(Washington, D.C.) 213, 1497–1501.
Rao, A. (1979) J. Biol. Chem. 254, 3503–3511.
Reed, D. W., Raveed, D., & Reporter, M. (1975) Biochim. Biophys. Acta 387, 368–378.
Rosen, D. (1979) Ph.D. Thesis, University of California, San Diego.
Rosen, D., Okamura, M. Y., Feher, G., Steiner, L. A., & Walker, J. E. (1977) Biophys. J. 17, 67a.
Rosen, D., Feher, G., & Steiner, L. (1980) Fed. Proc., Fed. Am. Soc. Exp. Biol. 39, 1801.
Sauer, R. T., Pan, J., Hopper, P., Hehir, K., Brown, J., & Poteete, A. R. (1981) Biochemistry 20, 3591–3598.
Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., & Ballantyne, D. L. (1971) Biochemistry 10, 4912–4921.
Steck, T. L., Koziarz, J. J., Singh, M. K., Reddy, G., & Köhler, H. (1978) Biochemistry 17, 1216–1222.
Steiner, L. A., Okamura, M. Y., Lopes, A. D., Moskowitz, E., & Feher, G. (1974a) Biochemistry 13, 1403–1410.
Steiner, L. A., Lopes, A. D., Okamura, M. Y., Ackerson, L. C., & Feher, G. (1974b) Fed. Proc., Fed. Am. Soc. Exp. Biol. 33, 1461.
Summers, M. R., Smythers, G. W., & Oroszlan, S. (1973) Anal. Biochem. 53, 624–628.
Sutton, M. R., Steiner, L. A., Abresch, E. C., & Feher, G. (1981) Biophys. J. 33, 19a.
Unwin, P. N. T., & Henderson, R. (1975) J. Mol. Biol. 94, 425–440.
Valkirs, G., & Feher, G. (1981) Biophys. J. 33, 18a.
Valkirs, G. E., & Feher, G. (1982) J. Cell Biol. (in press).
Valkirs, G., Rosen, D., Tokuyasu, K. T., & Feher, G. (1976) Biophys. J. 16, 223a.
Von Heijne, G. (1981) Eur. J. Biochem. 120, 275–278.
Waller, J.-P. (1963) J. Mol. Biol. 7, 483–496.
Williams, D. G., Jenkins, R. E., & Tanner, M. J. A. (1979) Biochem J. 181, 477–493.
Zürrer, H., Snozzi, M., Hanselmann, K., & Bachofen, R. (1977) Biochim. Biophys. Acta 460, 273–279.

Intramolecular Electron Transfer in *Chlorobium thiosulfatophilum* Flavocytochrome *c*†

Gordon Tollin,* Terrance E. Meyer, and Michael A. Cusanovich

ABSTRACT: The electron-transfer reactions of photoproduced lumiflavin semiquinone and fully reduced lumiflavin with oxidized *Chlorobium thiosulfatophilum* flavocytochrome *c* have been studied by using laser flash photolysis. The *Chlorobium* flavocytochrome *c* contains one heme and one flavin per M_r 50000, and thus the possibility exists for intramolecular electron transfer. We find a complex kinetic pattern which is consistent with the transient formation of a spectrally perturbed protein-bound flavin semiquinone which transfers an electron intramolecularly to the heme ($k = 1 \times 10^3$–1.8×10^3 s^{-1} for the neutral semiquinone, depending upon the pH). Evidence is presented that electron transfer from exogenous lumiflavin to the heme moiety occurs through the protein-bound flavin. We have also performed redox titrations which determine the midpoint potentials of the heme and flavin prosthetic groups at various pH values and the p*K* values for the semiquinone (6.4) and fully reduced flavin (6.1). Thus, at pH 7, the semiquinone is predominantly in the anionic form at equilibrium. The reactions of *Chlorobium* flavocytochrome *c* with photoreduced lumiflavin are similar to those previously found with *Chromatium vinosum* flavocytochrome *c* [Cusanovich, M. A., & Tollin, G. (1981) *Biochemistry 19*, 3343–3347] in that a protein-bound flavin semiquinone is an intermediate in the pathway of reduction. However, the rate constants are substantially different. As a class, the flavocytochromes *c* appear to operate by analogous mechanisms involving rapid intramolecular transfer between the heme and flavin moieties.

In a recent publication (Cusanovich & Tollin, 1980) kinetic studies of electron transfer from fully reduced lumiflavin (LFH^-) and lumiflavin semiquinone radical ($LFH\cdot$) generated by laser flash photolysis to *Chromatium vinosum* cytochrome *c*-552 (flavocytochrome *c*) were reported. This cytochrome is representative of a class of low-potential flavin-containing heme proteins which have been isolated from four species of purple and green phototrophic bacteria (Bartsch, 1978). It was found that both the heme and flavin moieties of *Chr. vinosum* flavocytochrome *c* were reduced simultaneously on a millisecond time scale, with the transient formation of a protein-bound flavin anion radical. Further, studies on the redox process following photolysis of the CO–ferrocytochrome complex in which the flavin was partly oxidized established that intramolecular electron transfer from ferrous heme to oxidized flavin occurred with a first-order rate constant of greater than 1.4×10^6 s^{-1}.

Cytochrome *c*-553 from *Chlorobium thiosulfatophilum* is also a member of the low-potential flavin-containing cytochrome class discussed above. However, this protein contains one heme and one flavin per *Mr* 50000, as opposed to two hemes and one flavin for the *Chr. vinosum* flavocytochrome (Bartsch et al., 1978). In addition, the midpoint oxidation–reduction potential of the *Chlorobium* flavocytochrome is approximately 100 mV compared to 35 mV for the *Chromatium* protein (Bartsch et al., 1978; Meyer et al., 1968; Vorkink, 1972). Recent studies (Kusai & Yamanaka, 1973;

† From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721 (G.T. and M.A.C.), and the Department of Chemistry, University of California at San Diego, La Jolla, California 92093 (T.E.M.). *Received November 12, 1981; revised manuscript received March 24, 1982.* This research was supported in part by U.S. Public Health Service Grants AM-15057 and GM-18528 and National Science Foundation Grant PCM-7804349.

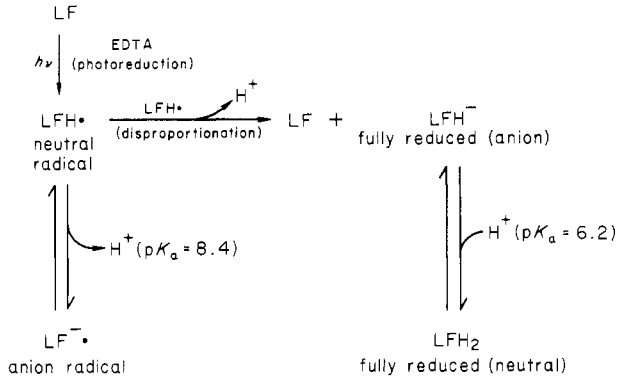
0006-2960/82/0421-3849$01.25/0

Fukumori & Yamanaka, 1979 have suggested that both the *Chromatium* and *Chlorobium* flavocytochromes can function as sulfide dehydrogenases.

In order to extend our previous studies (Cusanovich & Tollin, 1980) and to obtain additional information on intramolecular electron transfer in this class of enzymes, we have investigated the reduction of *Chlorobium* flavocytochrome *c* by photoreduced lumiflavin (both fully reduced and semiquinone). As will be demonstrated below these studies have allowed us to directly measure intramolecular electron transfer from the flavin to the heme moiety of this protein.

Experimental Procedures

Chlorobium thiosulfatophilum strain Tassajara cytochrome *c*-553 (flavocytochrome *c*) was prepared as described by Meyer et al. (1968). The cytochrome used had a flavin/heme absorbance ratio, A_{475}/A_{525}nm = 1.50, typical of the native protein (Meyer & Bartsch, 1976). All experiments were conducted anaerobically in cuvettes sealed with serum stoppers which were deaerated by bubbling with water-saturated argon gas. The buffer used for the experiments was 20 mM potassium phosphate, 10 mM ethylenediaminetetraacetic acid (EDTA), and 20 μM lumiflavin, pH 7.0, and the temperature was 25 °C. Laser photoexcitation was carried out with a nitrogen laser-pumped dye solution [2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene, λ_{max} 436 nm], as described previously (Cusanovich & Tollin, 1980). The detection apparatus was also as described previously (Tollin et al., 1979; Cusanovich & Tollin, 1980). The relevant redox and protonation reactions (Holmström, 1964; Vaish & Tollin, 1970, 1971) for free lumiflavin are as follows:



The generation of LFH· by the laser pulse occurs in <1 μs, whereas the half-time for the disproportionation reaction is several hundred microseconds (depending upon conditions). Thus, electron transfers from LFH· and LFH^- to the flavocytochrome are well separated in time and can be followed independently (Ahmad et al., 1981). For the experimental conditions used, the time resolution of the apparatus was 1.0 μs (limited by scattering and fluorescence artifacts), and typically eight flashes were averaged in each experiment. Oxidation–reduction titrations were carried out anaerobically by using the Fe–EDTA couple as described previously (Meyer, 1970).

Results

Figure 1 presents the reduced minus oxidized difference spectrum for *Chlorobium* flavocytochrome *c* (solid line). As can be seen, at wavelengths above 500 nm the difference spectrum is typical of *c*-type cytochromes. However, below 500 nm a loss of absorbance is observed due in large part to reduction of the flavin moiety. When a solution containing

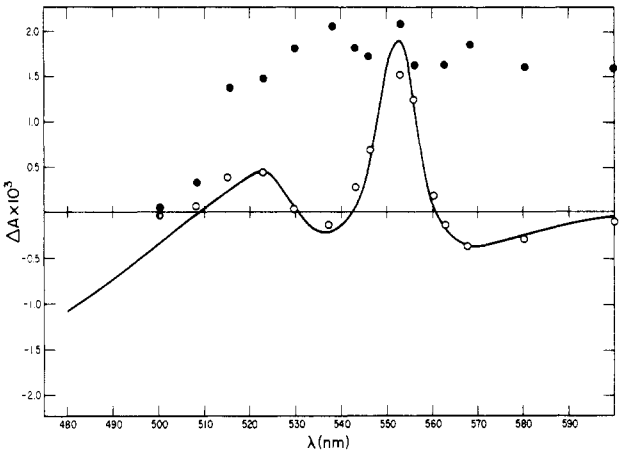


FIGURE 1: Flavocytochrome *c*, reduced minus oxidized spectra. (Solid line) Reduced minus oxidized spectra; (open circles) total absorbance change following flash (absorbance at t = infinity minus absorbance prior to flash); (filled circles) absorbance change just after flash minus absorbance prior to flash. Buffer: 20 mM potassium phosphate, 10 mM EDTA, 20 μM lumiflavin, pH 7.0, 25 °C. The difference spectrum was normalized to the total absorbance change observed at 567 nm.

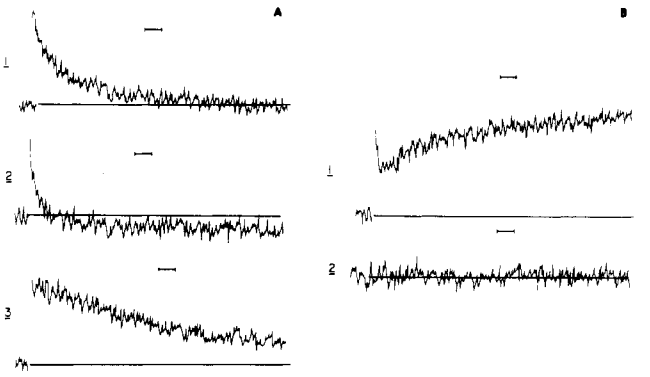


FIGURE 2: Reduction of flavocytochrome *c* by photoreduced lumiflavin. The signals were in volts and are presented on the same scale. Except for curve 3 in part A, the average of eight flashes is given. The buffer was 20 mM potassium phosphate , 10 mM EDTA, 20 μM lumiflavin, pH 7.0. Flavocytochrome *c*, 11 μM, 25 °C. (A) Kinetics at 600 nm. (Curve 1) Time increment 0.59 ms/division; (curve 2) time increment 5.9 ms/division; (curve 3) time increment 0.59 ms/division, no flavocytochrome, 16 flashes. (B) Kinetics at 553 nm. (Curve 1) Time increment 5.9 ms/division; (curve 2) lumiflavin omitted.

lumiflavin, EDTA, and the flavocytochrome is subjected to laser flash photolysis to produce lumiflavin semiquinone radical, complex kinetics are observed. Typical kinetic traces at pH 7.0 are presented in Figure 2 for the reaction monitored at two different wavelengths. At 600 nm, a species is formed during the flash (initial fast rise) which rapidly decays (Figure 2A, curve 1). This would be expected for the formation and disproportionation of the lumiflavin neutral semiquinone radical ($2LFH\cdot \rightarrow LF + LFH^- + H^+$) (Vaish & Tollin, 1971). Also, as would be expected if LFH^- reduction of flavocytochrome *c* were occurring (Ahmad et al., 1981), at later times a small net decrease in absorbance is observed (Figure 2A, curve 2) due to heme reduction (see Figure 1). However, laser flash photolysis of an identical solution but in the absence of flavocytochrome *c* yields a positive transient signal of smaller size and with a much slower decay (Figure 2A, curve 3) which does not terminate below the base line (note that the signal displayed in curve 3 of Figure 2A was obtained with twice as many flashes as that in curve 1; see below). Moreover, the decay observed in the absence of cytochrome is second order, consistent with disproportionation, whereas the decay observed

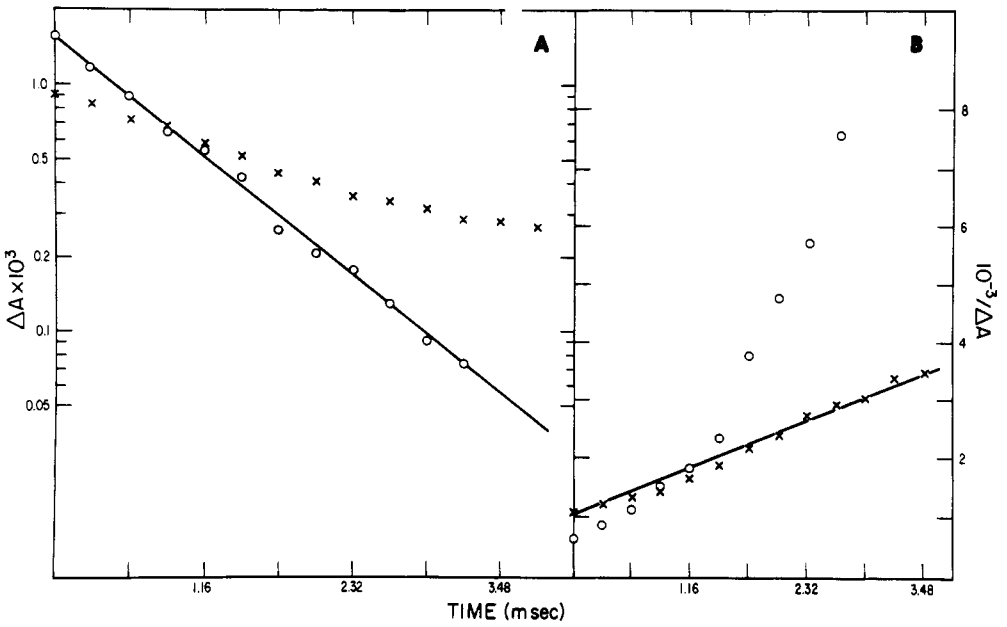


FIGURE 3: Kinetics at 600 nm. (A) Plot of ln ΔA vs. time. (O) Data in Figure 2A, curve 1; (×) data in Figure 2A, curve 3. (B) Plot of $1/\Delta A$ vs. time. Symbols as in part A.

in the presence of cytochrome is first order. These differences are shown in Figure 3 where the data from curves 1 and 3 from Figure 2A are plotted for both pseudo-first-order (Figure 3A) and second-order (Figure 3B) kinetic processes. It is apparent therefore that the rapidly decaying transient species which is generated by the laser flash (as observed at 600 nm) has a larger signal amplitude and altered decay kinetics compared with those of the lumiflavin semiquinone formed in the absence of flavocytochrome *c*. The size of the 600-nm transient is dependent upon the amount of heme reduced prior to the flash, i.e., the more heme reduction, the smaller the signal. By measuring the amount of signal produced by successive laser flash experiments on the same sample, and at the same time spectrophotometrically measuring the amount of heme reduction which occurred, we can obtain a value for the signal size at zero heme reduction by extrapolation. Correcting this for the decrease in the amplitude of the lumiflavin semiquinone signal caused by absorption of laser exciting light by the flavocytochrome (this was determined to be approximately 50% by attentuating the laser beam with an 11 μM solution of the flavocytochrome), we estimate that the size of the 600-nm transient signal is 5–6 times larger than that due solely to lumiflavin semiquinone. Since the concentration of this species cannot be larger than that of LFH· [and, in fact, must be smaller since sufficient free LFH· exists to allow disproportionation to form LFH^- to be a significant pathway (see Discussion)], its extinction coefficient must be at least 5–6 times larger than that of LFH·.

The fast decay observed at 600 nm has a first-order rate constant which is independent of flavocytochrome *c* concentration (11–50 μM). This demonstrates that the reaction leading to the disappearance of this species is truly first order, as opposed to pseudo first order as a consequence of an excess of cytochrome. This is consistent with an intramolecular process. Moreover, the rate constant shows only a slight pH dependence over the pH range 5–9.0. These results are summarized in Table I. The magnitude of the signal due to the 600-nm fast transient was pH dependent. Expressed as the ratio of ΔA at 600 nm to that at 553 nm to correct for experimental variations, the values varied from 0.32 at pH 7.7 to 2.07 at pH 5.0 (1.87 at pH 6.0 and 0.95 at pH 7.0). The behavior above pH 8 will be described below. These data,

Table I: Rate Constants for the Reduction of *Chlorobium thiosulfatophilum* Flavocytochrome *c* by Photoreduced Lumiflavin

pH	fast component[a] (s^{-1}) × 10^{-3}	slow component[a] (M^{-1} s^{-1}) × 10^{-6}
5.0	1.9	3.7
6.0	1.7	5.4
7.0	1.2 (1.1)[b]	8.0 (8.0)[b]
7.7	1.1	12.0
9.0	1.2[c]	13.3

[a] Buffer: 20 mM potassium phosphate (20 mM sodium acetate at pH 5.0), 10 mM EDTA, 20 μM lumiflavin. Given are average values for different samples and the same sample at different wavelengths (usually 600 and 553 nm). Estimated error ±10%. [b] Values in parentheses: same buffer as in footnote *a*, but also 10 mM in sodium sulfite. In this case the same rate constant was observed but with only 10% of the signal remaining. Moreover, the reaction was still biphasic at 553 nm, indicating that both the fast (first order) and slow (second order) processes were occurring. Estimated error ±10%. [c] See text for discussion of this component.

although not extensive, are consistent with a pK of approximately 6.8, taking the limiting ratio at high pH to be 0.25 (based on the LFH· and heme extinction coefficients). It should be noted that this pK is somewhat higher than that obtained by redox titration (see below) for the ionization of the protein-bound flavin semiquinone. However since this measurement refers to a transient species, whereas the latter refers to an equilibrium species, a different pK is not unreasonable (see below).

The kinetics observed at 553 nm on laser flash photolysis are shown in Figure 2B and also complex. An initial fast decaying transient is produced which has the same rate constant as the species seen at 600 nm. This kinetic process is followed by a relatively slow increase in absorbance at 553 nm, consistent with the reduction of the heme moiety. This second slow process is pseudo first order (Figure 4A) and dependent on cytochrome concentration (Figure 4B) as expected for a second-order reaction. Similar behavior was observed at all wavelengths and over the pH range 5–9.0. However, the second-order rate constant is clearly pH dependent (Table I).

In Figure 5 are shown kinetic data obtained at 553 nm at several pH values. At pH 5.0, the shape of the laser-induced absorbance change (Figure 5, curve 1) is similar to that ob-

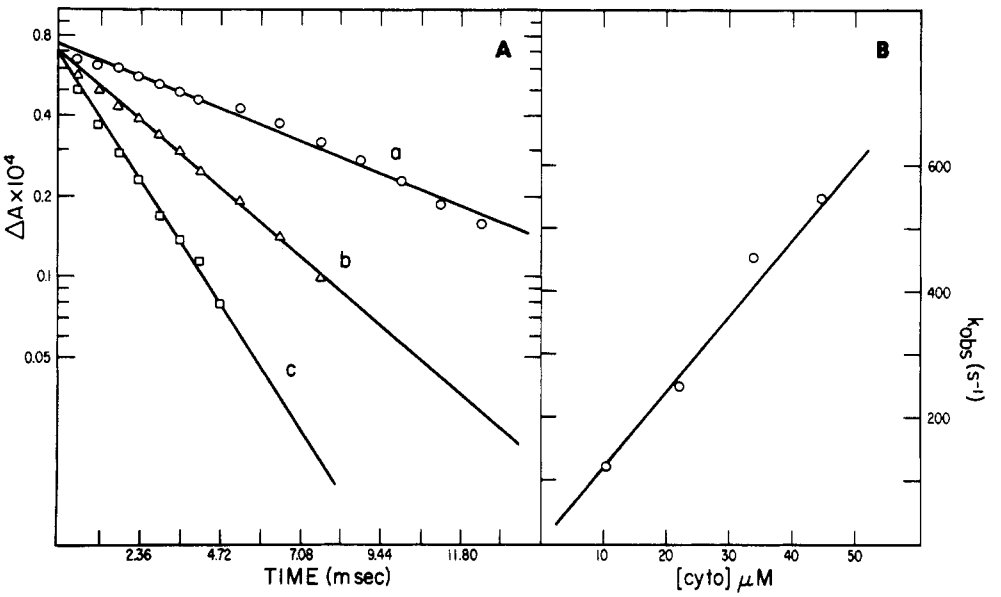


FIGURE 4: Heme reduction by photoreduced lumiflavin. Reactions were monitored at 553 nm. Conditions are as in the caption of Figure 2 except that the pH is 7.7. (A) Plot of ln ΔA vs. time. Flavocytochrome *c* concentration: (a) 11 μM, (b) 22 μM, and (c) 33 μM. (B) Plots of observed rate constants (k_{obsd}) from part A as a function of flavocytochrome *c* concentration.

tained at pH 7.0 (Figure 2B, curve 1). However, at pH 7.7 (Figure 5B, curve 2) the initial transient is no longer clearly evident. Rather, a flattening of the trace in this region is observed, suggesting a cancellation effect. Presumably, the magnitude of the initial transient signal is small enough at this pH so that the absorbance change due to its decay, as well as the decay of the free lumiflavin semiquinone, is cancelled by the rise in absorbance due to the fast phase of heme reduction. The fast transient can, however, be clearly seen at 563 nm, which is an isosbestic point for heme reduction. Unexpectedly, at pH values above 8, the initial transient reappears (Figure 5, curve 3 and 4) and its kinetic behavior is very much like that seen at pH values $\leq$7.0. This will be discussed further below.

By extrapolating the ln ΔA vs. time plots for the slow species (at 11 μM flavocytochrome) to zero time and measuring the total heme reduction at infinite time, we can estimate that 70–80% of the heme is reduced in the second slow process, with 20–30% of the heme reduced during the decay of the fast transient. This is the case over the entire pH range studied (cf. Figure 5). We attribute the second slow process, as we have previously (Cusanovich & Tollin, 1980; Ahmad et al., 1981), to the reduction of the heme by fully reduced lumiflavin generated by the semiquinone disproportionation. The more rapid heme reduction must then be due to an intramolecular electron transfer from the fast decaying transient to the heme.

One further point relative to the 600-nm intermediate needs to be commented upon. We observe that the species is present immediately following the laser flash, with no measurable lag time in its formation (<1 μs). This indicates that it must be generated within a preexisting lumiflavin–protein complex, rather than as a consequence of a second-order reaction between lumiflavin semiquinone and the flavocytochrome (at the concentrations involved, this would have to have an unreasonably large rate constant). Furthermore, the fact that only 20–30% of the heme is reduced during the rapid initial phase of the reaction suggests that this is a reflection of the amount of such a preexisting complex. It follows then from mass action considerations that the relative contribution of the fast phase should decrease with increasing flavocytochrome inasmuch as the amount of free flavocytochrome increases more rapidly than the complex under weak binding conditions. Although

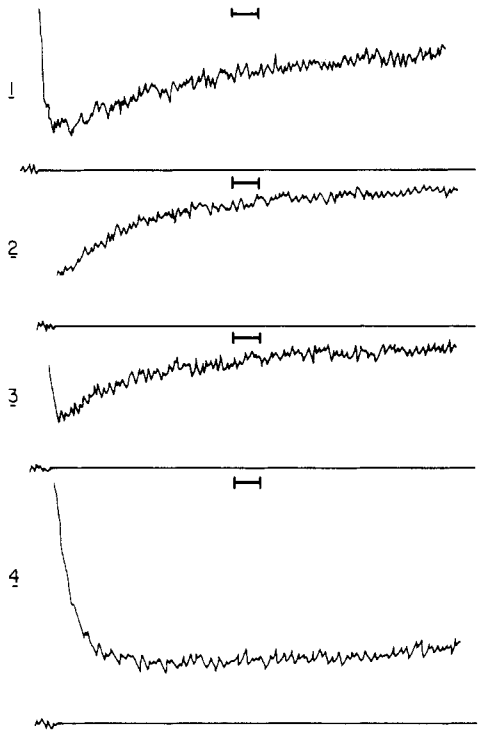


FIGURE 5: pH dependence of kinetics of reduction of flavocytochrome *c* by photoreduced lumiflavin. Conditions as in Figure 2 except where noted. Observation wavelength 553 nm; 11 μM flavocytochrome *c*. (1) pH 5.0; 20 mM acetate buffer; 5.9 ms/division; (2) pH 7.7; 20 mM phosphate buffer; 1.2 ms/division; (3) pH 9.0; 20 mM borate buffer; 2.9 ms/division; (4) pH 9.0; 20 mM borate buffer; 0.59 ms/division.

it is difficult to accurately quantitate the fraction of the fast phase, we do find that it decreases in relative contribution from 30% at 11 μM protein to 23% at 44 μM protein. This decrease is consistent with a binding constant of $(3.2 \pm 0.6) \times 10^4$ M^{-1} (pH 7.7) for lumiflavin. Inasmuch as we only have four data points over a rather narrow concentration range (technical considerations limit the accessible range), the binding constant given here can only be considered an estimate. Nevertheless, the trend is clear and provides support for the presence of a weak lumiflavin–protein complex.

Table II: Redox Properties of *Chlorobium thiosulfatophilum* Flavocytochrome *c*

pH	E_m^{heme} (mV)	E_m^{flavin} (mV)	K_f	E_{S-R} (mV)	E_{O-S} (mV)	% neutral semiquinone$_{max}$	% total semiquinone$_{max}$
5[a]		117	0.79	120	114	29.6	30.8
6	98	64	0.81	67	61	22.2	31.0
6.75	98	34	1.39	30	38	11.4	37.0
7.00	98	28	2.13	18	38	8.5	42.2
7.76[b]	93						

[a] All values in this row were calculated assuming no protein-linked ionizations affected the flavin potentials. For the calculations, the following equations were used: $E_{O-S} = E_0^{O-S} + 0.026 \ln ([H^+] + K_S)$, $K_S = 4 \times 10^{-7}$ M, $E_0^{O-S} = 0.412$ mV; $E_{S-R} = E_0^{S-R} + 0.026 \ln [[H^+](K_R + [H^+])]/(K_S + [H^+])$, $K_R = 8.6 \times 10^{-7}$ M, $E_0^{S-R} = 0.418$ mV [preceding equations derived on the basis of discussion given by Clark (1960)]; $E_m = (E_{O-S} + E_{S-R})/2$ and $K_f = \exp[-(E_{S-R} - E_m)]/0.013$ [preceding equations modified from Michaelis (1951)]. [b] At pH 7.76, the flavin parameters were not measured since the potential of the flavin was too much lower than that of the heme; the redox couple used (iron-EDTA) was inadequate.

In Figure 1 the open circles represent the total ΔA observed (absorbance at infinite time minus the absorbance prior to the flash) which accurately follows the reduced minus oxidized difference spectrum above 510 nm. A smaller absorbance change than expected is observed below 510 nm. However, the data presented were taken on samples for which the extent of heme reduction was small (20–40%), and thus no appreciable net flavin reduction was expected on themodynamic grounds (see below). The filled circles represent the ΔA observed approximately 1 μs after the flash. The data reported were taken on samples which were 20–40% reduced prior to the flash. Hence, the magnitude of the fast transient (filled circles) is somewhat less than its maximum value (extrapolated to zero heme reduction). As can be seen, a broad absorbance change is found between 515 and 600 nm similar to that expected for a neutral flavin semiquinone radical. However, this result is not consistent with oxidation–reduction titrations at pH 7 where no absorbance was observed (500–700 nm), which could be attributed to such a neutral semiquinone radical (see below; Meyer, 1970).

For further investigation of the nature of the fast kinetic component (i.e., Figure 1, solid circles), the effect of pH on the oxidation–reduction potentials of both the flavin and heme moieties of flavocytochrome *c* was studied. The apparent midpoint potential of the heme was found to be essentially independent of pH between 6 and 7.7 (Table II). However, analysis of the redox titration in the region of flavin absorbance indicated a more complex situation in that the apparent midpoint potential was pH dependent (Table II) and the slopes of the Nernst plots were not integral, that is, they fit neither $n = 1$ nor $n = 2$ processes (Figure 6). Such a situation can occur if a semiquinone intermediate exists and if the equilibrium formation constant for the generation of the semiquinone from fully reduced and fully oxidized flavin species (K_f) falls between approximately 0.1 and 16, as described by Michaelis (1951). As shown in Table II, this is indeed the case for flavocytochrome *c* [both the values of K_f and the corresponding potentials for the oxidized to semiquinone (E_{O-S}) and the semiquinone to the fully reduced (E_{S-R}) conversions are given]. The potentials for both one-electron steps vary as a function of pH and allow calculation of the apparent pK values for each transition. This analysis yields a pK value of 6.4 for the semiquinone (pK_S) and 6.1 for the fully reduced species (pK_R).[1] Thus, an anion radical is expected to predominate at equilibrium as proposed for *Chromatium* flavocytochrome *c* (Cusanovich & Tollin, 1980). This is consistent with recent studies of 8α-substituted flavins and flavoproteins (Edmondson et al., 1977, 1981). Moreover, the maximum amount of semiquinone formed at any pH value is dependent on the equilibrium constant K_f [% semiquinone$_{max}$ = 100 $K_f^{1/2}/(2 + K_f^{1/2})$; Michaelis, 1951]. Thus at pH 7, the maximum amount of neutral semiquinone radical that could be expected at equilibrium is 8.5% (based on K_f and pK_S). In the transient experiment, however, approximately 38% of the signal amplitude (correcting for the limiting value at high pH) for the 600-nm species which is observed at pH 5.0 remains at pH 7.0, consistent with a somewhat higher pK_S value (see above).

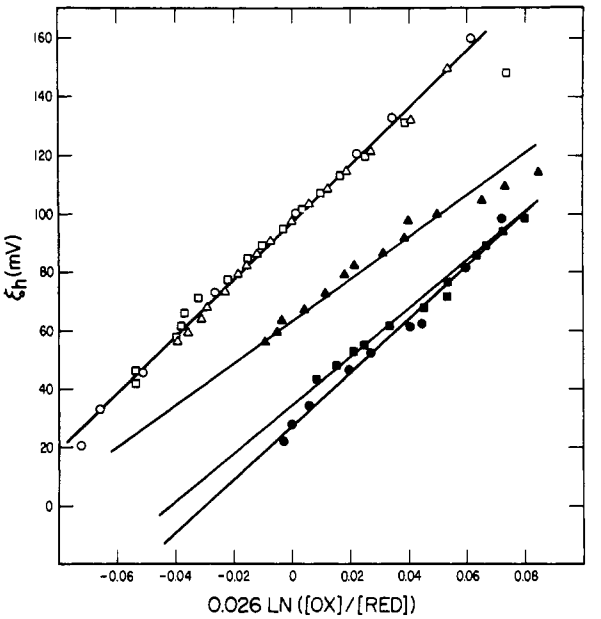


FIGURE 6: Oxidation–reduction titration of flavocytochrome *c*. (Open symbols) Data from 553 nm, representing heme reduction. (Solid symbols) Data from 480 nm corrected for heme contribution (20% of the absorbance change at 553 nm). (○, ●) pH 7.0; (□, ■) pH 6.76; (△, ▲) pH 6.0. Buffer: 20 mM potassium phosphate, 1 mM EDTA, 100 μM ferric chloride. Flavocytochrome *c*, 20 μM.

Photochemical titrations were carried out at pH 5 to further characterize the semiquinone species. A mixture of cytochrome (11 μM), lumiflavin (5 μM), and EDTA (10 mM) was irradiated for different periods of time and allowed to reach equilibrium, and the absorption spectra were determined at different extents of reduction. At this pH, significant neutral flavin radical should exist (% neutral semiquinone$_{max}$ = 30%, assuming no protein-linked ionizations influence the flavin; Table II). Figure 7A presents difference spectra, reduced minus oxidized and partially reduced minus oxidized, at pH 5. The formation of a species with an absorption maximum at about 613 nm, consistent with a neutral flavin semiquinone, can be seen. The results of the titration are shown in Figure 7B where the percent flavin reduced (observed at 480 nm) and

[1] It should be noted that the pK_S value for free lumiflavin semiquinone is 8.4 (Vaish & Tollin, 1971), whereas the pK_R for free $FMNH_2$ is 6.7 (Lowe & Clark, 1956).

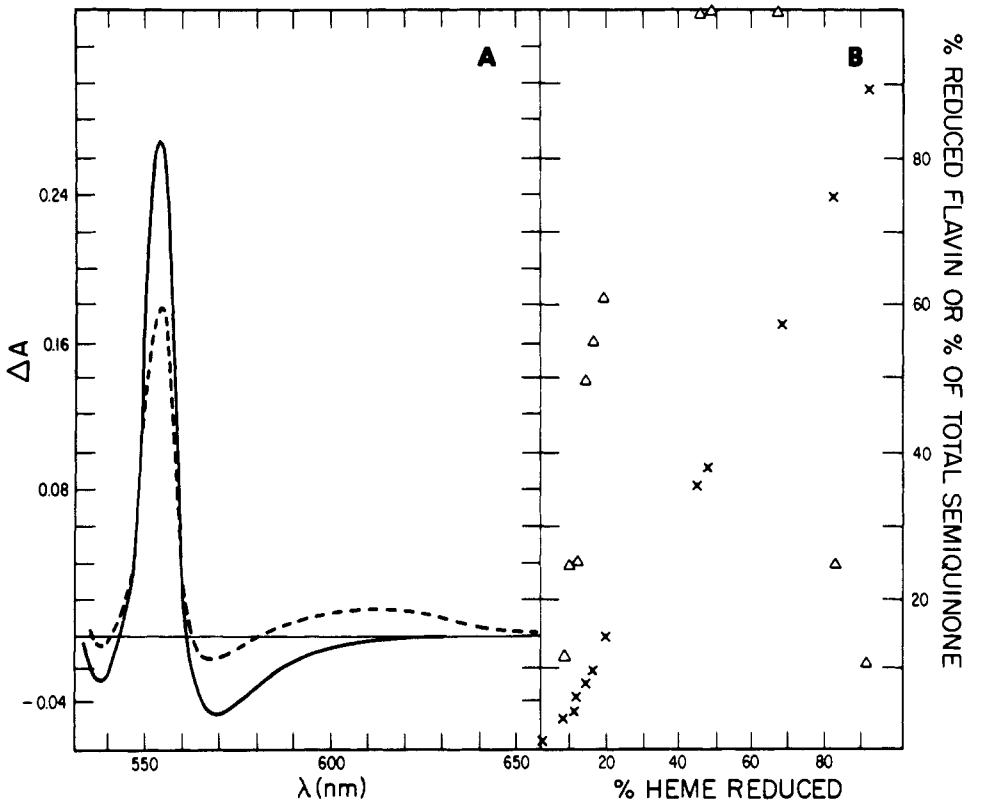


FIGURE 7: Oxidation–reduction titration of flavocytochrome *c*. Buffer: 20 mM sodium acetate, 10 mM EDTA, 5 μM lumiflavin, pH 5.0. Flavocytochrome *c*, 11 μM. Anaerobic samples were illuminated for various periods of time to reduce the cytochrome. (A) Fully reduced minus oxidized difference spectrum (solid line); partially reduced minus oxidized difference spectrum (dashed line). (B) Plotted is the % heme reduction (monitored at 553 nm) vs. the % flavin reduced (×) (monitored at 480 nm and corrected for heme contribution) and the % of total semiquinone produced (Δ) (measured at 613 nm).

the percent of total semiquinone formed (based on the increase in absorbance at 613 nm) are plotted against the percent heme reduced. Assuming a difference extinction coefficient of 5 mM^{-1} cm^{-1} for the neutral flavin radical (typical for such species), we obtain a maximum of 25% conversion, as compared to 30% calculated from the p*K* value for semiquinone ionization and the formation constant. Thus, there can be no doubt that a protein-bound neutral flavin radical with apparent normal spectral properties exists, but only at pH values below pH 7. It should be pointed out that the 600-nm transient, as observed at pH 5.0, in addition to exhibiting an anomalously high p*K*, is also spectrally perturbed, at least insofar as its extinction coefficient is appreciably larger than that expected for a flavin neutral semiquinone (see below).

Kinetic experiments were conducted in the presence of 10 mM sodium sulfite in order to determine the role of the bound flavin moiety in electron transfer to the heme. Under these conditions, the flavin moiety of flavocytochrome *c* reacts with sulfite to form a charge-transfer complex in which the flavin absorbance is bleached (Meyer & Bartsch, 1976). If the protein-bound flavin is an obligatory intermediate in the reduction of heme by both species of reduced lumiflavin, the formation of this sulfite adduct would be expected to block electron transfer. Typical results for the experiments just described are summarized in Table III. As can be seen, 10 mM sulfite has, within experimental error, no effect on the absorbance change due to the free lumiflavin semiquinone produced by the laser flash in the absence of flavocytochrome. However, in the presence of the flavocytochrome, sulfite suppresses approximately 80% of the expected heme reduction and decreases the magnitude of the fast transient observed at 600 nm near the level expected for free lumiflavin. Thus, it can be concluded that at least the bulk of the expected heme reduction requires the presence of uncomplexed protein-bound flavin and that reduction of the heme moiety by both lumiflavin semiquinone and fully reduced lumiflavin must occur through the endogenous flavin moiety.

Table III: Effect of Sodium Sulfite on the Reduction of *Chlorobium thiosulfatophilum* Flavocytochrome *c*[a]

sample	ΔA_{550} heme	ΔA_{600} transient
20 μM lumiflavin		1.15×10^{-3}
20 μM lumiflavin + 10 mM sodium sulfite		1.24×10^{-3}
20 μM lumiflavin + 11 μM flavocyt. *c*	2.0×10^{-3}	1.89×10^{-3}
20 μM lumiflavin + 11 μM flavocyt. *c* + 10 mM sodium sulfite	0.37×10^{-3}	1.35×10^{-3}

[a] pH 7.0, 25 °C; buffer: 20 mM potassium phosphate, 10 mM EDTA. Numbers represent averages of two to four experiments and typically show a variation of ±15%.

Discussion and Conclusions

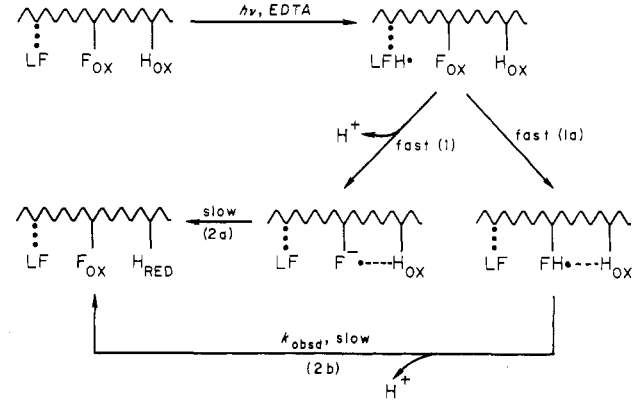
The results presented above establish that both lumiflavin semiquinone and fully reduced lumiflavin are capable of reducing *Chlorobium* flavocytochrome *c* and that an unmodified protein-bound flavin is an obligatory participant in the process. We have also shown that a protein-bound intermediate is produced during the course of the reaction and that the decay of this species results in heme reduction, presumably by an intramolecular electron transfer. We propose that this transient intermediate is the semiquinone radical of the flavin prosthetic group of flavocytochrome *c*. Thus, we suppose that an electron is transferred from either $LFH\cdot$ or LFH^- to the protein-bound flavin and that this is followed by an intra-

molecular reduction of the protein-bound oxidized heme (see below for detailed reaction scheme). The justification for this proposal follows.

Below pH 7.0, it is clear from both the ordinary redox titration behavior and the photochemical titration that a neutral protein-bound flavin semiquinone with apparently normal spectral properties is present whose pK is approximately 2 pH units below that of free flavin. However, at pH 7.0, where the anionic form would be expected to predominate, laser photolysis produces a rapidly decaying transient which has spectral properties (larger extinction coefficient and appreciable absorption in the 600-nm region) quite different from what would be anticipated for an anionic semiquinone (Yagi, 1975). Furthermore, such a long wavelength absorbing intermediate cannot be observed in an equilibrium experiment at pH 7.0. We suggest, therefore, that this transient species is indeed a protein-bound neutral flavin semiquinone which transfers an electron intramolecularly to the heme but that its absorption spectrum is significantly perturbed and its pK (as observed in a transient experiment) is raised by protein interaction, most likely involving the oxidized heme and possibly via a charge-transfer mechanism. In an equilibrium situation where the pK for the neutral to anion semiquinone conversion is 6.4 and where the heme is largely reduced because of its higher redox potential (Table II), the spectrum of this species would be that of a normal anionic flavin semiquinone, which has no appreciable absorption at 600 nm. Furthermore, this would make its observation considerably more difficult due to overlapping of the oxidized flavin and reduced heme spectra. Such an interpretation is consistent with the fact that the magnitude of the 600-nm transient decreases with the extent of heme reduction at pH 7.0. This type of unusual spectral behavior is not unprecedented for this protein, inasmuch as the sulfite adduct of the flavin moiety exhibits a unique long wavelength absorption which has been attributed to a charge-transfer interaction (Meyer & Bartsch, 1976). According to this hypothesis, the pK value obtained in the flash experiments (6.8; see above) represents the neutral to anion semiquinone transformation under transient conditions in which the heme is largely oxidized. The fact that the magnitude of the 600-nm transient decreases as the pH is raised from 5.0 to 7.7 without any corresponding decrease in the amount of heme reduced during this initial fast phase of the reaction indicates that both the neutral and anionic semiquinone species are capable of transferring an electron to the heme. However, we are only able to measure the rate constant for the neutral semiquinone reaction. This is because at pH values above 8, where the anion radical should in principle predominate and where the kinetics of the fast phase of heme reduction should be measurable at 553 nm, we find an anomalous reappearance of a neutral semiquinone transient. This precludes any such direct observation of heme reduction. It should be pointed out that the presence of this semiquinone species is only observable at these pH values in a transient experiment; steady-state phototitration provides no evidence for the equilibrium formation of a neutral semiquinone (data not shown). The reason for this unusual behavior is not clear at present, but it suggests a pH-induced change in protein properties which decreases the rate of proton loss from the semiquinone intermediate, thereby transiently stabilizing the neutral form. This requires further investigation.

We have presented evidence above for the existence of a weak lumiflavin–protein complex which participates in the photochemical reduction of the flavocytochrome heme via LFH·. Since only 20–30% of the heme reduction occurs via this rapid route, a significant quantity of uncomplexed lumiflavin semiquinone must also be present which can disproportionate and produce the fully reduced species. The domination of the observed 600-nm decay by the intramolecular process is evidently a consequence of the abnormally high extinction coefficient of the protein-bound semiquinone.

This raises the possibility that the rapidly decaying species is actually a protein-bound *lumiflavin* radical which is spectrally perturbed and has its pK lowered by interaction with the oxidized heme. However, we observe that the formation of a protein flavin–sulfite adduct virtually eliminates heme reduction by *both* LFH· and LFH^-. One would expect that, if the lumiflavin radical interacts strongly enough with the heme to have its spectrum and pK so drastically altered, it would still be able to transfer an electron even if the protein flavin were modified. One could, of course, argue that sulfite binding by the flavin disrupts the LFH·–heme interaction, thereby eliminating both the perturbations and the electron transfer. This would require that the protein-bound flavin is not involved at all in the pathway of electron transfer from LFH· to heme. In order to account for the suppression of LFH^- reduction of heme by sulfite under this hypothesis, one would have to assume either that this reaction does occur via the endogenous flavin moiety, in contrast to the LFH· reaction, or that it proceeds via protein-bound oxidized lumiflavin as an intermediate electron carrier and is prevented for the same reason that LFH· reduction is eliminated. Although we cannot completely rule out this alternative interpretation, it seems rather unlikely. In a steady-state photoreduction experiment, the amount of oxidized lumiflavin present during the course of the reaction would be quite small, inasmuch as the rate-limiting step is the oxidation of LFH^- by the protein. This would argue against a role for bound oxidized lumiflavin as an intermediate. It also is improbable that LFH· and LFH^- reductions follow totally different pathways, i.e., one transferring directly to the heme and the other via the endogenous flavin. Finally, it is more reasonable to believe that the rather large spectral and pK effects that we observe are produced upon the specifically bound endogenous flavin rather than upon nonspecifically bound exogenous lumiflavin. We conclude, therefore, that the mechanism for the fast phase of the reaction either above (step 1) or below (step 1a) the pK_s value is as follows:



According to this scheme, a weakly bound lumiflavin radical rapidly transfers an electron to the protein-bound flavin (F) to generate an anionic or neutral semiquinone [interacting with the oxidized heme (H)], followed by heme reduction at the experimentally observed first-order rate. Thus, in this sequence, reactions 2a and 2b are the rate-limiting steps. It is interesting that the rate constant for reaction 2b (Table IV) is at least 3 orders of magnitude slower than that observed for

Table IV: Cytochrome Reduction by Photoreduced Lumiflavin[a]

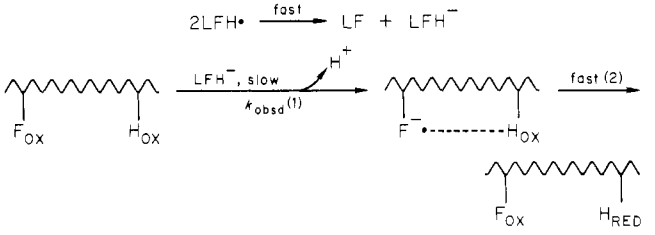
cytochrome	k (M^{-1} s^{-1}) for reductant: LFH·	LFH⁻	app intramolecular rate constant (s^{-1})
Chromatium flavocytochrome[b]	~1.9×10^8	1.4×10^6	≥1.4×10^6
Chlorobium flavocytochrome	TFM[c]	8.0×10^6	1.2×10^3[d]
horse heart cytochrome *c*[e]	6.2×10^7	2.9×10^7	

[a] pH 7.0, 25 °C. [b] Cusanovich & Tollin (1980). [c] Too fast to measure. [d] Rate constant for electron transfer from neutral semiquinone to heme at pH 7.0. [e] Ahmad et al. (1981).

intramolecular electron transfer with the *Chromatium* flavocytochrome (Cusanovich & Tollin, 1980). The structural basis for this large difference is obscure at present but when established should have interesting implications for the mechanism of biological electron-transfer processes.

At pH values above 8, it appears that the rate of proton loss in reaction 1 slows down sufficiently so that electron transfer again proceeds via the neutral semiquinone.

The slow phase of the reaction (i.e., LFH⁻ reduction) proceeds via the disproportionation of free LFH· as follows (written for the sequence occurring above pK_s):



Reduction of protein-bound flavin by LFH⁻ is sufficiently slow so that no 600-nm intermediate can be observed in the flash experiment; i.e., reaction 1 is the rate-limiting step.

Both second-order rate constants (Table IV) for the reduction of the *Chlorobium* flavocytochrome are appreciably faster than for the *Chromatium* protein. Again, in the absence of structural information, this difference cannot be definitively rationalized, although it must ultimately reflect the specific environments of the redox cofactors, e.g., solvent exposure and/or the factors which result in a higher redox potential for the *Chlorobium* heme. It is also noteworthy that the rate constants for LFH⁻ reduction of the flavocytochromes are appreciably smaller than for horse heart cytochrome *c* (Table IV), even though the LFH· rate constants are larger. This may relate to the nature of the electrical charge adjacent to the prosthetic group, since the horse heart protein is known to possess a positive charge cluster in this region (cf. Salemme, 1977).

References

Ahmad, I., Cusanovich, M. A., & Tollin, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6724–6728.

Bartsch, R. G. (1978) in *The Photosynthetic Bacteria* (Clayton, R, K., & Sistrom, W. R., Eds.) pp 249–279, Plenum Press, New York.

Bartsch, R. G., Meyer, T. E., & Robinson, A. B. (1978) in *Structure and Function of Cytochromes* (Okanaki, K., Kamen, M. D., & Sekuzei, I., Eds.) pp 443–451, University of Tokyo Press, Tokyo.

Clark, W. M. (1960) *Oxidation-Reduction Potentials of Organic Systems*, p 196ff, Williams and Wilkins, Baltimore, MD.

Cusanovich, M. A., & Tollin, G. (1980) *Biochemistry 19*, 3343–3347.

Edmondson, D. E., Rizzuto, F., & Tollin, G. (1977) *Photochem. Photobiol. 25*, 445–450.

Edmondson, D. E., Ackrell, B. A. C., & Kearney, E. B. (1981) *Arch. Biochem. Biophys. 208*, 69–74.

Fukumori, Y., & Yamanaka, T. (1979) *J. Biochem. (Tokyo) 85*, 1405–1414.

Holmström, B. (1964) *Photochem. Photobiol. 3*, 97–114.

Kusai, K., & Yamanaka, T. (1973) *Biochim. Biophys. Acta 325*, 304–314.

Lowe, H. J., & Clark, W. M. (1956) *J. Biol. Chem. 221*, 983–992.

Meyer, T. E. (1970) Ph.D. Thesis, University of California at San Diego.

Meyer, T. E., & Bartsch, R. G. (1976) in *Flavins* and Flavoproteins (Singer, T. P., Ed.) pp 312–317, Elsevier, Amsterdam.

Meyer, T. E., Bartsch, R. G., Cusanovich, M. A., & Mathewson, J. H. (1968) *Biochim. Biophys. Acta 153*, 854–861.

Michaelis, L. (1951) in *The Enzymes* (Sumner, J. B., & Myrbäck, K. T., Eds.) Vol. 2, Part 1, Chapter 44, Academic Press, New York.

Salemme, F. R. (1977) *Annu. Rev. Biochem. 46*, 299–329.

Tollin, G., Chan, R. I., Malefyt, T. R., & Bruice, T. C. (1979) *Photochem. Photobiol. 29*, 233–243.

Vaish, S. P., & Tollin, G. (1970) *J. Bioenerg. Biomembr. 1*, 181–192.

Vaish, S. P., & Tollin, G. (1971) *J. Bioenerg. Biomembr. 2*, 61–72.

Vorkink, W. P. (1972) Ph.D. Thesis, University of Arizona.

Yagi, K., Ed. (1975) *Reactivity of Flavins*, pp 29–35, University of Tokyo Press, Tokyo.