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Effect of Specific Lysine Modification on the Reduction of Cytochrome *c* by Succinate–Cytochrome *c* Reductase†

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ABSTRACT: The reduction of cytochrome *c* by succinate–cytochrome *c* reductase was studied at very low cytochrome *c* concentrations where the reaction between cytochrome c_1 and cytochrome *c* was rate limiting. The rate constant for the reaction was found to be independent of ionic strength up to 0.1 M chloride, and to decrease rapidly at higher ionic strength, suggesting that the interaction between cytochrome c_1 and cytochrome *c* was primarily electrostatic. The reaction rates of cytochrome *c* derivatives modified at single lysine residues to form trifluoroacetylated or trifluoromethylphenylcarbamylated cytochromes *c* were studied to determine the role of individual lysines in the reaction. None of the modifications affected the reaction at low ionic strength, but at higher ionic strength the reaction rate was substantially decreased by modification of those lysines surrounding the heme crevice, lysine-8, -13, -27, -72, and -79. Modification of lysine-22, -25, -55, -99, and -100 had no effect on the rate. These results indicate that the binding site on cytochrome *c* for cytochrome c_1 overlaps considerably with that for cytochrome oxidase, suggesting that cytochrome *c* might undergo some type of rotational diffusion during the electron-transport process.

The mechanism by which cytochrome *c* transfers electrons from cytochrome c_1 to cytochrome oxidase is not well understood. Several lines of evidence suggest that there is some difference between the reaction sites on cytochrome *c* for cytochrome c_1 and cytochrome oxidase. Smith et al. (1976) have compared the reactivity of several cytochromes *c* with several different oxidases and reductases and have concluded that the two reaction sites are different but both close to the heme crevice. Smith et al. (1973) found that a purified cytochrome *c* specific antibody completely inhibited the cytochrome oxidase activity of cytochrome *c*. The antibody significantly decreased the rate of reduction of cytochrome *c* by succinate–cytochrome *c* reductase but did not completely prevent it.

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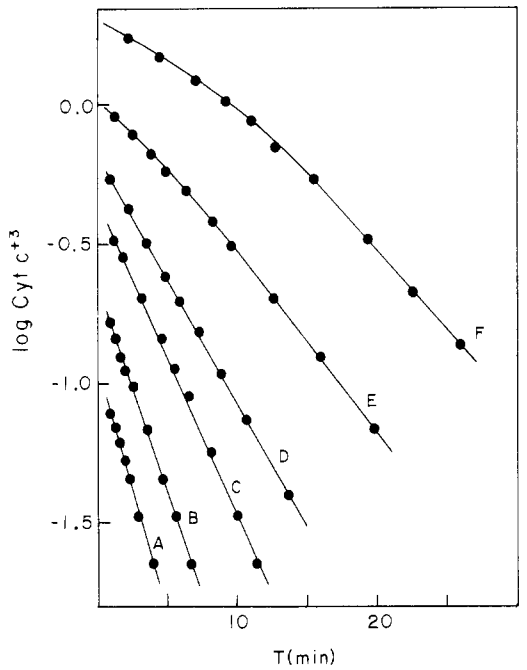

FIGURE 1: Time course for the reduction of cytochrome *c* by succinate–cytochrome *c* reductase in pH 7.5 Tris-Cl buffer containing 0.2 M chloride, 10 mM succinate at 25 °C. The cytochrome c_1 concentration was 0.20 nM, and the total cytochrome *c* concentration was 0.1 (A), 0.2 (B), 0.5 (C), 0.7 (D), 1.0 (E), and 2 μM (F).

Margoliash et al. (1973) reported that modification of lysine-13 at the top of the heme crevice of cytochrome *c* decreased the oxidase activity without affecting the reductase activity. Erecinska et al. (1975) have used a photoaffinity label to cross-link an unspecified lysine on cytochrome *c* to cytochrome oxidase. The complex was found to be partially active in transporting electrons from succinate–cytochrome *c* reductase to O_2, indicating that cytochrome *c* probably does not undergo long-range diffusion during electron transport. However, Bisson et al. (1977) have found that a very similar complex was completely inactive in the absence of free cytochrome *c*. Recently, cytochrome *c* derivatives containing singly modified lysine residues have been used to establish that the reaction between cytochrome *c* and cytochrome oxidase involves electrostatic interactions between the highly conserved lysines surrounding the heme crevice of cytochrome *c* and negatively charged residues on cytochrome oxidase (Smith et al., 1977; Ferguson-Miller et al., 1978; Staudenmayer et al., 1976, 1977). In this paper, we report the effect of modification of specific cytochrome *c* lysines on its reactivity with succinate–cytochrome *c* reductase.

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI), cholic acid, and ammonium sulfate were obtained from Sigma Chemical Co. Tris[1] was obtained from Schwarz/Mann. The specifically trifluoroacetylated (TFA) derivatives were prepared according to the procedure of Staudenmeyer et al. (1976, 1977), while the trifluoromethylphenylcarbamoyl (TFC) derivatives were prepared by the procedure of Smith et al. (1977). All derivatives were chromatographed a final time on a 1.5 × 10 cm column of Whatman CM32 carboxymethylcellulose eluted with 0.08 M phosphate buffer (pH 6.0). The derivatives were passed through a Bio-Gel P-60 column immediately prior to the enzyme kinetic studies to remove any possible polymeric material. The derivatives were never lyophilized at any point in the purification. Beef heart succinate–cytochrome *c* reductase was prepared according to the method of Yu et al. (1974). Cytochrome *c* depleted Keilin–Hartree particles were prepared by the method of Smith and Camerino (1963). Protein concentration was determined by the biuret method after solubilization of the Keilin–Hartree particles as described by Jacobs et al. (1956).

Methods. The reductase preparation was treated with 5% cholate (1 mg/mg of protein) and diluted with 0.2 M Tris-Cl (pH 7.5) buffer containing 10 mM succinate immediately prior to use. The assay medium routinely contained 0.1 to 12 μM ferricytochrome *c*, 10 mM succinate, and 0.2 M Tris-Cl (pH 7.5) buffer. The rate of reduction of cytochrome *c* following the addition of reductase was monitored at 420 nm on a Cary 14 spectrophotometer equipped with a 0–0.05 absorbance slide-wire. A 4-cm cell was used for lower concentrations of cytochrome *c*, while a 1-cm cell was used for the higher concentrations.

Results

Smith et al. (1974) have reported that the reduction of cytochrome *c* by the succinate–cytochrome *c* reductase activity of Keilin–Hartree particles is mixed zero and first order and suggested that the zero-order rate is probably limited by the reduction of cytochrome *c* by earlier redox components and not by the rate of the reaction between cytochrome *c* and cytochrome c_1. They used cyanide to inhibit the oxidase activity in the preparation. We chose to use a purified succinate–cytochrome *c* reductase preparation to obviate any possible effects of cyanide on the cytochrome *c* derivatives. The kinetics of cytochrome *c* reduction by the purified reductase were mixed zero and first order at cytochrome *c* concentrations down to 1 μM (Figure 1), in agreement with the results of Smith et al. (1974). At low cytochrome *c* concentrations (<0.5 μM) the reaction became purely first order in cytochrome *c*, indicating that the reaction between cytochrome c_1 and cytochrome *c* was rate limiting. The rate constant of the first-order component of the reaction decreased somewhat at higher cytochrome *c* concentrations, indicating that the product ferrocytochrome *c* might inhibit the reaction, as discussed by Smith et al. (1974). To avoid the complexities of product inhibition, we measured the initial velocity of the reaction as a function of cytochrome *c* concentration, recognizing that the apparent maximum velocity of the reaction was not related to the cytochrome c_1–cytochrome *c* reaction. The parameter $k_v = (v/S)_{v \to 0}$ obtained by extrapolating v/S to $v = 0$ on an Eadie–Hofstee plot (Figure 2) is probably the most unambiguous kinetic parameter that can be measured under these conditions. It was equal to the first-order rate constant for the reaction in the limit of zero cytochrome *c* concentration. Furthermore, the constant k_v/E_0 obtained by dividing k_v by the cytochrome c_1 concentration E_0 was found to be 3.4×10^7 M^{-1} s^{-1} (0.1 M phosphate, pH 7.0, 25 °C), in reasonable agreement with the second-order rate constant for the reaction between purified cytochrome c_1 and cytochrome *c* measured by stopped-flow techniques (3.3×10^6 M^{-1} s^{-1} in 0.1 M phosphate, pH 7.0, 10 °C; Yu et al., 1973). The effect of ionic strength was rather unusual (Figures 2 and 3). The rate constant k_v was nearly independent of anion concentration up to 0.1 M chloride or 0.07 M phosphate but then decreased rapidly. The apparent maximum velocity increased somewhat as the anion concentration was increased up to 0.15 M chloride,

[1] Abbreviations used are: TFA, trifluoroacetyl; TFC, trifluoromethylphenylcarbamyl; Tris, tris(hydroxymethyl)aminomethane.

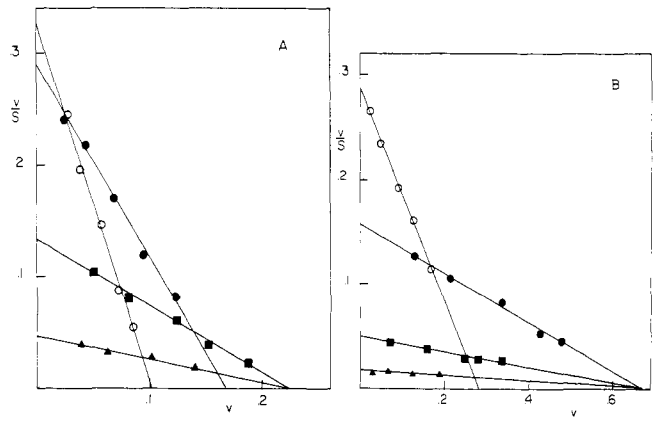

FIGURE 2: The effect of ionic strength on the reduction of cytochrome *c* by succinate-cytochrome *c* reductase. The cytochrome c_1 concentration was 0.08 nM and the temperature 25 °C. The initial rates of reduction, *v*, were measured in μM cytochrome *c* reduced per minute, and the concentration of cytochrome *c*, *S*, was in μM. (A) The chloride concentration in the pH 7.5 Tris-Cl buffer was 0.02 (○), 0.1 (●), 0.2 (■), and 0.3 M (▲). (B) The total phosphate concentration in the pH 7.0 sodium phosphate buffer was 0.01 (○), 0.10 (●), 0.2 (■), and 0.3 M (▲).

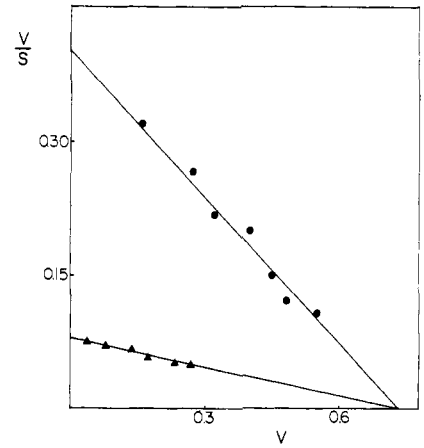

FIGURE 4: The rate of reduction of native cytochrome *c* (●) and TFA-Lys-13 cytochrome *c* (▲) by succinate-cytochrome *c* reductase in pH 7.5 Tris-Cl buffer containing 0.2 M chloride and 10 mM succinate at 25 °C. The cytochrome *c* concentration *S* was in μM, and the initial velocity *v* was measured in μM cytochrome *c* reduced per minute. The cytochrome c_1 concentration was 0.20 nM.

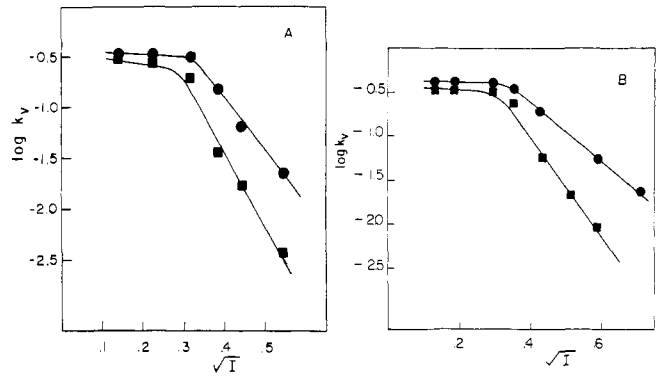

FIGURE 3: The effect of ionic strength on the rate constant k_v for the reduction of cytochrome *c* by succinate-cytochrome *c* reductase at 25 °C. k_v was measured in min^{-1} for native cytochrome *c* (●) and TFA-Lys-13 cytochrome c_1 (■). The total cytochrome c_1 concentration was 0.08 nM. (A) Tris-Cl buffer, pH 7.5. The ionic strength *I* was calculated assuming the pK_a of Tris was 8.0. (B) Sodium phosphate buffer, pH 7.0. The ionic strength *I* was calculated assuming the pK_a of phosphate was 7.2.

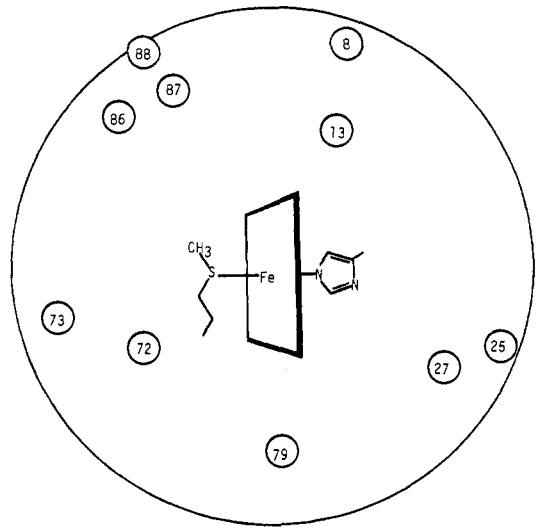

FIGURE 5: A schematic diagram of horse heart cytochrome *c* showing the approximate positions of the lysine groups surrounding the heme crevice. The view is from the front of the heme crevice (Swanson et al., 1977).

or 0.1 M phosphate, and then stabilized. Above the plateau region, log k_v decreased approximately linearly as the square root of the ionic strength increased. At low ionic strength, the rate constant for the TFA-Lys-13 derivative was nearly the same as that for native, but at higher ionic strength it was about fivefold less than that for native cytochrome *c* (Figures 3 and 4). At 0.2 M chloride, none of the lysine modifications of cytochrome *c* affected the apparent maximum velocity of reduction, but there was a substantial decrease in the rate constant k_v upon modification of lysine-8 and -13 at the top of the heme crevice (Figure 5) and lysine-72 at the left side of the heme crevice (Table I). Modification of lysine-27 at the right side of the heme crevice and lysine-79 at the bottom of the heme crevice led to similar decreases in the rate constant. The reduction rate was unaffected by modification of lysine-22 and -25 at the right side of cytochrome *c*, lysine-99 and -100 at the back of cytochrome *c*, and lysine-55 at the bottom left. None of the modifications affected k_v at low ionic strength. The pH dependence of k_v at 0.2 M chloride is shown in Figure 6.

The lysine modifications and ionic strength had nearly the same effect on the reductase activity of cytochrome *c* when a Keilin-Hartree preparation was used (Staudenmeyer et al., 1976; Millett, et al., 1976), indicating that the properties of the succinate-cytochrome *c* reductase were not affected by the purification procedures and also that cytochrome oxidase present in the Keilin-Hartree preparation did not perturb the reaction between cytochrome c_1 and cytochrome *c*. Potassium cyanide (1 mM) was used in these latter studies to inhibit the oxidase activity.

Discussion

The kinetics of the reduction of cytochrome *c* by succinate-cytochrome *c* reductase are complicated by a number of factors, the two most important being the presence of a rate-limiting step prior to the electron transfer between cytochrome c_1 and cytochrome *c* and product inhibition of cytochrome c_1 by ferrocytochrome *c* (Smith et al., 1974). These complications can both be circumvented by measuring the rate of reduction at low cytochrome *c* concentrations where the cytochrome

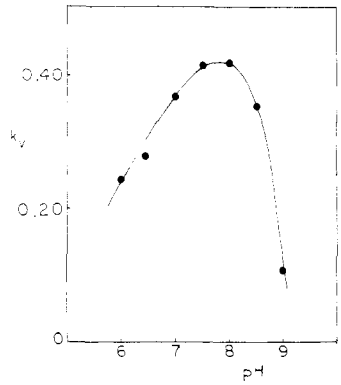

FIGURE 6: The pH dependence of the rate constant k_v for the reduction of cytochrome c by succinate-cytochrome c reductase at 25 °C. The buffers all contained 0.2 M chloride and enough Tris to adjust the pH to the required value. The cytochrome c_1 concentration was 0.20 nM.

TABLE I: Enzymatic Activity of Cytochrome c Derivatives.

Derivative	Cytochrome c_1 act. k_v/E_0 (M^{-1} s^{-1})[e]	Cytochrome oxidase act. K_m (μM)	Cytochrome b_5[d] act. k_v/E_0 (M^{-1} s^{-1})
Native	3.4×10^7	0.051[a]	3.0×10^8
TFA-Lys-13	0.8	0.25[a]	1.1
TFA-Lys-22	3.4	0.057[b]	2.9
TFA-Lys-25	3.6	0.14[a]	1.7
TFA-Lys-55	3.5	0.051[b]	2.9
TFA-Lys-99	3.3	0.046[a]	3.1
TFC-Lys-8	2.0	0.12[c]	2.1
TFC-Lys-13	0.9	0.35[c]	1.1
TFC-Lys-27	1.6	0.080[c]	1.7
TFC-Lys-72	1.6	0.14[c]	1.2
TFC-Lys-79	1.5	0.22[c]	1.6
TFC-Lys-100	3.4	0.048[c]	3.0

[a] Staudenmayer et al. (1977). [b] Staudenmayer et al. (1976). [c] Smith et al. (1977). [d] Ng et al. (1977). [e] Measured as described in the text in Tris-Cl buffer containing 0.2 M Cl^-, pH 7.5, 10 mM succinate, 25 °C. E_0, the cytochrome c_1 concentration of the succinate-cytochrome c reductase complex, was 0.20 nM.

c_1-cytochrome c reaction becomes rate limiting. We feel that the rate parameter $k_v = (v/S)_{v \to 0}$ is the most unambiguous parameter that can be measured which relates directly to the cytochrome c_1-cytochrome c reaction. Although there is insufficient data to determine a specific mechanism for the reduction of cytochrome c, the involvement of an active enzyme-substrate complex is suggested by the fact that cytochrome c_1 and cytochrome c form a stable complex in both their fully oxidized and fully reduced states (Chiang et al., 1976). The dissociation constant of the complex rapidly increases as ionic strength increases, indicating that electrostatic interactions are important in maintaining the complex. It is useful to consider the following hypothetical mechanism which is similar to the Minnaert type IV mechanism (Minnaert, 1961) in which the regeneration of reduced cytochrome c_1 by the earlier redox components is not assumed to occur infinitely rapidly but with a first-order rate constant k_r.

$$c_1^{2+} + c^{3+} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} c_1^{2+}c^{3+} \xrightarrow{k_3} c_1^{3+} + c^{2+} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} c_1^{3+} + c^{2+}$$
$$c_1^{3+} \xrightarrow[k_r]{} c_1^{2+} \qquad (1)$$

At low cytochrome c concentrations the first two steps become rate limiting and steady-state assumptions lead to the result $k_v/E_0 = (k_3k_1)/(k_2 + k_3)$, where E_0 is the total cytochrome c_1 concentration. This result also holds for a number of alternative mechanisms involving complex formation. The unusual effect of ionic strength can be explained if it is assumed that the dissociation rate constant k_2 increases with ionic strength, while k_1 is independent of ionic strength and possibly diffusion limited. At low ionic strength, k_2 would be negligible compared to k_3, and $k_v/E_0 \approx k_1$. k_v will remain constant as the ionic strength is increased until k_2 becomes comparable to k_3, and then k_v will decrease. The ionic-strength dependence of the reaction demonstrates that the interaction between cytochrome c_1 and cytochrome c is largely electrostatic, in agreement with the equilibrium binding studies (Chiang et al., 1976). In fact, the cytochrome c_1-cytochrome c reaction depends nearly as strongly on ionic strength above the plateau region as does the cytochrome c-cytochrome oxidase reaction (Ferguson-Miller et al., 1976). Log k_v was found to be approximately proportional to the square root of the ionic strength above the plateau region, as suggested by a simple mechanism involving complementary charge interactions between cytochrome c_1 and cytochrome c (Wherland and Gray, 1976). The decrease in the apparent maximum velocity at low ionic strength might be because the dissociation rate constant of the cytochrome c_1-cytochrome c complex, k_4, becomes small enough to be rate limiting, rather than k_r. Alternatively, ionic strength might affect some earlier step in electron transport and thus affect k_r.

Although the above mechanism and explanation of the ionic-strength effects are not necessarily completely correct, they do serve to point out the complexities of the system. We feel that the succinate-cytochrome c reductase reaction is optimally sensitive to the interaction between cytochrome c_1 and cytochrome c when the buffer contains at least 0.2 M chloride or 0.1 M phosphate, and low concentrations of cytochrome c are used to obtain the limiting rate parameter k_v. At low ionic strength the reaction is not sensitive to ionic strength or charge modifications, possibly because the rate-limiting step is a diffusion-controlled formation constant k_1. The finding that modification of cytochrome c lysine-13 with 4-nitrobenzo-2-oxa-1,3-diazole did not affect the reductase activity in the presence of 0.1 M chloride (Margoliash et al., 1973) is in agreement with the present results. At intermediate cytochrome c concentrations the rate equation derived from mechanism 1 is rather complex, and the first-order component of the reaction might be limited by the formation of the substrate complex, its conversion to product complex, or the dissociation of the product complex, depending on the total cytochrome c concentration and the ionic strength. Since the effect of ionic strength on the formation and dissociation rate constants would be expected to be opposite, a very complex effect on the overall rate might occur. Although it is perhaps questionable whether the reaction between particulate succinate-cytochrome c reductase and soluble cytochrome c in 0.1 M phosphate buffer mimics the reaction in intact mitochondria under physiological conditions, the main goal of our assay system is to measure the interaction of cytochrome c_1 and cytochrome c independent of interactions with other proteins, such as cytochrome oxidase.

The effect of ionic strength on the rate constant k_v of the TFA-Lys-13 derivative suggests that the modification increased the dissociation rate k_2 without affecting the formation rate k_1, probably because a complementary charge interaction between lysine-13 and a negatively charged group on cytochrome c_1 was removed by the modification. An alternative possibility that the modified group sterically interfered with

the binding is less likely, since both the bulky TFC group and relatively small TFA group caused a similar decrease in the rate constant. The results obtained under optimal conditions for all the derivatives indicate that the binding site on cytochrome *c* for cytochrome c_1 involves lysine-8 and -13 at the top of the heme crevice, lysine-72 at the left side of the heme crevice, lysine-27 at the right side of the heme crevice, and lysine-79 at the bottom of the heme crevice (Figure 5). Modification of lysine-13, -27, -72, or -79 immediately surrounding the heme crevice decreased the redox potential of cytochrome *c* slightly, from 260 to 245 mV in each case (Smith et al., 1977). It is possible that the decreased redox potential difference between cytochrome c_1 and cytochrome *c* might itself decrease the reaction rate somewhat. We used relative Marcus theory to predict a rate reduction of about 30% due to this effect (Wherland and Gray, 1976). None of the modifications led to any changes in the visible absorption spectrum, including the ligand-sensitive 695-nm band, or in the proton NMR spectrum (Smith et al., 1977; Staudenmayer et al., 1976, 1977), showing that the native conformation of cytochrome *c* is not significantly perturbed.

It has previously been shown that the binding site for cytochrome oxidase involves all of the lysines immediately surrounding the heme crevice of cytochrome *c*, lysine-8, -13, -25, -27, -72, -79, and -87 (Table I; Smith et al., 1977; Staudenmayer et al., 1976, 1977; Ferguson-Miller et al., 1978). The present results indicate that the binding site for cytochrome c_1 overlaps considerably with the cytochrome oxidase binding site, involving the highly conserved lysines surrounding the upper part of the heme crevice of cytochrome *c*, lysine-8, -13, and -72, and also lysine-27 and -79 at the lower part of the heme crevice. This suggests that cytochrome *c* might transfer electrons from cytochrome c_1 to cytochrome oxidase by rotational or translational diffusion (Chance, 1956). An important objection to this mechanism is that the overall rate of electron transfer in mitochondria is much larger than the rate of complete dissociation of cytochrome *c* from cytochrome oxidase (Ferguson-Miller et al., 1976). However, if the cytochrome c_1–cytochrome oxidase complex (Chiang and King, 1976) forms a single continuous binding site for cytochrome *c* with two closely adjacent subsites provided by cytochrome c_1 and cytochrome oxidase, respectively, then cytochrome *c* could undergo a low-amplitude rotational diffusion between the two subsites without the necessity for a complete rate-limiting dissociation. The association constant of cytochrome *c* for each subsite would be expected to be about the same, $3 \times 10^7\ M^{-1}$, in agreement with the data for the separate complexes (Chiang et al., 1976; Ferguson-Miller et al., 1976).

Although the present study does not give evidence for a particular mechanism for electron transfer to and from cytochrome *c*, the location of the binding sites for both cytochrome c_1 and cytochrome oxidase at the heme crevice region is consistent with a mechanism involving electron transfer through the exposed edge of the cytochrome *c* heme, as suggested by studies with small molecule oxidants and reductants (Wherland and Gray, 1976). It is significant that cytochrome b_5 also reacts with cytochrome *c* at the heme crevice region (Ng et al., 1977), since this supports a model complex proposed by Salemme (1976) in which the heme groups of cytochrome b_5 and cytochrome *c* are coplanar and about 8-Å apart. This separation distance is consistent with a thermally activated tunneling mechanism for electron transfer (Hopfield, 1974).

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