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Laser Flash Photolysis Studies of Electron Transfer between Semiquinone and Fully Reduced Free Flavins and the Cytochrome *c*-Cytochrome Oxidase Complex†

Iqbal Ahmad, Michael A. Cusanovich, and Gordon Tollin*

ABSTRACT: Laser flash photolysis has been used to determine the rate constants for the reduction of bovine cytochrome oxidase and the cytochrome *c*-cytochrome oxidase complex by the semiquinone and fully reduced forms of various flavin analogues ($FH^{\cdot}$ and FH^{-}, respectively). Under the conditions used, the reaction of $FH^{\cdot}$ with free cytochrome oxidase is too slow to compete with $FH^{\cdot}$ disproportionation whereas FH^{-} reacts measurably. Both $FH^{\cdot}$ and FH^{-} are effective in reducing the complex. The reduction of heme *a* in the complex is shown to proceed via cytochrome *c*, and a limiting first-order rate is observed in the case of FH^{-} at high complex concentrations. The data indicate that the interaction site for electron transfer to cytochrome *c* is the same in the complex as with the free protein, and although a tight complex exists, at least small reactants like the flavins are not sterically hindered in their access to the bound cytochrome *c*. Moreover, the results also establish that intramolecular electron transfer between cytochrome *c* and cytochrome oxidase within the complex occurs with a first-order rate constant of greater than 700 s^{-1}. Thus, the presence of cytochrome *c* greatly enhances electron transfer from reduced flavins to cytochrome oxidase.

Although cyt oxidase[1] has been intensively investigated, many aspects of its molecular mechanism remain to be elucidated [cf., for example, Malmström (1979)]. This results in part from a lack of structural information. A large number of studies have also been carried out on the interaction of cyt *c* with cyt oxidase, but in spite of the extensive structural information available concerning cyt *c*, the details of the interaction are still not well understood (Malmström, 1979; Hill & Nicholls, 1980). This results in part from limitations inherent in the methodology used. Thus, the reaction rate is at the borderline of the stopped-flow technique ($k \sim 4 \times 10^7$ M^{-1} s^{-1}), and the interpretation of steady-state studies is difficult inasmuch as a mechanism must be assumed and in general individual rate constants are not easily resolved.

Recently, we have applied a rather different kinetic approach to the study of biological oxidation-reduction reactions (Cusanovich & Tollin, 1980; Ahmad et al., 1981). Since free flavins can be photochemically reduced with a short pulse of laser light, a direct means of producing a strong reducing agent in a short time period (<1 μs) is available. Moreover, substantial information has been accumulated concerning the physical-chemical and kinetic properties of free flavins, as well as a wide variety of flavin analogues. We have used this approach to study intramolecular electron transfer in *Chromatium vinosum* cytochrome *c*-522, a complex cytochrome containing two hemes and a flavin moiety, and to derive information about the electrostatics, steric restrictions, and nonpolar interactions at the site of electron transfer between ferricyt *c* and fully reduced and semiquinone flavins. We report here on a similar study of the reduction of cyt oxidase and the cyt *c*-cyt oxidase complex with a variety of photochemically reduced flavins.

Materials and Methods

Riboflavin, FMN (sodium salt), and cytochrome *c* (horse heart, ferri form, type VI) were obtained from Sigma Chemical Co. Lumiflavin, 10-methylisoalloxazine, and 7,8-dichlororiboflavin were synthesized as previously described (Guzzo & Tollin, 1963; Shiga & Tollin, 1976). 8α-[*S*-(*N*-Acetyl)-L-cysteinyl]tetraacetylriboflavin was a gift from Dr. D. B. McCormick. The structures of the flavin derivatives used in this work are shown in Figure 1. Cytochrome oxidase was prepared from beef hearts as described previously (Yonetani, 1960). All other materials were analytical grade or of the purest form available from Fisher Scientific Co. and Mallinckrodt Chemical Co.

Solutions for flash photolysis, which contained 0.1 mM flavin, 5 mM EDTA, and suitable concentrations of cyt oxidase (concentration calculated on the basis of two hemes per

† From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721. *Received September 25, 1981; revised manuscript received March 3, 1982.* Supported in part by research grants from the National Institutes of Health (AM 15057 and GM 21277) and the National Science Foundation (PCM 7804349).

[1] LF, lumiflavin; RF, riboflavin; FMN, flavin mononucleotide; 10-MI, 10-methylisoalloxazine; Cl_2RF, 7,8-dichlororiboflavin; Ac-Cys-TARF, 8α-[*S*-(*N*-acetyl)-L-cysteinyl]tetraacetylriboflavin; $FH^{\cdot}$, neutral flavin semiquinone; FH^{-}, fully reduced flavin; cyt *c*, horse heart cytochrome *c*; cyt oxidase, beef heart cytochrome oxidase; EDTA, ethylenediaminetetraacetic acid.

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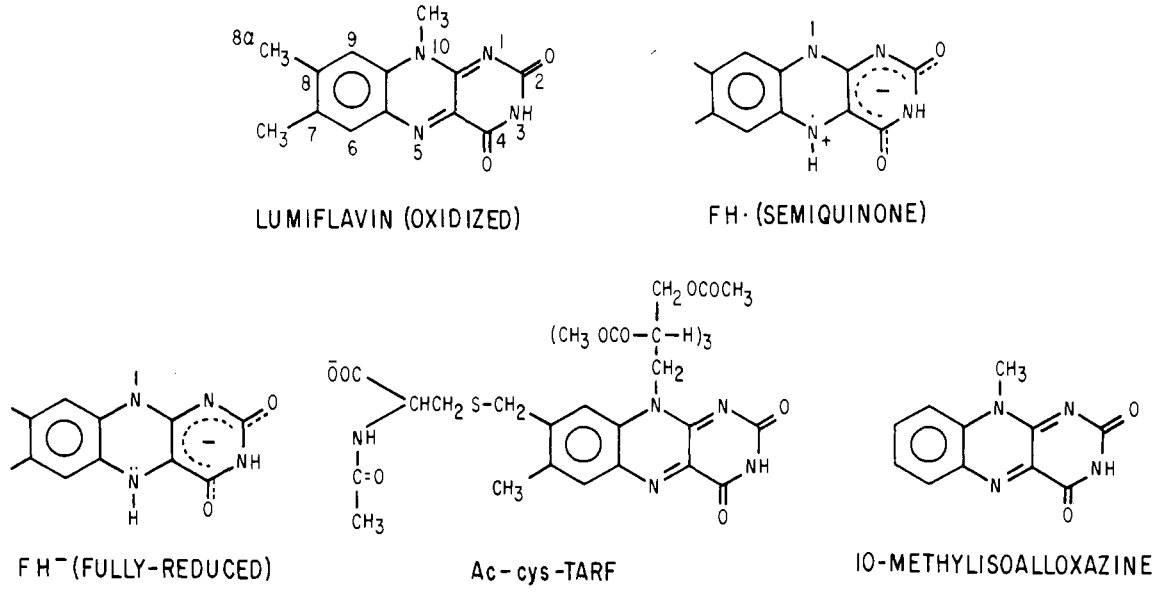

FIGURE 1: Structures and charge states of flavin analogues.

molecule) or cyt *c* and cyt oxidase (5–50 μM each) dissolved in 100 mM potassium phosphate buffer (pH 7.0), were placed in 10-mm Thunberg tubes and subjected to several evacuation cycles under nitrogen so as to remove oxygen. In all experiments, 1% Tween 80 [poly(oxyethylene)sorbitan monooleate] was added to the samples in order to minimize light scattering effects by maintaining cyt oxidase in solution. It was determined that the presence of this detergent had no effect on the kinetics of reduction of cyt *c* in the absence of cyt oxidase. Some experiments were carried out in pH 8.0 buffer in the presence of 10 mM KCN. In certain cases cyt *c* plus cyt oxidase mixtures containing LF and EDTA (pH 7.0) were subjected to steady-state irradiation (500-W Sylvania lamp, white light) to achieve half-reduction of cyt oxidase (absorbance changes at 605 nm monitored spectrophotometrically) and then studied by laser photolysis. All experiments were conducted at 25 °C, and care was taken to keep the samples in the dark prior to laser excitation.

Flash photolysis of the various samples was carried out with a nitrogen laser-pumped dye solution [1 mM 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene in benzene, λ_{max} emission 436 nm] in a 4-cm path length rectangular cuvette (Tollin et al., 1979). Flavin triplet-state formation occurred in approximately 10 ns. Flavin semiquinone (FH·) was formed by reaction of the triplet with EDTA, and fully reduced flavin (FH^-) was subsequently generated by semiquinone disproportionation ($2FH\cdot \rightarrow F + FH^- + H^+$). The amount of FH· generated per flash was typically 5×10^{-7} M, which corresponds to about 0.5% of the total flavin initially present. Since the cytochrome concentrations were in the 5–50 μM range, the reduction reactions proceeded under pseudo-first-order conditions. Under the experimental conditions used, the time resolution of the apparatus was 0.3–1.0 μs (limited mainly by fluorescence and scattering artifacts). Kinetic data were obtained by averaging four flashes with a Nicolet 1072 signal averager.

The kinetics of the reaction of FH· with the cyt *c*–cyt oxidase complex was followed at 553–554 nm. This is an isosbestic point for cyt *c* reduction under the present conditions. Cyt oxidase has a very small absorption change at this wavelength on reduction, thus allowing us to follow the rate of disappearance of FH·. The reduction of cyt oxidase by FH· was too slow to compete effectively with the disproportionation, and thus the rate constants for this reaction could not be determined. The reaction of FH^- with cyt oxidase and with the complex was followed at 605 nm, where an increase in absorbance occurs upon reduction. Some experiments were also monitored at 550 and 570 nm to permit the detection of cyt *c* redox changes.

The molar concentrations of reduced cyt *c* and reduced cyt oxidase in the laser experiments, alone or in the complex, were calculated by using $\Delta\epsilon_{570}$(cyt *c*) = 1000 M^{-1} cm^{-1} and $\Delta\epsilon_{605}$(cyt oxidase) = 25000 M^{-1} cm^{-1}, respectively. The wavelength used for cyt *c* allowed a more convenient separation of the kinetics of its reduction by FH^- from FH· disproportionation.

Subsequent to the laser flash, complex kinetics were observed. An initial fast absorbance change was seen ($t_{1/2}$ = 2–6 ms), which corresponded to the pseudo-first-order reduction of the cytochrome by FH· superimposed on FH· disproportionation. A second slower change was also observed ($t_{1/2}$ = 20–60 ms), which resulted from the pseudo-first-order reduction of the cytochrome by FH^- produced from FH· disproportionation (Ahmad et al., 1981). The pseudo-first-order rate constants for the reaction of FH· with the cyt *c*–cyt oxidase complex using various flavin analogues (the initial fast portion of the reaction) were determined by digitizing the kinetic traces at a particular cytochrome concentration and analyzing the data in terms of parallel pseudo-first-order (FH· + cyt) and second-order (FH· disproportionation) reactions with a Nova 2 minicomputer as described previously (Ahmad et al., 1981). The reaction of FH· with free cyt oxidase was too slow to resolve in this way. Plots of computed pseudo-first-order rate constants (k_{obsd}) vs. cytochrome concentrations were used to determine the second-order rate constants by a least-squares calculation of the slopes. On the basis of multiple determinations, the estimated error in the second-order rate constants is ±10%. The pseudo-first-order and second-order rate constants for the reduction of cyt oxidase and the cyt *c*–cyt oxidase complex by FH^- (the second slower phase of the reaction) were determined by a least-squares fitting procedure in terms of the mechanism given by eq 1. Other possible

$$FH^- + \text{cyt } c(Fe^{3+}) \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C \xrightarrow{k_{23}} FH\cdot + \text{cyt } c(Fe^{2+}) \qquad (1)$$

mechanisms will be considered below. The mechanism given by eq 1 was used, as opposed to a simple bimolecular mechanism, inasmuch as plots of the observed pseudo-first-order

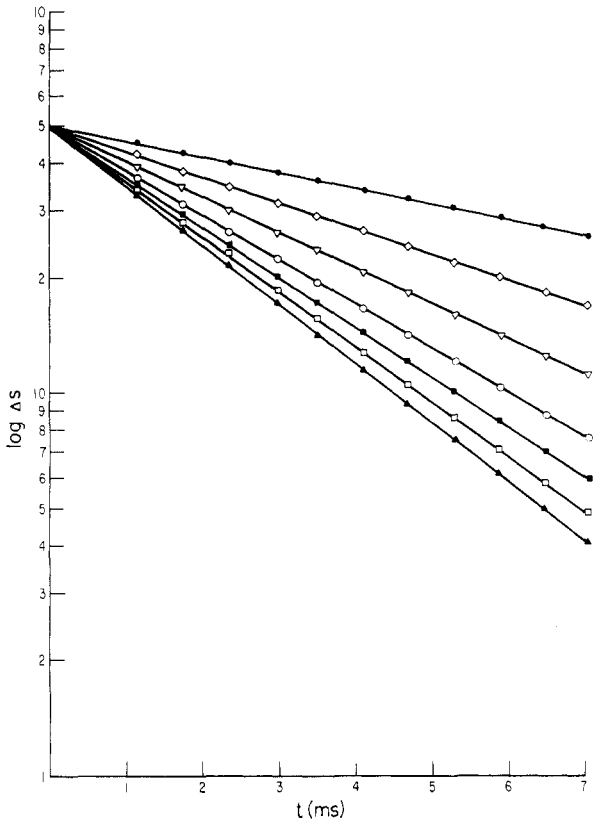

FIGURE 2: Pseudo-first-order plots for reduction of the cyt *c*–cyt oxidase complex by fully reduced lumiflavin. The signal (ΔS) is in arbitrary units. [Cytochrome] = 5 (●), 10 (◇), 15 (▽), 20 (○), 25 (■), 30 (□), and 35 μM (▲); [flavin] = 0.1 mM; [EDTA] = 5 mM; 100 mM phosphate buffer, pH 7.0, containing 1% Tween 80.

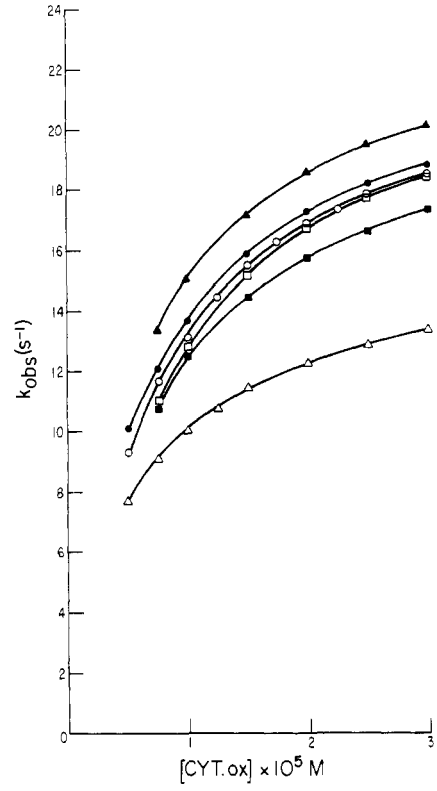

FIGURE 3: Plots of k_{obsd} vs. cyt oxidase concentrations for cyt oxidase reduction by fully reduced flavin. LF (○); RF (●); FMN (△); 10-MI (▲); Cl_2RF (□); Ac-Cys-TARF (■). Lines represent theoretical curves (see the text). Conditions are as in Figure 2.

rate constant (k_{obsd}) became independent of cytochrome concentration at high cytochrome concentrations, consistent with a change in the rate-limiting step. Since all reactions were conducted under pseudo-first-order conditions, eq 1 can be treated as two consecutive first-order reactions with the second step irreversible. Thus an explicit mathematical solution of the rate equations can be written (Frost & Pearson, 1961) and programmed into the computer. Importantly, within experimental error, plots of ln ΔA vs. time were linear, restricting possible solutions to the equation describing the mechanism. For analysis of a particular data set, ln ΔA vs. time data and corresponding cytochrome concentrations (typically 6–10 data sets) were input to the computer and initial estimates of k_{12}, k_{21}, and k_{23} provided. The program then used a steepest descents procedure to obtain a minimum least-squares error in fitting the experimental data. The only restriction applied was that the fitted solution had to be 90% or greater monophasic, inasmuch as this represented our experimental accuracy. It was found that K_1 (k_{12}/k_{21}) and k_{23} could be determined directly. However, for k_{12} we could only specify a minimum value, with any larger value giving identical fits within experimental error (with K_1 constant). These parameters (K_1, k_{23}, and the minimum k_{12}) will be reported below. The flavin semiquinone decay does not interfere in this case since the FH^- reduction reaction is slower than FH· disproportionation.

Results

In order to demonstrate that photoreduction of cyt oxidase occurs in the presence of free flavin and EDTA, we carried out preliminary studies anaerobically using steady-state irradiation with lumiflavin as the reductant. Virtually complete reduction was rapidly obtained, as indicated by the extent of the increase in absorbance at 605 nm (LFH^- does not absorb at this wavelength). A more detailed experiment of this type will be described later (cf. Figure 6).

As mentioned above, laser photolysis studies of the kinetics of reduction of cyt oxidase and the cyt *c*–cyt oxidase complex were performed under pseudo-first-order conditions; i.e., the oxidant was present in large excess over the reductants (FH· and FH^-). As an example of the type of data obtained in these experiments, a set of first-order plots for the reduction of the cyt *c*–cyt oxidase complex by LFH^- is shown in Figure 2. It can be seen that these are linear over at least three half-lives. Similar results were obtained for all of the reactions studied and will not be shown explicitly.

We will first describe the results of experiments on the reduction of free cyt oxidase by photoreduced flavins. The minimum second-order rate constants and K_1 (as defined for eq 1) for the reduction of cyt oxidase by the FH^- form of various flavin analogues, determined by laser photolysis at pH 7.0, along with the first-order limiting rate constants (k_{23}), are reported in Table I. The k_{obsd} vs. concentration plots for the flavin analogues used are shown in Figure 3, with the solid line the computer calculated curve for the parameters given in Table I. As can be seen, the fits are quite good. The close agreement in the values of the rate constants obtained with the different flavins suggests that redox potential and structural variations among the analogues do not appreciably influence reactivity. This is in contrast to our previous results with cyt *c* (Ahmad et al., 1981). Another difference between the oxidase and cyt *c* systems is that limiting first-order rates were not observed with the latter.

Cyt *c* is known to form a tight complex ($K_a \sim 10^8$ M^{-1}) with cyt oxidase (Petersen & Cox, 1980). In order to examine the role of cyt *c* in facilitating the reduction of the oxidase within

Table I: Kinetic Parameters for Reduction of Cytochrome Oxidase by Fully Reduced Flavin at pH 7.0[a]

flavin	$k_{12} \times 10^{-7}$ (M^{-1} s^{-1})[b] (min)	$K_1 \times 10^{-5}$ (M^{-1})[c]	k_{23} (s^{-1})[c]
LF	1.3	1.5 ± 0.1	22.6 ± 0.1
RF	1.3	1.7 ± 0.1	22.5 ± 0.1
FMN	1.1	2.2 ± 0.1	15.2 ± 0.1
10-MI	1.3	1.9 ± 0.1	23.7 ± 0.1
Cl_2RF	1.2	1.3 ± 0.1	23.2 ± 0.1
Ac-Cys-TARF	1.2	1.5 ± 0.1	21.2 ± 0.1

[a] The absolute error in the individual observed rate constants is estimated to be ±10%. Solutions contained 0.1 mM flavin, 5 mM EDTA, and 5–50 μM cytochrome in 100 mM phosphate buffer, pH 7.0; 1% Tween 80 was also present. [b] k_{12}(minimum) was obtained as described under Materials and Methods. [c] The standard deviations were derived from fits using different values of k_{12}, ranging from the minimum value to approximately 3×10^8 M^{-1} s^{-1}, and demonstrate the insensitivity of K_1 and k_{23} to k_{12} above the minimum value of k_{12}.

Table II: Kinetic Parameters for Reduction of Cytochrome *c* and the Cytochrome *c*–Cytochrome Oxidase Complex[a] by Flavin Semiquinone and Fully Reduced Flavin at pH 7.0

	FH· reduction[b]		FH⁻ reduction[b]			
	$[k_{12} \times 10^{-7}$ (M^{-1} s^{-1})]		$k_{12} \times 10^{-7}$ (M^{-1} s^{-1})		$K_1 \times 10^{-4}$	
flavin	cyt *c*[c]	complex	cyt *c*[c]	complex	(M^{-1}), complex[d]	k_{23} (s^{-1}), complex[d]
LF	6.2	11.0	2.9	8	3.8 ± 0.5	650 ± 9
RF	6.2	5.6	3.1	9	4.3 ± 0.4	511 ± 6
FMN	7.0	5.6	3.0	9	3.1 ± 0.3	596 ± 6
10-MI	7.6	12.0	5.3	12	4.1 ± 0.5	658 ± 6
Cl_2RF	6.3	5.6	1.6	4	3.4 ± 0.3	216 ± 4
Ac-Cys-TARF	3.3	2.8	1.6	5	3.4 ± 0.4	294 ± 6

[a] The molar ratio of cyt *c* to cyt oxidase was 1:1. Conditions were as in Table I. [b] k_{12} for the complex is the minimum value determined as described under Materials and Methods. [c] Cyt *c* data from Ahmad et al. (1981). [d] The standard deviations were determined as described in footnote *c*, Table I.

such a complex, we performed laser photolysis studies with different flavin analogues. The rate constants for the reduction of the complex by FH· and FH^- are reported in Table II, along with some corresponding data for free cyt *c* (Ahmad et al., 1981). The rate constants for the FH· reactions were obtained by least-squares analysis of the computer-derived k_{obsd} values at different cytochrome concentrations (Figure 4) as described above. The k_{obsd} vs. concentration plots for the FH^- reactions are shown in Figure 5. Again, the fits are excellent. As was the case with free cyt *c* (Ahmad et al., 1981), the reactions of the FH· forms of the various flavin analogues with the cyt *c*–cyt oxidase complex do not show any saturation effects at high oxidant concentrations. Thus, the limiting first-order rate constants must be large. On the other hand, the FH^- reactions do exhibit saturation, as was found with the free oxidase. The second-order rate constants for FH· reduction of the complex are much larger than is the case for free oxidase. Similarly, the second-order rate constants for FH^- reduction are 3–10 times larger, and the limiting first-order rate constants are 10–30 times larger. These results suggest either that the reduction of cyt oxidase in the complex proceeds via cyt *c* or that electrons enter directly into cyt oxidase and complex formation appreciably modifies its properties. The former conclusion is supported by the fact that the rate constants for FH· reduction are very similar to those found previously for free cyt *c* reduction (Ahmad et al., 1981), especially with

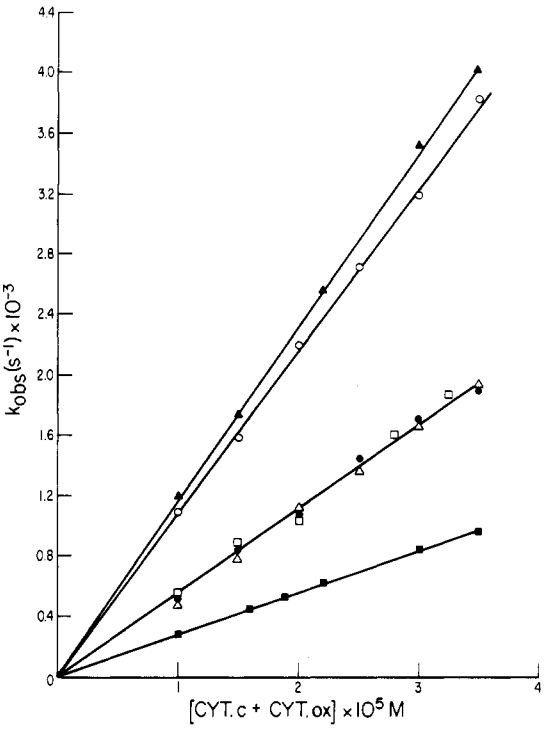

FIGURE 4: Plots of k_{obsd} vs. cytochrome concentrations for the cyt *c*–cyt oxidase complex reduction by flavin semiquinone. Symbols are as in Figure 3. Conditions are as in Figure 2.

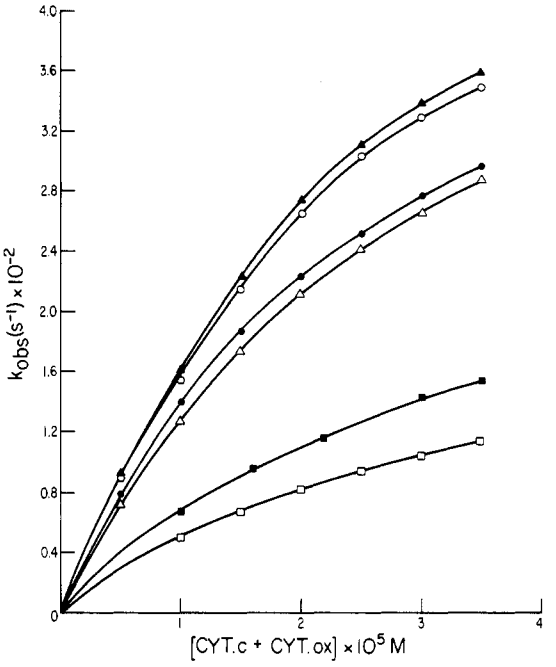

FIGURE 5: Plots of k_{obsd} vs. cytochrome concentrations for the cyt *c*–cyt oxidase complex reduction by fully reduced flavin. Symbols are as in Figure 3. Lines represent theoretical curves (see the text). Conditions are as in Figure 2.

regard to the relative values for the various analogues (with the possible exception of LF). A similar pattern is found for the FH^- reaction. Further evidence for the intermediary role of cyt *c* in these reactions was obtained by studies performed in the presence of 10 mM CN^-. It was found (Table III) that the extents of LFH^- reduction (at pH 8.0) of free cyt *c* and the complex were decreased by approximately the same amounts (60% and 71%, respectively). However, the reduction of the free oxidase was decreased by an appreciably smaller amount (20%). Inasmuch as CN^- is known to complex with

Table III: Effect of Cyanide on Cytochrome Reduction by Photoreduced Lumiflavin at pH 8.0[a]

sample	concn of reduced heme ($\times 10^6$ M) no CN^-	plus 10 mM CN^-	% decrease due to CN^-
cyt *c*	5.3	2.1	60
cyt oxidase	6.1	4.9	20
cyt *c*–cyt oxidase complex	6.9	2.0	71

[a] Initial cytochrome concentration was 30 μM. Estimated error was ±10%. Conditions were as in Table I, except the pH was 8.0.

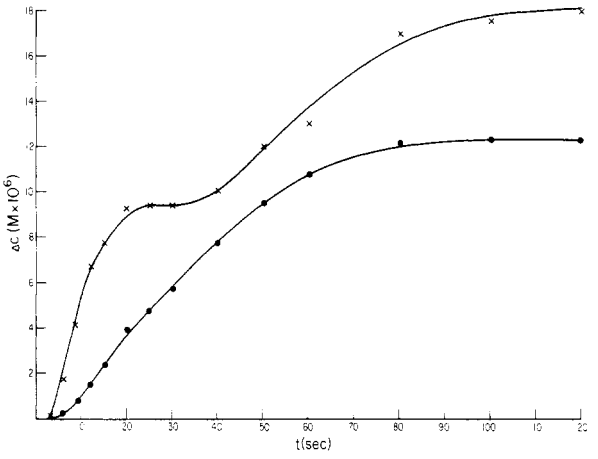

FIGURE 6: Phototitration plots of concentration changes (Δc) vs. time determined at 550 (●) and 605 nm (×) for steady-state photoreduction of the cyt *c*–cyt oxidase complex by lumiflavin. Cytochrome concentrations were 10 μM. Conditions are as in Figure 2.

cyt *c* (Sutin & Yandell, 1972) and with the cyt a_3 component of cyt oxidase (Gibson et al., 1965), these results are consistent with a mechanism involving electron transfer from FH^- to cyt *c* and then to the cyt *a* component of cyt oxidase.

When laser photolysis of the complex was observed at 550 nm (a wavelength at which cyt *c* oxidation–reduction can be seen), no transient was found beyond that which could be ascribed to cyt oxidase reduction. This indicates that the electron transfer between cyt *c* and cyt oxidase is rapid compared to the rate-limiting step in the overall reaction. This is consistent with the results of a steady-state phototitration experiment in which the absorption spectra of a mixture of LF, EDTA, and the cyt *c*–cyt oxidase complex were measured at various stages of photoreduction. A plot of the concentration changes (determined at 550 and 605 nm) obtained in such an experiment is shown in Figure 6. It is seen that the increase at 605 nm (cyt oxidase reduction) precedes that at 550 nm (cyt *c* reduction) up to approximately half-reduction. This is followed by a steep increase in the absorbance at 550 nm, probably due to back electron transfer to cyt *c*. This result is consistent with the redox potentials of cyt *c* and cytochromes *a* and a_3 (Wilson et al., 1972; Leigh et al., 1974; Tiesjema et al., 1973). When laser photolysis was carried out by using a sample that was at the plateau region with respect to oxidase reduction, again no transient due to cyt *c* could be observed at 550 nm. Thus, even at this stage of the reaction, cyt *c*–cyt oxidase electron exchange is rapid.

In Table IV a series of determinations of the stoichiometry of the laser photolysis reaction between flavins and the cyt *c*–cyt oxidase complex is presented. It can be seen that, in all cases, a ratio of approximately 2:1 is found for the concentrations of FH· produced by the laser flash and the final

Table IV: Stoichiometry of Flavin Reduction of the Cytochrome *c*–Cytochrome Oxidase Complex[a]

cyt oxidase concn × 10^5 M	lumiflavin FH· × 10^7 M	lumiflavin reduced heme *a* × 10^7 M	riboflavin FH· × 10^7 M	riboflavin reduced heme *a* × 10^7 M	FMN FH· × 10^7 M	FMN reduced heme *a* × 10^7 M
1.0	7.0	3.3	4.0	1.8	4.0	1.8
1.5	6.4	3.3	3.7	1.7	3.6	1.6
2.0	5.3	2.7	3.2	1.5	3.0	1.5
2.5	4.6	2.5	2.6	1.4	2.8	1.5
3.0	3.8	2.0	2.5	1.3	2.6	1.3
3.5	3.8	2.1	2.6	1.3	2.8	1.3

[a] Conditions were as in Table I; no net cytochrome *c* reduction occurred under these conditions.

concentration of reduced heme in the cyt oxidase molecule. This demonstrates that most of the reduction occurs as a consequence of the FH^- reaction (the disproportionation reaction produces one FH^- per two FH·), and only a relatively small amount of reduction proceeds via reaction with FH·. Furthermore, it indicates that the FH^- reaction is a one-electron transfer; otherwise, one would expect a 1:1 stoichiometry with respect to FH·.

Discussion

It is clear that free cyt oxidase is only slowly reduced by photochemically reduced flavins (Table I); moreover, the structure, net charge, and oxidation–reduction potential of the free flavins do not appear to influence the observed kinetics. This is in sharp contrast to the reduction of cyt *c* by photochemically reduced flavins, where the kinetics are dependent on the structural features of the flavin used (Ahmad et al., 1981). The kinetic differences between cyt *c* and cyt oxidase may be a consequence of the fact that the second-order rate constants for the oxidase reactions are somewhat smaller than those for cyt *c*, allowing more time for the reactants to sample a variety of mutual orientations. This could act to minimize steric constraints upon complex formation. The insensitivity of the limiting first-order rate constants to flavin structure and redox potential suggests a rate-limiting protein conformation change rather than the actual electron transfer step. However, it is important to note that we have no direct evidence that the mechanism given by eq 1 is operative in this case. Thus a mechanism that involves the rate-limiting interconversion of two cyt oxidase conformers, with only one of these reactive with FH^-, cannot be excluded. Whatever the correct mechanism, the addition of cyt *c* clearly has a profound effect on the kinetics of reduction of cyt oxidase (see Figures 3 and 5). This will be further discussed later.

Reduction of the cyt *c*–cyt oxidase complex by photoreduced flavins has rate constants as large or larger than those measured for cyt *c* alone (Table II). In the case of FH· reduction, with the exception of LF, the relative rate constants for the different flavin analogues are similar for the two systems. Thus the rate constants for RF, FMN, and Cl_2RF are about the same, 10-MI somewhat larger, and Ac-Cys-TARF substantially less. From these data we can conclude that the presence of cyt oxidase has little effect on the reduction of cyt *c* by the semiquinone form of the various flavins.

Analysis of FH^- reduction of the cyt *c*–cyt oxidase complex is somewhat less straightforward and requires some background prior to discussion. It is well established (Gibson et al., 1965) that the reaction of free ferrocyt *c* with cyt oxidase is rapid ($k \simeq 4 \times 10^7$ M^{-1} s^{-1}); moreover, steady-state analysis of the cyt *c*–cyt oxidase interaction suggests that two binding

sites (high and low affinity) exist. Although the cyt *c*–cyt oxidase high-affinity association constant has not been measured under the present experimental conditions (100 mM phosphate buffer, pH 7.0), it is expected to be large ($>1 \times 10^6$ M^{-1}), and thus the two proteins should be substantially complexed, consistent with the observed kinetics (see below). For example, Errede & Kamen (1978) report binding constants for the high-affinity site of 1.2×10^7 M^{-1} and 6.2×10^6 M^{-1} at 4 and 44 mM ionic strength, respectively. Thus, the ionic strength dependence is not large enough to negate the above expectation. The bulk of the studies to date have been carried out by using either steady-state methods, which presume a mechanism and require that the relative values of the rate constants permit application of the steady-state assumption, or mixing methods, which are dominated by the collision process. The studies reported here are inherently different in that preexisting cyt *c*–cyt oxidase complexes are rapidly exposed to electrons provided by the laser-generated reduced flavins. Thus, in the present situation the analysis becomes relatively simple in that inactive complexes do not affect the observed kinetics. Moreover, in all cases studied the reactions were accurately first order for three to four half-lives, indicating that the cyt *c* and cyt oxidase, which undergo reduction, are kinetically homogeneous (true for both FH· and FH^- reduction). In view of the facts that the rate constants at any particular cyt *c* concentration are different for free and bound cyt *c* and that in the case of FH^- reduction of the cyt *c*–cyt oxidase complex a change in the rate-limiting step is observed, we have to conclude that for the conditions of our experiment only the cyt *c*–oxidase complex exists [i.e., no appreciable (>10–20%) free cyt *c* is present]. In view of the stoichiometry used (1 mol of cyt *c* to 1 mol of cyt oxidase), we must also conclude that only the high-affinity cyt oxidase site is occupied. Nevertheless, as with FH· reduction, we find that the relative values of the measured rate constants are similar for free and bound cyt *c* (Table II) with FMN, LF, and RF approximately the same, 10-MI somewhat larger, and Cl_2RF and Ac-Cys-TARF substantially less. As a consequence, we must argue that, although cyt *c* is bound to cyt oxidase, it undergoes reduction by FH^- in the same general region on its molecular surface as it does in the free state. This conclusion is not unprecedented as Hill & Nicholls (1980) have found that *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine and diaminodurene reduce both free cyt *c* and bound cyt *c*. Indeed, they found, just as reported here (Table II), that the bound form is more rapidly reduced. Thus, it may be that what is being affected by complex formation is the molecular cross section for effective reaction with the reductant, perhaps as a consequence of a larger active-site region in the cyt *c*–cyt oxidase system. Whatever the case, as with free cyt *c*, steric and charge effects appear to be playing a role (Ahmad et al., 1981). It is important to note that under no conditions could we kinetically observe the transient formation of ferrocyt *c*. Thus electron transfer from ferrocyt *c* to heme *a* in the complex must be rapid (>700 s^{-1}).

The available data are consistent with the view that heme *a* reduction is mediated by cyt *c*. This is derived from three observations. First, formation of the cyt *c*–cyt oxidase complex substantially alters the observed rate constants, suggesting the participation of cyt *c*. Second, the kinetic differences between the various flavin analogues parallel those observed when reaction occurs with cyt *c* in the absence of cyt oxidase and not those observed with oxidase by itself. Third, cyanide reduces the extent of reduction of cyt *c* and heme *a* in a parallel fashion. Since under the conditions of the experiments reported cyt *c* and cyt a_3, but not cyt *a*, should be complexed with cyanide, it is evident that electron transfer to cyt *a* must occur via cyt *c* when a cyt *c*–cyt oxidase complex is present. Such a conclusion is identical with that of Hill & Nicholls (1980), who found that at low ionic strength, when the cyt *c*–cyt oxidase complex is formed, the rate-limiting step is the reduction of cyt *c*. Hence, direct reduction of cyt *c* in its tight complex with the oxidase is followed by rapid intramolecular electron transfer to both cyt *a* and its associated copper atom (Hill & Nicholls, 1980).

When a change in the rate-limiting step is observed (as shown by Figures 3 and 5), two simple mechanisms must be considered. First, as given by eq 1, complex formation occurs, followed by some first-order process (electron transfer or conformational change). Second, an equilibrium between two conformers exists with only one reactive with the reducing agent. These two mechanisms are mathematically equivalent and cannot be separated on the basis of kinetic data alone. However, in the case of reduction of the cyt *c*–cyt oxidase complex, it can be concluded that eq 1 applies since the limiting rate constant (k_{23}) is dependent on the nature of the particular flavin analogue used. Thus, a protein-linked equilibrium between two conformers can be excluded since this would require that the first-order process be independent of the reactant. However, the limiting first-order process cannot be ascribed to an intrinsic property of the cyt *c* (for example, a conformational change following complex formation or electron transfer) since this should also be independent of the flavin analogue used. A possible rationalization of the observed results may be given in terms of a rate-limiting rearrangement of the initial collision complex, which allows FH^- to gain access to the heme crevice of cyt *c* prior to electron transfer. Thus Ac-Cys-TARF, which has a negative charge at the 8α position, and Cl_2RF, which may be more polar than RF because of the electronegativity of the chlorine atoms (Ahmad et al., 1981), have substantially smaller limiting rate constants. This could be a consequence of the positively charged cyt *c* surface. Consistent with this interpretation is the fact that FH· reduction of the complex shows no change in the rate-limiting step, suggesting that it is the net negative charge on the flavin ring in FH^- that is in part responsible for the observed change in the rate-limiting step. The analysis given here requires that the dimethylbenzene ring of the flavin moiety must be the dominant structural feature in the interaction of reduced flavins with the complex. This conclusion is identical with that derived from a study of the reaction of the various flavin analogues with uncomplexed cyt *c* (Ahmad et al., 1981).

In conclusion, the studies reported here establish that photochemically reduced free flavins can be used to transfer electrons to the cyt *c*–cyt oxidase complex and that subsequent reactions can be studied. Although only FH^- will measurably reduce free cyt oxidase, both FH^- and FH· are effective in reducing the complex. The data presented establish that reduction of heme *a* in the cyt *c*–cyt oxidase complex proceeds through cyt *c* and, most importantly, that the apparent interaction site on cyt *c* is the same in the complex as with free cyt *c* and that, although a tight complex is formed, at least small reactants like the flavins are not sterically restricted in their interaction with bound cyt *c*. The data also show that the rate at which electrons can be transferred from reduced flavins to cyt oxidase is greatly enhanced in the presence of cyt *c*.

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Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System: Role of Divalent Metals in the Dimerization and Phosphorylation of Enzyme I†

Henk Hoving, Johan H. Koning, and George T. Robillard*

ABSTRACT: The function of divalent metal ions (Mg^{2+} and Mn^{2+}) in the dimerization and phosphorylation of enzyme I has been studied. Only a dimeric form of the enzyme can be phosphorylated [Misset, O., Brouwer, M., & Robillard, G. T. (1980) *Biochemistry 19*, 883–890; Hoving, H., Lolkema, J. S., & Robillard, G. T. (1981) *Biochemistry 20*, 87–93]. Kinetic studies of phosphoryl-group exchange between phosphoenolpyruvate and pyruvate and measurements of initial enzyme I phosphorylation rates revealed that a divalent metal ion must be bound to the enzyme to render the dimer active. Mn^{2+} binding experiments by means of electron paramagnetic resonance showed binding of at least one Mn^{2+} per unphosphorylated dimer with a binding constant comparable to the activation constant found in the kinetic studies and a 10-fold tighter binding of only one Mn^{2+} per phosphorylated dimer. Gel filtration experiments provided evidence that divalent metals produce about a 10-fold stabilization of the dimers, in addition to their effect on the specific dimer activity. The stability of the dimer was also strongly dependent on salts such as LiCl, NaCl, KCl, and a series of tetraalkylammonium chlorides. The relative effects of these salts suggest that hydrophobic interactions possibly play a significant role in enzyme I dimerization.

Enzyme I, a component of the bacterial PEP[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phospho-carrier protein HPr. This is the first step in a process that ultimately leads to the phosphorylation and concomitant transport of PTS sugars into the bacterial cell (Roseman, 1969; Postma & Roseman, 1976; Hengstenberg, 1977; Saier, 1977; Hays, 1978). During the last few years increasing evidence has been presented that the enzyme I catalyzed reaction proceeds via a phosphoenzyme I intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981). It has also been shown that the active dimeric form of the enzyme reversibly dissociates into inactive monomers (Waygood et al., 1979; Misset et al., 1980):

$$2E_I^m \rightleftharpoons E_I^d \quad (1a)$$

$$PEP + E_I^d \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_I^dP + Pyr \quad (1b)$$

$$E_I^dP + HPr \rightleftharpoons PHPr + E_I^d \quad (1c)$$

In the following paper (Misset & Robillard, 1982) data are presented indicating that the reactions in the above sequence, although basically correct, are a simplification because there is also an interaction between HPr and unphosphorylated enzyme I. It is also shown in the following paper that only one phosphoryl group can be bound per enzyme I dimer.

The phosphorylation of enzyme I is dependent on divalent metals, a property that this enzyme has in common with many other phosphoryl-group transferring enzymes. In a recently published study of enzyme I catalyzed phosphoryl-group exchange between PEP and pyruvate, we showed that Mg^{2+} shifted the monomer–dimer equilibrium of enzyme I toward the dimer side (Hoving et al., 1981). In a similar study Saier et al. (1980) demonstrated that Mn^{2+} and Co^{2+} can substitute for Mg^{2+}.

The purpose of the study presented here was to provide more insight into the role of divalent metal ions in enzyme I phosphorylation. Since phosphorylation can only proceed in

† From the Department of Physical Chemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands. *Received August 11, 1981.* This research has been supported by The Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from The Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

[1] Abbreviations: PTS, phosphotransferase system; PEP, phosphoenolpyruvate; Pyr, pyruvate; DTT, dithiothreitol; TMA^+, tetramethylammonium; TEA^+, tetraethylammonium; TBA^+, tetrabutylammonium; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

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