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Chromatium vinosum Cytochrome *c*-552. Reduction by Photoreduced Flavins and Intramolecular Electron Transfer[†]

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ABSTRACT: Cytochrome *c*-552 from *Chromatium vinosum* is an unusual heme protein in that it contains two hemes and one flavin per molecule. To investigate whether intramolecular electron transfer occurs in this protein, we have studied its reduction by external photoreduced flavin by using pulsed-laser excitation. This approach allows us to measure reduction kinetics on the microsecond time scale. Both fully reduced lumiflavin and lumiflavin semiquinone radical reduce cytochrome *c*-552 with second-order rate constants of approximately $1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Kinetic and spectral data and the results of similar studies with riboflavin indicate that both the flavin and heme moieties of

cytochrome *c*-552 are reduced simultaneously on a millisecond time scale, with the transient formation of a protein-bound flavin anion radical. This is suggested to be due to rapid intramolecular electron transfer. Further, steric restrictions play an important role in the reduction reaction. Studies were conducted on the redox processes following photolysis of CO-ferrocyanochrome *c*-552 in which the flavin was partly oxidized to resolve the kinetics of electron transfer between the heme and flavin of cytochrome *c*-552. Based on these results, we conclude that intramolecular electron transfer from ferrous heme to oxidized flavin occurs with a first-order rate constant of greater than $1.4 \times 10^6 \text{ s}^{-1}$.

Chromatium vinosum cytochrome *c*-552 is a low-potential flavin containing *c*-type cytochrome (Bartsch, 1978). Its molecular weight is 72 000, consisting of a flavin-containing subunit ($M_r \sim 45\,000$) and two heme-containing subunits ($M_r \sim 15\,000$ each) (Kennel, 1971; Brown and Cusanovich, unpublished observations). The native protein has an absorption spectrum which is consistent with that of a mixture of flavin

and low-spin heme *c*. The flavin moiety has been shown to be FAD (Hendriks & Cronin, 1971), with the 8 α -methylene group of the flavin covalently bound to the sulfur of a protein cysteinyl side chain (Walker et al., 1974; Kenney et al., 1974; Kenney & Singer, 1977). *Chromatium vinosum* cytochrome *c*-552 can catalyze the oxidation of sulfide with a variety of electron acceptors (Fukumori & Yamanaka, 1979), suggesting that its physiological function is that of sulfide dehydrogenase.

The oxidation-reduction potential of both the heme and flavin moieties is approximately 30 mV (Vorkink, 1972; Brown and Cusanovich, unpublished observations), and there is no evidence for formation of a flavin semiquinone during redox titrations. The circular dichroism (CD) spectrum of *Chro-*

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matium cytochrome *c*-552 is unusual for *c*-type cytochromes and has led to the suggestion that the two hemes interact (Bartsch et al., 1968). More recently, electron paramagnetic resonance (EPR) studies (Strekas, 1976) have suggested that the two hemes are in nonidentical environments and that a heme-flavin interaction exists. The ferrohemes of cytochrome *c*-552 bind carbon monoxide, but with extinction coefficients which suggest only one CO complex per protein molecule. On the basis of this observation and the CD properties, it has been proposed that the CO molecule may be shared by the two hemes (Bartsch et al., 1968). Further, the oxidized flavin moiety of cytochrome *c*-552 can bind a variety of ligands, including CN^- , HSO_3^- , and $\text{S}_2\text{O}_3^{2-}$, leading to a loss of flavin absorbance below 500 nm and formation of a charge-transfer band above 650 nm (Meyer, 1970; Meyer & Bartsch, 1976; Yamanaka & Kusai, 1976).

In summation, cytochrome *c*-552 is an atypical *c*-type cytochrome with unusual properties. Studies reported to date indicate that the heme and flavin moieties are oriented to permit interactions between them, suggesting the possibility that rapid electron transfer between chromophores could take place. In this communication, we report studies on the reduction of cytochrome *c*-552 which provide information on the kinetics and mechanism of electron transfer to and within this cytochrome. For this work, we have utilized photoreduced free flavin as the reducing agent as it can be formed in less than 1 μs by a pulse of light, thus avoiding the time limitations imposed by mixing methods. As will be documented below, the free flavin redox potentials are such as to make this reduction a thermodynamically favorable reaction. In addition, pulsed-light excitation was used to photodissociate the CO-heme complex of partially reduced cytochrome *c*-552 and thus initiate intramolecular electron transfer.

Experimental Procedures

Chromatium vinosum cytochrome *c*-552 was isolated as previously described (Cusanovich, 1967). As prepared, the oxidized protein has absorbance ratios of 0.54 (A_{278}/A_{410}) and 1.26 (A_{480}/A_{523}) which have been established as values consistent with those of purified cytochrome *c*-552 containing its normal complement of chromophores (Bartsch, 1978; Vorkink, 1972). Figure 1 presents the oxidized and reduced absorption spectra of cytochrome *c*-552. All experiments were conducted anaerobically in cuvettes sealed with serum stoppers with buffer deaerated by bubbling for at least 20 min with water-saturated argon gas. Subsequent additions were made via the septum with a syringe. Photoreduction of cytochrome *c*-552 was studied in a buffer consisting of 20 mM potassium phosphate, 10 mM EDTA, and 20 μM lumiflavin or riboflavin, pH 7.0. For experiments in the presence of CO, the buffer was as described above except that the added flavin concentration was 0.5 μM . All experiments were conducted at 25 $^\circ\text{C}$, and care was taken to keep the samples in the dark prior to laser excitation.

Laser photoexcitation was carried out with a nitrogen laser pumped dye solution to excite either flavin or heme [dyes used were either 2,5-bis[2-(5-benzoxazolyl)thiophene], λ_{max} 436 nm, or *p*-bis(*o*-methylstyryl)benzene, λ_{max} 420 nm]. As the heme absorption dominates at 420 nm, excitation at this wavelength was used to drive dissociation of CO from the heme-CO complex. For photoreduction of exogenous flavin, the 436-nm excitation wavelength was used, as heme has significantly less absorption at this wavelength. The photoproduced exogenous flavin radical (via reaction of the flavin triplet with EDTA) produced fully reduced exogenous flavin by disproportionation. At the pH values studied, free lumiflavin has an ab-

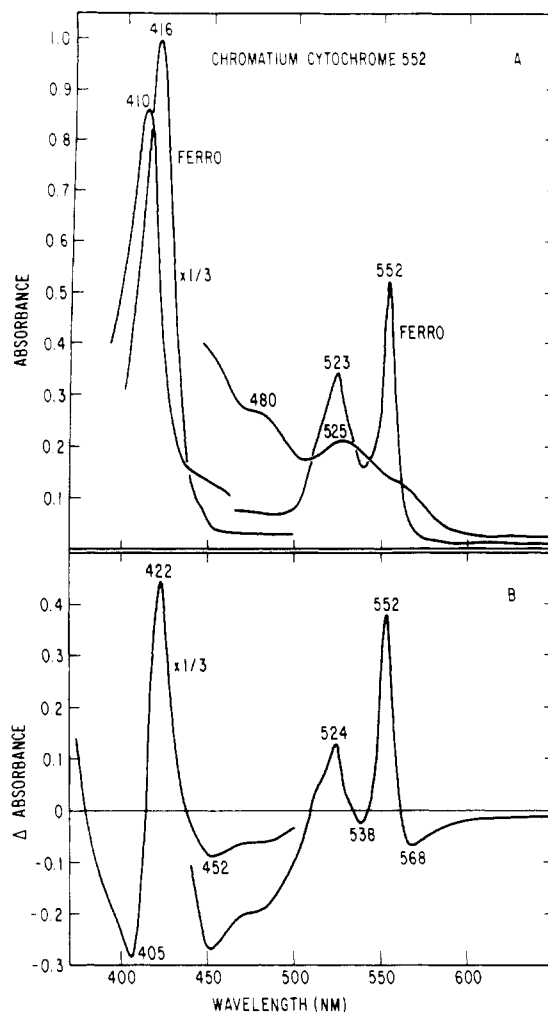


FIGURE 1: Absorption spectrum of cytochrome *c*-552. (A) Oxidized and reduced cytochrome *c*-552. Buffer: 20 mM potassium phosphate, pH 7.0. The fully reduced cytochrome was obtained via reduction with a slight excess of sodium dithionite. (B) Reduced minus oxidized difference spectrum. Conditions were as described in (A).

sorption maximum at 445 nm. The lumiflavin radical is predominately in its neutral form and absorbs maximally at 560 nm (Vaish & Tollin, 1971). Fully reduced lumiflavin has only minimal absorption beyond 400 nm. The detection apparatus was as described previously (Tollin et al., 1979) with the exception that a Biomation 8100 waveform recorder was used as a buffer between the detector and the signal averager. Under the experimental conditions used, the time resolution of the apparatus was 1.0 μs (limited mainly by scattering artifacts). Data were obtained by averaging 2–4 flashes for reduction or 500–1000 flashes for photolysis of CO.

Results

Flavin Reduction of Oxidized Cytochrome *c*-552. Figure 2A presents a typical reaction trace for the reduction of cytochrome *c*-552 by laser-photoreduced lumiflavin. The corresponding log (ΔA) vs. time plot is given in Figure 2B. In the absence of added lumiflavin, no detectable absorption changes take place on laser excitation of the sample. The reduction of cytochrome *c*-552 was biphasic, with an initial phase occurring rapidly, followed by a second slower phase which was first order for four half-lives at all cytochrome concentrations used. The kinetic nature of the rapid phase could not be determined due to the overlapping of the decay of the lumiflavin radical transient (L^{\cdot}H). Figure 3 presents

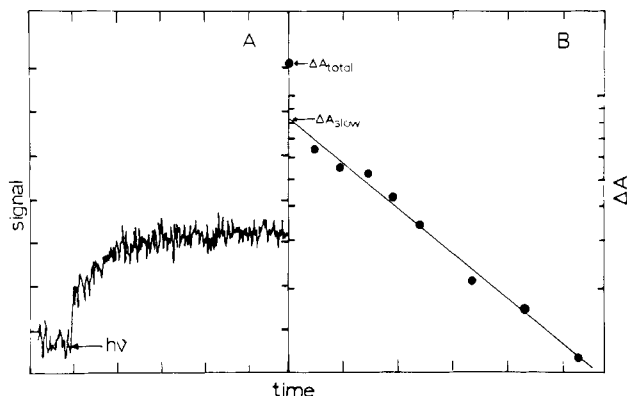


FIGURE 2: Reduction of cytochrome *c*-552 by photoreduced lumiflavin. (A) The signal was in volts and is presented in arbitrary units. The time increment is 7.7 ms/division. The cytochrome *c*-552 concentration was 26 μ M, and the buffer was 20 mM potassium phosphate, 10 mM EDTA, and 20 μ M lumiflavin, pH 7.0, 25 $^{\circ}$ C. The reaction was monitored at 552 nm with excitation at 436 nm; four flashes were averaged. (B) Semilog plot of the data given in Figure 2A after conversion of the signal to ΔA . All conditions were as described in Figure 2A.

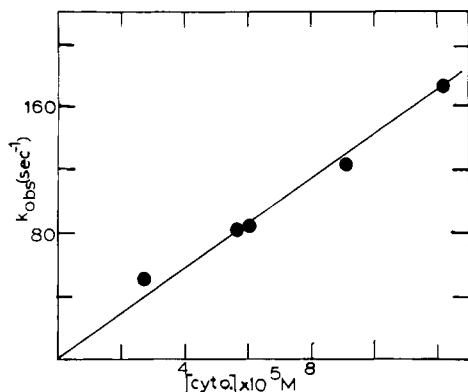


FIGURE 3: Second-order plot for the reduction of cytochrome *c*-552 by photoreduced lumiflavin, slow phase. Conditions were as described in Figure 2 except that the cytochrome concentration was varied to obtain different values of k_{obsd} .

a plot of the observed rate constant (k_{obsd}) for the second phase against the cytochrome *c*-552 concentration. Variation of the observed rate constant at any cytochrome concentration was found to be less than 10%. On the basis of these data, it can be concluded that this portion of cytochrome reduction is a second-order process with a rate constant of $1.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. Further, the observed rate constant was independent of the wavelength at which the reaction was monitored, with the fraction of the initial phase typically 20–40% of the total absorbance change.

Figure 4 presents a time vs. voltage trace for the decay of the flash-produced lumiflavin radical in the absence of cytochrome *c*-552. This decay results from disproportionation with the formation of fully reduced lumiflavin (LfH_2) and is rapid (Vaish & Tollin, 1971), with a $t_{1/2}$ of approximately 38 μ s as opposed to $t_{1/2}$ values of 15.4–3.3 ms for cytochrome *c*-552 reduction (slow phase), depending on cytochrome concentration. These observations establish that the slow phase for cytochrome *c*-552 reduction is due to reaction with LfH_2 ($E_{m,7} = -220 \text{ mV}$; Clark, 1960) and not $\text{LfH}\cdot$. Thus, it can be concluded that the fast phase of cytochrome *c*-552 reduction must reflect reaction with $\text{LfH}\cdot$ ($E_{m,7} = -240 \text{ mV}$; Draper & Ingraham, 1968). Quantitation of the size of the lumiflavin radical signal and the coupled fast phase indicate that the amount of heme reduced during the lifetime of the radical is only about 3% of the initial radical concentration. As the

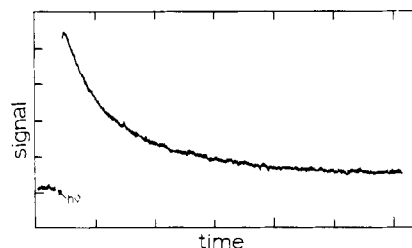


FIGURE 4: Decay of lumiflavin radical. The signal is in arbitrary units. The time increment is 77 μ s/division. The reaction conditions were as described in Figure 2 except that cytochrome *c*-552 was omitted.

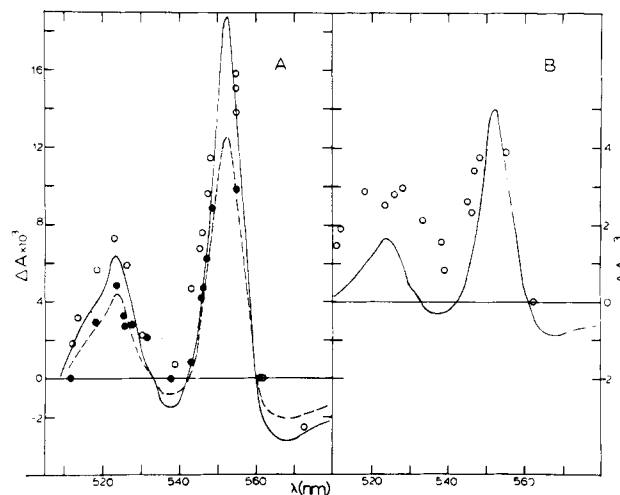


FIGURE 5: Difference spectra for the reduction of cytochrome *c*-552 by photoreduced lumiflavin. (A) Open circles represent the total absorbance change at a particular wavelength, closed circles the absorbance change due to the second kinetic species. Conditions were as given in Figure 2. The solid and dashed lines were drawn by normalizing a reduced minus oxidized difference spectrum for cytochrome *c*-552 to the observed absorbance change at 555 nm. (B) The open circles are the difference in ΔA between the total absorbance change and that due to the second kinetic species. Conditions were as described in Figure 5A.

second-order rate constant for $\text{LfH}\cdot$ decay by disproportionation is $6.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Vaish & Tollin, 1971), the rate constant for the fast reduction can be estimated to be 3% of this or $1.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 5 presents difference spectra for the reduction of cytochrome *c*-552 by photoreduced lumiflavin. Figure 5A includes data for both the total absorbance change and the absorbance change for the slow kinetic phase. In each case, the line is derived from the reduced–oxidized difference spectrum and normalized to the kinetic data at 555 nm, a wavelength at which a sizable absorbance change occurs but off the peak to minimize bandwidth problems. Within experimental error, the kinetic data and the oxidized–reduced difference spectrum agree well for the slow kinetic phase. However, the total absorbance change is larger at wavelengths below 550 nm than is expected from the difference spectrum. This is seen more clearly in Figure 5B where the absorption change due to the fast phase is given, with the solid line calculated as described for figure 5A. These results demonstrate that, in addition to a component due to heme reduction, the fast spectral change contains a component with an absorption maximum below 540 nm. As this spectral change is observed during the lifetime of the lumiflavin radical, a one-electron reductant, the absorption below 540 nm not accounted for by heme could reflect a protein-bound flavin radical. Since this species does not have absorbance above 560 nm as expected for a flavin neutral radical (Vaish &

Tollin, 1971), a flavin anion radical is suggested (Yagi, 1975).

Some studies were conducted monitoring the reduction at 475 nm, a wavelength at which flavin makes a major contribution to the absorbance change on reduction (Figure 1). Reaction with photoreduced lumiflavin occurred as described for the heme wavelengths, with an initial fast phase followed by a second slower phase having an apparent rate constant identical with that found at higher monitoring wavelengths. The 450–500-nm wavelength region could not be studied extensively as the monitoring beam reduced the added lumiflavin. Nevertheless, no evidence for any kinetic species different from those observed at higher wavelengths was obtained, as might be expected if flavin was being reduced at a rate different from the heme. In addition, illumination of cytochrome *c*-552 with white light for various periods of time in the presence of 0.5 μ M lumiflavin and 10 mM EDTA yielded an equivalent extent of reduction of the heme and flavin moieties.

Experiments were conducted with riboflavin as the exogenous flavin to extend the studies described to this point. The distribution between fast and slow phase and the wavelength dependence were identical with those described for lumiflavin. However, the apparent second-order rate constant for fully reduced riboflavin reduction of cytochrome *c*-552 (slow phase) was approximately 70% of that found for lumiflavin ($1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$).

Intramolecular Electron Transfer between the Flavin and Heme Moieties of Cytochrome *c*-552. The results described above bear on the transfer of electrons between free flavins and the heme and flavin moieties of cytochrome *c*-552 and were consistent with either rapid intramolecular electron transfers or essentially identical rate constants for heme and flavin reduction. To distinguish between these possibilities, we took advantage of the fact that the ferroheme of cytochrome *c*-552 will bind CO. Thus, a solution of cytochrome *c*-552 was illuminated (white light) in the presence of 1 atm of carbon monoxide and a trace amount of lumiflavin (0.5 μ M) relative to cytochrome (50 μ M). In this manner, a cytochrome *c*-552 solution could be obtained with the heme and flavin moieties 25–50% reduced and with the reduced heme partially complexed with CO. By using *p*-bis(*o*-methylstyryl)benzene for laser excitation (λ 420 nm), we then determined if the ferrous heme formed on photodissociation of CO was capable of undergoing oxidation coupled to the reduction of protein-bound flavin. As will be described more completely below, if internal reequilibration of electrons occurs, it would be expected that about 63% of the absorbance change observed on photolytic release of the carbon monoxide would result from formation of ferrous heme, and approximately 37% of the absorption change would be due to a subsequent oxidation of ferrous heme coupled to reduction of flavin. If the latter change occurred in a time longer than 1 μ s, we would expect to see a biphasic signal.

Voltage vs. time traces for the photolysis of CO–cytochrome *c*-552 demonstrated that the reaction is extremely rapid, with a maximum $t_{1/2}$ of approximately 0.5 μ s (instrument limited). No significant slower absorbance changes could be detected. Thus, the kinetics are not resolvable into two separate steps. Figure 6 presents the observed total absorbance change (open circles) as a function of wavelength. (Under the conditions of this experiment, excitation wavelength 420 nm and lumiflavin concentration 0.5 μ M, no transients due to exogenous LfH $^{\cdot-}$ could be detected.) The solid line through the data points was hand drawn for clarity, and the dashed line is the spectrum expected if the only reaction taking place was that due to the formation of ferrous cytochrome *c*-552 on CO release. For

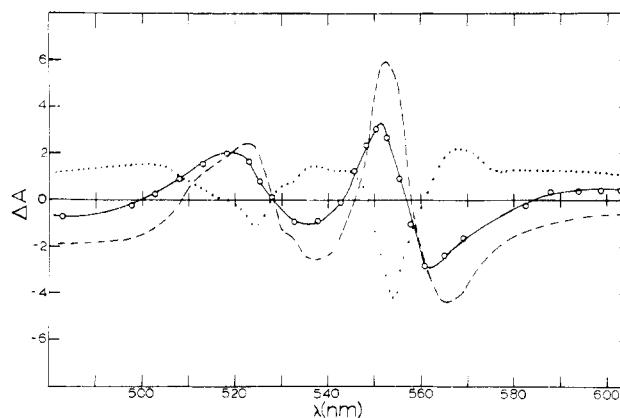


FIGURE 6: Difference spectrum for the photolysis of CO–ferrocyanochrome *c*-552. The ΔA values are times 10^3 ; the cytochrome *c*-552 concentration was 26 μ M; the buffer was 20 mM potassium phosphate, 10 mM EDTA, and 0.5 μ M lumiflavin, pH 7.0, 25 °C. The reaction was initiated by excitation at 420 nm, and 720 flashes were averaged at each wavelength where data were obtained. Open circles are the data points with the solid line hand drawn for clarity. The dashed line represents a CO–ferrocyanochrome *c*-552 difference spectrum obtained by normalizing the spectral data to the flash photolysis data at 561 nm. The dotted line presents the difference between the observed (solid line) and calculated (dashed line) spectra.

this calculation, the difference spectrum was normalized to the experimental data at 561 nm, as this wavelength is an isosbestic point for the oxidation of ferrocyanochrome *c*-552. If internal electron transfer occurs, subtraction of the calculated spectrum from the observed spectrum should yield the ferrocyanochrome–ferriheme *c*-552 difference spectrum. The dotted line in Figure 6 gives the difference between the curve expected if only CO release were occurring (dashed line) and the observed (solid line) difference spectrum. This spectrum has a general shape consistent with a ferroheme–ferriheme difference spectrum (see Figure 1), as well as an absorbance change below 500 nm corresponding to protein-bound flavin reduction. Thus, we conclude that an internal redox reaction took place upon CO release.

For the data given in Figure 6 (dotted line), the β (523 nm) to α (522 nm) peak ratio is 0.28, which is less than the 0.34 expected (see Figure 1). This suggests that a component absorbing below 550 nm is present which reduces the magnitude of the decrease in absorbance at 523 nm. Such a component could be a protein-bound flavin anion radical, as would be expected from the previous results. Unfortunately, the quality of the data resulting from the experimental errors involved and the difficulties inherent in subtracting calculated and observed spectra preclude a precise quantitative analysis of this component. Nevertheless, the data presented support the conclusion that a fraction ($\sim 20\%$ based on known extinction coefficients) of the ferrous heme formed on photolysis is oxidized with a coupled reduction of protein-bound flavin and that this process has a $t_{1/2}$ of 0.5 μ s or less.

Discussion

The results presented here establish that both LfH $^{\cdot-}$ and LfH $_2$ are capable of reducing cytochrome *c*-552. Reduction of both heme and protein-bound flavin by LfH $^{\cdot-}$ is rapid ($\sim 1.9 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$). This is in sharp contrast to horse heart cytochrome *c* for which the rate constant for reduction by LfH $^{\cdot-}$ is less than $6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Vaish & Tollin, 1971). Thus, either the cytochrome *c*-552 flavin is reduced rapidly by LfH $^{\cdot-}$, with the electron equilibrating between the protein-bound flavin radical and the heme in an intramolecular process, or the heme moiety of cytochrome *c*-552 is much more available

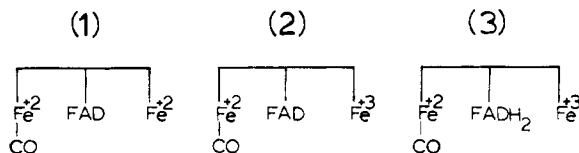


FIGURE 7: CO-ferrocyanochrome *c*-552 species existing at equilibrium. FAD and FADH₂ represent oxidized and reduced protein-bound flavin. Fe²⁺ and Fe³⁺ represent reduced and oxidized heme.

to the LfH[•] than that of horse heart cytochrome *c*. The reduction of horse heart cytochrome *c* by LfH₂ has a rate constant of $2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Jung and Tollin, unpublished observations), which is an order of magnitude larger than the rate constant which we have obtained for cytochrome *c*-552 reduction. This result demonstrates that LfH₂ finds the heme moiety of cytochrome *c* more accessible than that of cytochrome *c*-552 and would argue for the first alternative mentioned above, i.e., a rapid internal equilibrium of electrons upon LfH[•] reduction. The rate constants for both horse heart cytochrome *c* and cytochrome *c*-552 reduction by LfH₂ are well below the diffusion-controlled limit and suggest that steric constraints play a role in both reactions. Indeed, the experiments reported here for reduction of cytochrome *c*-552 by reduced riboflavin (RfH₂) support this view as the rate constant for RfH₂ reduction is approximately 30% less than for LfH₂ reduction. Thus, we conclude that steric factors involving the ribityl side chain are important in the interaction of the reduced flavin molecule and the cytochrome.

It is important to note that for reduction by LfH₂, no evidence was obtained that protein-bound flavin is reduced with a rate constant different from that for heme reduction. This means either that electrons equilibrate rapidly between the heme and flavin moieties or that the heme and flavin react with LfH₂ with identical rate constants. Hence, assuming the former is true, we can put a lower limit on the rate constant for electron transfer between the heme and flavin of cytochrome *c*-552 of approximately 200 s^{-1} . Further, the difference spectra suggest that during the lifetime of LfH[•] some protein-bound flavin radical is formed, as there is a significantly larger increase in absorbance at wavelengths below 540 nm than expected from the oxidized minus reduced difference spectra. The lack of "extra" absorbance above 550 nm argues that a protein-bound flavin anion radical is formed transiently. An accurate quantitative estimate of the amount of this species is not possible due to uncertainties in the extinction coefficients.

Photolysis of heme-bound CO, under the conditions used here, i.e., approximately 50% reduced cytochrome *c*-552, will lead to a mixture of molecular species. As the exact number of CO molecules bound per molecule of cytochrome *c*-552 is not known, some uncertainties exist. Nevertheless, with the proposal that one CO is bound per two hemes (Bartsch et al., 1968), the photoactive species (i.e., those with Fe²⁺-CO) capable of intramolecular electron transfer (i.e., with oxidized flavin) which are expected to predominate are as given in Figure 7. No evidence exists for protein-bound flavin radicals at equilibrium; hence, such species are not included. Based on the fact that the protein was approximately 50% reduced for these experiments, we would expect that half of the molecules capable of intramolecular electron transfer prior to photolysis would be as species 1 in Figure 7 (since this is the only half-reduced species), with the other half distributed between species 2 and 3. Thus, immediately following photolysis, 75% of the molecules formed by CO release would contain oxidized flavin. A maximum of 37% of the ferrous heme formed on photolysis would be oxidized in a spectrally detectable fashion based on the distribution of species described

above, assuming that electrons distribute equally among the chromophores, as would be expected if the heme and flavin potentials are equal. The data reported are qualitatively consistent with that expectation, but with only approximately 20% of the ferrous heme oxidized and coupled to flavin reduction. This amount of heme oxidation is not unreasonable, considering the assumptions made and the difficulties involved in subtracting the calculated and measured spectra (Figure 6). Thus, we conclude that intramolecular electron transfer occurs with a minimum first-order rate constant of $1.4 \times 10^6 \text{ s}^{-1}$. This value is quite large and is thus consistent with the proposal that heme and flavin moieties are in close proximity (Strekas, 1976).

To the best of our knowledge, the electron transfer between the heme and flavin moieties reported here is the most rapid of any known biological system in which electron transfer occurs between the normal ground-state forms of electron carriers. In the absence of any structural information, nothing can be said concerning the distance or relative orientation of the chromophores of cytochrome *c*-552. However, the data presented suggest that this protein provides a most interesting system for studying the factors which control biological electron-transfer processes.

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