaction conditions are as given in legend to Figure 3. (a and b) Lumiflavin at pH 5 and pH 5 + 10 mM sulfite; (c) riboflavin at pH 7; (d) dichlororiboflavin at pH 7.

& Tollin, 1980; Tollin et al. 1982). Figure 2 presents typical slow-phase data obtained with *Chromatium* FC and LF for comparison. In this example, an increase in absorbance at 550 nm is shown that is consistent with heme reduction. Generally, similar results were obtained for *Chlorobium* FC (data not shown, see below).

In order to further elucidate the nature of the kinetic species observed, time-resolved difference spectra were measured for both the fast and slow phases with a variety of flavin analogues. These difference spectra are presented in Figures 3 and 4 for *Chromatium* FC and in Figure 5 for *Chlorobium* FC. Data are presented for measurements made at pH 7, pH 5, and in some cases pH 5 in the presence of HSO_3^-. For the present, the discussion will focus on the pH 7 results. In Figures 3–5 the open circles represent the signal change due to the fast kinetic species, obtained by extrapolating the signal for the slow kinetic species (Figure 2) to zero time and determining the difference between this point and the preflash base line. The solid circles represent the difference spectra for the slow kinetic species and were obtained from the difference between the extrapolated $t = 0$ point and a point at an arbitrary but relatively long time (typically 40 ms). In principle, this last point should be taken at $t = \infty$, but this was inconvenient inasmuch as it would be necessary to obtain data in two separate experiments in order to accurately determine both the $t = 0$ and $t = \infty$ points. Although the approach used precludes quantitative comparison of the absolute magnitudes of the fast and slow phases, it does not affect the shape of the spectra nor the relative contributions of the components observed under a particular set of conditions. Direct quantitative comparison of the signal amplitudes in the spectra at different pH values or with different flavin analogues is not generally possible because of day to day variations in the data acquisition system. However, in some cases (see below), spectra under different conditions were obtained during a single experiment and, hence, are quantitatively comparable. The solid lines in Figures 3–5 are smooth curves drawn through the difference spectral data, and the dashed lines were obtained by subtracting the spectrum of the heme moiety (obtained from a dithionite-reduced minus oxidized difference spectrum) from these smooth curves. Because of the experimental uncertainties, in most cases these latter curves were simply drawn as horizontal lines.

Table I: Analysis of Reduced Minus Oxidized Transient Difference Spectra

flavin	flavin concn (μM)	pH	addition	ΔA_{552} (heme)/ ΔA_{552} (nonheme) fast	slow
Chromatium FC					
lumiflavin	75	7		0.56	>20
lumiflavin	47	5		0.59	4.6
lumiflavin	45	5	HSO_3^-	0.24	7.9
riboflavin	50	7		0.29	>20
riboflavin	52	5		0.36	9.0
riboflavin	52	5	HSO_3^-	0	>20
dichlororiboflavin	39	7		2.4	>20
dichlororiboflavin	40	5		1.4	5.7
dichlororiboflavin	58	5	HSO_3^-	0.5	>20
10-methylisoalloxazine	55	7		1.6	25
10-methylisoalloxazine	50	5		0.67	4.4
Chlorobium FC					
riboflavin	20	7		0.48	−9.3
dichlororiboflavin	20	7		8.6	−15
lumiflavin	50	5		0.42	>20
lumiflavin	50	5	HSO_3^-	0.50	−6.9

As can be seen in Figure 3a, the fast phase consists of two components, a small contribution due to heme reduction and a relatively large non-heme transient with a broad spectrum typical of a neutral flavin semiquinone. Using average extinction coefficients (6 mM^{-1} cm^{-1} for neutral flavin semiquinone and 18 mM^{-1} cm^{-1} for heme at 552 nm), it can be calculated that only 15% of the reducing equivalents reached the heme. Since the kinetic measurements indicate that the decay of the exogenous flavin semiquinone is independent of the presence of flavocytochrome, these results establish that the non-heme transient is LFH· that has not reacted with FC to a sufficient extent to alter the observed disproportionation decay kinetics. It follows then that lumiflavin semiquinone is not an effective reducing agent for the FC's studied (we estimate that the second-order rate constant must be less than 10^7 M^{-1} s^{-1}). As can be seen (Figures 3–5) within experimental error the difference spectra are generally similar for both *Chromatium* and *Chlorobium* FC with LF or RF as the exogenous flavins. Moreover, the observed ratios of heme to non-heme absorbance changes in the fast phase are independent of pH between 5 and 7. These data are summarized in Table I. In contrast, diClRF and 10MI give a much larger ratio of heme to non-heme transient, although the 10MI results at pH 5 (with *Chromatium* FC) are similar to those obtained with RF and LF. These results can be explained in terms of the relative exogenous flavin semiquinone p*K* values, if one assumes that the anion semiquinone is more reactive than the neutral semiquinone (see below). For RF and LF, this p*K* is 8.4, and thus, the dominant species at pH 5 and 7 is the neutral semiquinone. For diClRF the p*K* is 6.4, so that at pH 7 the dominant species is the anion semiquinone. Thus, the heme to non-heme transient ratio for diClRF (Table I) is large at pH 7 but decreases as the pH is decreased to 5. However, the pH 5 value is larger than that for lumiflavin. The p*K* value for 10MI is 7.7, and hence, a larger heme to non-heme transient ratio than that for lumiflavin is observed at pH 7. At pH 5, little anion exists, and hence, a ratio similar to that found with RF and LF is obtained. As might be expected from the foregoing, lumiflavin radical gives a much larger relative heme contribution at pH 8.5 (data not shown). A greater reactivity of the anion semiquinone is consistent with a recent study in which we found that lumiflavin anion semiquinone was 54-, 37-, and 10-fold more reactive with *Chromatium* high-potential iron sulfur protein, *Clostridium pasteurianum* rubredoxin, and horse heart cytochrome *c*, respectively (Przysiecki et al., 1985).

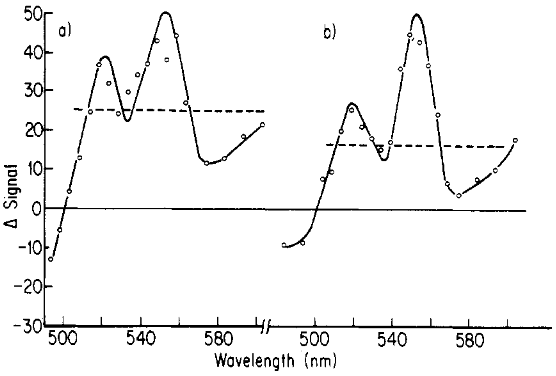


FIGURE 6: Time-resolved reduced minus oxidized difference spectra of flavocytochrome *c* in the presence of deazariboflavin. Buffer was 10 mM sodium acetate, 10 mM EDTA, and 64 μM deazariboflavin, pH 5.0. (a) *Chromatium* FC; (b) *Chlorobium* FC.

Summarizing to this point, we can conclude that although exogeneous neutral flavin semiquinone is capable of reducing both *Chromatium* and *Chlorobium* FC, the reaction is quite slow ($k_2 < 10^7$ M^{-1} s^{-1}) and does not effectively compete with disproportionation. The exogeneous anion semiquinone is a kinetically superior reducing agent, although even in this case the rate constant is not determinable because of the large contribution of the disproportionation reaction. We have previously proposed that the reduction of *Chlorobium* FC by LFH· involved a preformed exogenous flavin–protein complex and a first-order endogenous flavin to heme electron-transfer step ($k \sim 1200$ s^{-1}; Tollin et al., 1982). This clearly is not the case, as the data just presented indicate that in fact we cannot directly measure the kinetics of flavin semiquinone reduction of the FC. Our earlier analysis of the fast kinetic process was complicated by several experimental factors that contributed to our incorrect assignment. First, the observed rate of decay during the fast kinetic process is dependent on the exogenous flavin concentration. This is the case because at higher concentrations more semiquinone is formed during the laser flash and the second-order disproportionation reaction makes a larger contribution to the observed decay, thus obscuring any first-order processes. Our recent experience suggests that ~20 μM exogenous flavin is optimum for kinetic measurements of semiquinone reactions. Second, since the slow phase is second order, as the flavocytochrome concentration is increased, the fully reduced flavin reaction can influence the observed kinetics at early times. Thus, only a narrow flavocytochrome concentration range can be used to investigate the fast kinetic process (~5–30 μM). Finally, depending on the wavelength monitored, the kinetics can become more or less complicated as the number of detectable species and their contributions to the total signal vary.

In order to effectively reduce flavocytochrome *c* with flavin semiquinone, a stronger reductant is apparently needed. To test this possibility, deazariboflavin (dzRF) was used since its semiquinone has a much lower midpoint potential (ca −600 mV) than the other flavin analogues. Moreover, dzRF has much less absorption above 500 nm and thus permits us to determine more easily if endogenous flavin is being reduced. Figure 6 presents the time-resolved reduced minus oxidized difference spectra for dzRF reduction of both *Chromatium* and *Chlorobium* FC. These data were taken at pH 5 in order to produce the more easily detected neutral endogenous flavin semiquinone radical (see below). As can be seen, substantially more heme is reduced in the fast phase than with the other analogues (the slow phase was not studied inasmuch as the

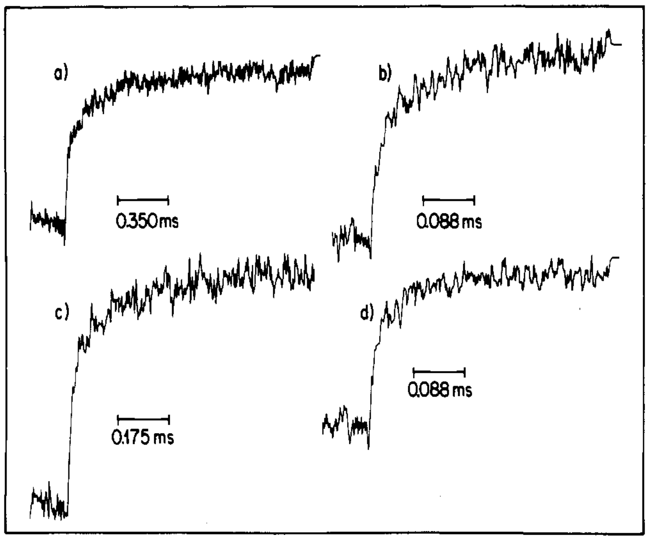


FIGURE 7: Kinetics of reduction of flavocytochrome *c* by deazariboflavin semiquinone. Buffer was 10 mM sodium acetate and 10 mM EDTA, pH 5.0. For *Chromatium* FC and 40 μM dzRF at 550 nm: (a) 2-ms sweep, 27 μM heme; (c) 1-ms sweep, 40.3 μM heme. For *Chlorobium* FC and 64 μM dzRF at 550 nm: (b) 0.5-ms sweep, 13 μM heme; (d) 0.5-ms sweep, 19.4 μM heme. Identical kinetics were observed at 600 nm (data not shown).

disproportionation products of dzRF are not well characterized), and substantial endogenous neutral flavin semiquinone is produced. Importantly, the kinetics were pseudo first order and dependent on FC concentration (Figure 7). Second-order rate constants of 3×10^8 M^{-1} s^{-1} and 1.2×10^9 M^{-1} s^{-1} were estimated for *Chromatium* and *Chlorobium* FC, respectively. These rate constants were independent of the observation wavelength (550 vs. 600 nm), indicating either that the heme and flavin moieties of FC are reduced with the same rate constant within experimental error or that one moiety is reduced preferentially followed by rapid intramolecular electron transfer (>3×10^4 s^{-1} based on measured pseudo-first-order rate constants). This last point will be addressed in more detail below.

Returning to the slow kinetic phase of reaction of FC with various flavin analogues, as shown in Figure 2 and as reported previously, this process is clearly second order and is consistent with reduction by fully reduced exogenous flavin. The reduced minus oxidized difference spectra for the slow phase are given in Figures 3–5. At pH 7, the kinetics are dominated by heme reduction, although we cannot exclude a small participation of a non-heme species (Figure 3d for example). However, at pH 5 with *Chromatium* FC it is clear that a non-heme transient is present with all of the flavin analogues. This apparently is due to the presence of a neutral endogenous flavin semiquinone, inasmuch as a pH titration monitoring the signal change at 600 nm has a p*K* of 5.7 (Figure 8). The p*K* value for this endogenous flavin is substantially different from that of LF (p*K* = 8.4) and is modified in the same direction as the corresponding transient observed with *Chlorobium* FC (p*K* = 6.8; Tollin et al., 1982).

The pH 5 studies with *Chromatium* FC indicate that the heme to non-heme signal ratio at 552 nm varies from 4.4 to 9.0, average 5.9 (Table I), with different flavin analogues. Using typical extinction coefficients for flavin neutral semiquinone and heme (see above), we obtain an average molar ratio of heme reduced to flavin reduced of 2.0 at pH 5.0. If we correct this for the p*K* (5.7) and assume that the semiquinone anion does not absorb at 552 nm, we calculate that the heme midpoint redox potential is approximately 13 mV higher than that of the oxidized flavin/flavin semiquinone redox potential. By the same arguments, at pH 7 the endogenous anionic semiquinone form should predominate, consistent with the large heme to non-heme signal ratio observed (typically >20, Table I). Comparable studies with *Chlorobium* FC indicate a much larger heme to non-heme ratio for lumiflavin at pH 5 (>20 vs. 4.4–9.0 for *Chromatium* FC and flavin analogues) (compare Figure 3b with Figure 5a). This is consistent with a greater difference between the heme and flavin redox potentials for this protein. Previous measurements (Meyer et al., 1968) place the heme potential for *Chlorobium* FC approximately 60 mV higher than that of *Chromatium* FC. Thus, on the basis of the heme to non-heme ratios, we would expect the flavin potentials for the two proteins to be approximately equal.

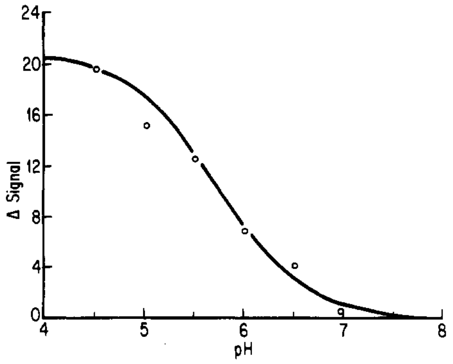


FIGURE 8: pH titration of transient signal at 600 nm. Buffer was 20 mM Tris, sodium acetate, potassium phosphate, glycine, 10 mM EDTA, and 20 μM lumiflavin, pH adjusted to indicated value. Plot of Δ signal vs. pH. The solid line was calculated with a p*K* of 5.7, $n = 1$ with an acid limit of 21 and a base limit of 0. The reaction was monitored at 600 nm where the signal actually went below the preflash base line at pH values above 6. The magnitude of the signal was constant at pH 8 and 9, and this value was subtracted from the signal at lower pH values to yield Δ signal.

Table II: Effect of pH on Rate Constant for Reduction of *Chromatium* and *Chlorobium* FC by Fully Reduced Lumiflavin

pH	*k* for *Chromatium* FC ($\times 10^{-6}$ M^{-1} s^{-1})	*k* for *Chlorobium* FC ($\times 10^{-6}$ M^{-1} s^{-1})
5	7.2	3.7
6	7.4	5.4
7	7.6	5.5 (8.0)
8	11.8	ND
9	14.6	13.3
10	16.4	ND

A more detailed examination of the effect of pH on the reduction of *Chromatium* FC by LFH^- has been carried out. The results are summarized in Table II where they are compared to the corresponding data for *Chlorobium* FC. There is no effect of hydrogen ion concentration between pH 5 and 7, and a slight increase in the rate constant is seen at pH 8 and above. Generally, similar results are obtained with *Chlorobium* FC, indicating no participation of protein-linked ionizations below pH 8 with either flavocytochrome. In a related experiment, the rate constant for reduction of *Chromatium* FC by fully reduced FMN was determined at pH 7.0 at three ionic strengths (10, 96, and 256 mM). The second-order rate constants were found to be 4.9×10^6, 5.6×10^6, and 5.2×10^6 M^{-1} s^{-1}, respectively. Since FMN has a charge of −1.9 at pH 7.0, the reaction should have been ionic-strength dependent if the interaction site on the flavocytochrome with exogenous FMN was charged. Thus, we can conclude that the site of interaction does not contain charged groups that influence the observed kinetics. This is quite different from previous observations with 18 different *c*-type cytochromes

Table III: Rate Constants for Reduction of Flavocytochrome *c* by Fully Reduced Flavins

flavin	pH	*k* for *Chromatium* FC ($\times 10^{-6}$ M^{-1} s^{-1})	*k* for *Chlorobium* FC ($\times 10^{-6}$ M^{-1} s^{-1})
lumiflavin	5.0	7.6	ND
	7.0	7.6	5.5
riboflavin	7.0	2.8	ND
	5.0	3.6	5.1
riboflavin + HSO_3^-	5.0	3.6	ND
dichlororiboflavin	5.0	2.4	ND
	7.0	3.6	5.6
10-methylisoalloxazine	5.0	8.0	ND
	7.0	7.2	ND
FMN[a]	7.0	4.8	5.7

[a] At 16 mM ionic strength.

(Meyer et al., 1984), where appreciable ionic strength effects were found.

Table III presents the rate constants for reduction of both *Chlorobium* and *Chromatium* FC by fully reduced flavins. *Chlorobium* FC has a rate constant that is independent of the flavin analogue used. On the other hand, *Chromatium* FC has a rate constant that is dependent on flavin structure, with LF and 10 MI giving similar rate constants that are larger than the values obtained with those flavins having a ribityl side chain (FMN, RF, and diClRF). This last observation suggests that some steric restrictions exist at the interaction site, giving a smaller rate constant with the more bulky exogenous flavins. Within experimental error, the rate constants for reduction by fully reduced flavins are independent of pH between pH 5 and pH 7 for *Chromatium* FC with all flavin analogues studied. It is important to note that the second-order rate constants for reduction of *Chromatium* FC are calculated with the total protein concentration instead of the heme concentration. If the heme moiety is reduced directly at independent but equivalent sites, then the rate constants for the *Chromatium* protein given in Table III should be divided by 2 (however, see below).

A variety of anions including HSO_3^- bind to the flavin moiety of FC, forming adducts that have a charge-transfer band centered in the 650–700-nm region and little or no absorbance in the 450–500-nm region (Cusanovich & Meyer, 1982). In a previous study of *Chlorobium* FC (Tollin et al., 1982), we found that HSO_3^- suppressed heme reduction, suggesting that heme reduction was mediated through the flavin moiety. This point has been reinvestigated and expanded, with data for *Chlorobium* FC summarized in Figure 5 and the corresponding data for *Chromatium* FC given in Figure 3 and Tables I and III (in these cases, the spectra were determined in the same experiment and can be directly compared). These experiments were carried out at pH 5 where the bulk of the endogenous flavin semiquinone would be in the neutral form (see above) and the adduct would be stable (Cusanovich & Meyer, 1982). As shown in Figure 5, sulfite suppresses heme reduction in both the fast and slow phases with *Chlorobium* FC, consistent with the heme being reduced through the flavin as previously reported. A very similar result is obtained when *Chromatium* FC reacts with both LF and RF (Figure 3); i.e., the extent of heme reduction is decreased in both phases by HSO_3^-. In addition, the formation of endogenous neutral semiquinone is suppressed in the slow phase (Figure 3c,f). It is important to compare the spectra given in Figure 3c,f and Figure 5b to the corresponding spectra at pH 7 where no endogenous neutral semiquinone is formed. It can be seen that, in the presence of HSO_3^-, the absorbance difference at wavelengths beyond 560 nm is much more negative (Figure 3c,f, Figure 5b). This is consistent with a reduction of the sulfite adduct occurring in the slow phase. Reduction of the adduct may also occur in the fast phase, where a decrease in the amplitude of the non-heme transient is observed. However, due to the large background contribution from exogenous neutral semiquinone, this cannot be quantitated. To test this further, we looked at the phototitration of FC by lumiflavin with steady illumination and found that adduct reduction lagged behind that of heme, consistent with a redox potential difference of about 50 mV (data not shown). Taken together, the results presented indicate that reduction of the heme moiety occurs via intramolecular electron transfer from the endogenous flavin with both *Chromatium* and *Chlorobium* flavocytochrome *c*. Thus, HSO_3^- apparently alters the distribution of electrons between the heme and flavin moieties but does not completely prevent reduction of the flavin.

CONCLUSIONS

It is clear from the results presented that some of the conclusions of our previous investigation (Tollin et al., 1982) of *Chlorobium* FC reduction kinetics require some revision. In particular, we have now demonstrated that both flavocytochromes are rather unreactive toward neutral exogenous flavin semiquinones (except for dzRF). This is unexpected inasmuch as a variety of *c*-type cytochromes and high-potential iron–sulfur proteins with oxidation–reduction potentials in the same range as those of the flavocytochromes *c* are considerably more reactive with lumiflavin semiquinone (Meyer et al., 1983). We have shown previously, by comparing different classes of redox proteins, that the exposure of the redox center at the protein surface correlates with the magnitude of the observed rate constant (Meyer et al., 1983). On this basis, we can conclude that both the heme and flavin moieties of FC are in sterically restricted environments that are not freely accessible to molecules the size of flavins. dzRF semiquinone, due to its larger thermodynamic driving force, does allow second-order reduction of the flavin and/or heme, although even in this case the reaction is relatively slow. For example, *Rhodopseudomonas gelatinosa* cytochrome *c*-550 (Em_7 = 28 mV) has a second-order rate constant (3.1×10^9 M^{-1} s^{-1}) with dzRF semiquinone that is approximately 10-fold greater than that found for *Chromatium* FC. Thus, even in the case of dzRF, steric restrictions are apparent. Reduction of FC by fully reduced flavins does take place although again the reactions are relatively slow [$(3–8) \times 10^6$ M^{-1} s^{-1}, Table III] compared to *c*-type cytochromes ($k = 2.9 \times 10^7$ M^{-1} s^{-1} for fully reduced lumiflavin reduction of horse cytochrome *c*). This range of rate constants for FC is comparable to those observed for high-potential iron–sulfur proteins that do not have solvent accessible chromophores (Meyer et al., 1983). Combining the discussion to this point with the facts that no protein-linked ionizations with p*K* values below 8 affect the observed kinetics and that ionic strength has no effect on the kinetics of FMN semiquinone reduction of *Chromatium* FC, it appears that the FC interaction domain is relatively buried (solvent inaccessible) and in an uncharged region of the protein surface.

We have shown that formation of a sulfite adduct of the flavin moiety in FC blocks ~50% of the heme reduction in the fast phase and 15–30% in the slow phase (Figures 3 and 5). This is consistent with heme reduction occurring via intramolecular electron transfer from the protein-bound flavin. Since we have shown that exogenous flavin can reduce the sulfite adduct, sulfite would not be expected to completely prevent heme reduction.

We have previously shown that with *Chromatium* FC (Cusanovich & Tollin, 1980) intramolecular electron transfer is quite fast ($k > 10^6$ s^{-1}). The present studies are consistent with this conclusion for both *Chlorobium* and *Chromatium* FC inasmuch as the dzRF results indicate that the rate constant for intramolecular electron transfer must be greater than 3×10^4 s^{-1}.

In summary, the studies reported establish that the mechanism of electron transfer from exogenous flavins is very similar for both *Chromatium* and *Chlorobium* FC, as would be expected for functionally related proteins. Although definitive conclusions are dependent upon acquiring detailed structural information, the available data are consistent with a sequential electron transfer from exogenous reducing agents to the protein-bound flavin moiety, followed by rapid intramolecular electron transfer to the heme.

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Spectroscopic Examination of the Active Site of Bovine Ferrochelatase[†]

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ABSTRACT: Spectrofluorometric techniques have been employed to examine the active site of the terminal enzyme of the heme biosynthetic pathway, ferrochelatase (protoheme ferrolyase, EC 4.99.1.1). The fluorescence of both endogenous tryptophan and exogenous 2-(4-maleimidylanilino)naphthalene-6-sulfonic acid (MIANS) has been examined. The fluorescence emission of the enzyme's active site bound MIANS is at 428 nm while the enzyme tryptophan(s) yielded a single fluorescence emission maximum at 347 nm. These values are characteristic of a polar environment for tryptophan and a relatively nonpolar environment for the MIANS. The dynamic fluorescence quenching constants for acrylamide of MIANS and tryptophan are 3.00 M^{-1} and 1.85 M^{-1}, respectively. Quenching constants for KI of both fluorescent centers were approximately 1 M^{-1}. These data suggest that both fluorophores are poorly accessible to the external anionic contact quencher but that an unchanged quencher, while larger, is still better able to penetrate the enzyme's active site. The extrapolated anisotropies (r_0) for ferrochelatase-bound MIANS and tryptophan are 0.198 and 0.307. The dissociation constant (K_D) determined by fluorescence anisotropy of protoporphyrin was 1.5 μM with the calculated number of porphyrin binding sites as 1.0 per 40000 daltons. A model is presented for the active site of ferrochelatase based upon the data presented here and previously. This model proposes that the active site is a hydrophobic pocket similar in nature to the heme binding crevices found in many hemoproteins.

The terminal step of the heme biosynthetic pathway is the insertion of ferrous iron into the porphyrin macrocycle to form protoheme IX. Ferrochelatase (protoheme ferrolyase, EC 4.99.1.1), the enzyme that catalyzes this step, is bound to the inner mitochondrial membrane in eukaryotes (Jones & Jones, 1969; Harbin & Dailey, 1985) and to the cytoplasmic membrane of prokaryotes (Dailey, 1982). Recently, the enzyme has been purified to homogeneity from a variety of sources (Taketani & Tokunaga, 1981, 1982; Dailey, 1982; Dailey & Fleming, 1983; Hanson & Dailey, 1984). With the availability of pure enzyme, the kinetic mechanism (Dailey & Fleming, 1983) and the role of enzyme sulfhydryl residues (Dailey,

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