Low-Temperature Studies of Electron Transfer between Different Cytochromes *c* and Cytochrome *c* Oxidase†

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ABSTRACT: The ability of various native and modified cytochromes *c* to transfer electrons to cytochrome oxidase is compared in cytochrome *c* depleted beef heart mitochondrial particles. The kinetics are followed at −49 °C after the reaction is initiated by photolysis of the CO compound of cytochrome oxidase in the presence of oxygen. Horse, human, yeast iso-2, and carboxydinitrophenyl (CDNP)-lysine-60 horse cytochromes *c* all give initial rates of electron transfer that are equal to those observed in whole beef mitochondria. *Euglena*, CDNP-lysine-72, and CDNP-lysine-13 horse cytochromes *c* give rates about one-tenth that of whole mitochondria. These rates were independent of the concentration of cytochrome *c*. Since the inhibited cytochromes *c*, but not the active proteins, had previously been shown to have lowered affinity for cytochrome oxidase, the results indicate that the structural characteristics important for the association of cytochrome *c* and oxidase are also essential for achieving normal rates of electron transfer within the complex once formed.

Steady-state kinetic measurements performed with mitochondrial cytochrome *c* oxidase have shown that cytochromes *c* from different eukaryotes have different activities that relate to changes in their affinity for the enzyme. These observations are readily interpreted in terms of changes in the positively charged residues on the front surface of cytochrome *c* in the vicinity of the exposed heme edge (Ferguson-Miller et al., 1976, 1978a; Margoliash et al., 1976). Confirmation of the importance of certain residues in the electrostatic interaction with cytochrome oxidase was obtained by studying the effect of changing the charge of individual lysine side chains, using pure mono-CDNP[1] derivatives of cytochrome *c* (Brautigan et al., 1978a,c). In all cases, the change from a positively to a negatively charged side chain lowered the activity of cytochrome *c* with oxidase (Ferguson-Miller et al., 1978b). But only when the modification was located at the front and top of the molecule was there a large decrease in activity, expressed as a greatly increased apparent K_m. The differences in degree of inhibition caused by introducing negative charges at various positions on the molecule have permitted the definition of a high affinity contact area between cytochrome *c* and oxidase (Ferguson-Miller et al., 1978b).

The steady-state kinetics and the binding measurements demonstrate changes in affinity of the CDNP-cytochromes *c* for oxidase, but do not provide any evidence concerning the rate of electron transfer within the enzyme-substrate complex once formed. In order to investigate this latter aspect of activity, cytochrome *c* depleted heart muscle mitochondrial particles were repleted with various native and modified cytochromes *c*. The kinetics of electron transfer from cytochrome *c* to the oxidase were followed at −49 °C, after the reaction was initiated by photolysis of the CO compound of cytochrome aa_3 in the presence of oxygen (Chance et al., 1975b,c).

The results show that, in those cases where the affinity of cytochrome *c* for the oxidase is greatly lowered, the rate of electron transfer within the complex is also inhibited, indicating that the structural characteristics that are important for correct complex formation are also needed to achieve physiological rates of electron transfer.

Experimental Procedure

Horse and human cytochromes *c* were prepared according to the method of Margoliash and Walasek (1966) as modified by Brautigan et al. (1978b). The preparation of *Euglena* cytochrome *c* has been described (Ferguson-Miller et al., 1976). Yeast iso-2 cytochrome *c* was prepared according to Sherman et al. (1968). CDNP-lysine derivatives of horse cytochrome *c* at residues 13, 60, and 72 were prepared as previously described (Brautigan et al., 1978a,c). Prior to use, all cytochromes *c* were gel-filtered in the reduced state on Sephadex G-75 Superfine (Pharmacia) to remove polymeric forms (Brautigan et al., 1978b).

Cytochrome *c* depleted beef heart muscle mitochondrial particles (Keilin–Hartree preparation) were made according to King (1967) with minor modifications (Ferguson-Miller et al., 1976) and stored in 50% glycerol at −20 °C (25 mg of protein per mL). For the experiments described in this paper, 2.6 mL of Keilin–Hartree preparation was diluted with 2 mL of 7 mM sodium phosphate buffer (pH 7.5) containing 30% ethylene glycol and 20 mM succinate. The final protein concentration was 10 mg/mL. Protein was determined by the biuret procedure described by Jacobs et al. (1956).

Preparation of Mitochondrial Particles for Low-Temperature Measurements. The Keilin–Hartree particle suspension, diluted in ethylene glycol buffer as described above, was kept at room temperature for several minutes to achieve anaerobiosis, bubbled with CO for 5 min, taken up in a 5-mL syringe and stored on ice. An aliquot (0.2 mL) of the CO-saturated solution was transferred to a 2-mm optical path cuvette, and the desired cytochrome *c* was mixed in (1 to 12 μM final concentration) with minimum disruption of the surface layer to prevent excessive aeration. The sample was again kept at room temperature for 2 to 10 min to ensure anaerobiosis. The longer period was required for the less active cytochromes *c*. The cuvette was then transferred to a bath at −22 °C and equili-

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[1] Abbreviation used: CDNP, 4-carboxy-2,6-dinitrophenyl.

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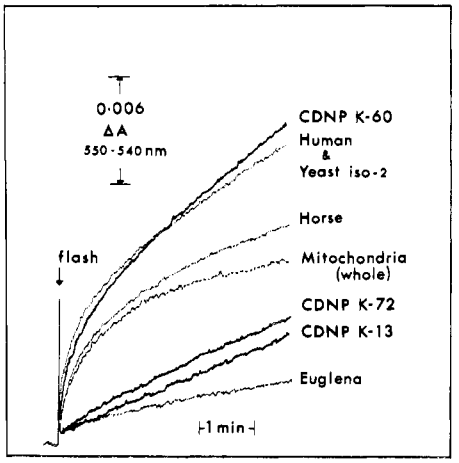

FIGURE 1: The oxidation of different cytochromes *c* by mitochondrial particles compared with that of endogenous cytochrome *c* by whole beef heart mitochondria, at −49 °C. The conditions are described in Experimental Procedure. The final concentration of Keilin-Hartree particles was 2.5 mg of protein per mL in 7 mM phosphate buffer containing 30% ethylene glycol and 600 μM oxygen. Cytochrome aa_3 was 1 μM and cytochrome *c* concentrations were (μM): horse, 2.2; human, 1.8; yeast iso-2, 1.9; *Euglena*, 2.5; CDNP-lysine-60 horse (CDNP K-60), 7.3; CDNP-lysine-72 horse (CDNP K-72), 4.1; CDNP-lysine-13 horse (CDNP K-13), 4.8. Beef heart mitochondria were present with 4 mg of protein per mL containing 1.2 μM cytochrome aa_3. The optical pathlength was 2 mm and the scattering enhancement factor was 7.

TABLE I: Rates of Oxidation of Different Cytochromes *c* by Keilin-Hartree Particles, Compared with Whole Beef Heart Mitochondria.[a]

Mitochondrial preparation	Type of cytochrome *c*	Cytochrome *c* concn (μM)	Initial rate of oxidation 5 s after flash (μM/min)	Extent of reaction at 10 min (μM)
Whole beef heart mitochondria	Endogenous		0.51	0.49
Beef heart particles (KHP)	Horse	2.2	0.58	0.66
	Human	1.8	0.52	0.99
	Yeast iso-2	1.9	0.52	0.98
	CDNP-lysine-60	7.3	0.58	0.99
	CDNP-lysine-72	4.1	0.077	0.49
	CDNP-lysine-13	4.8	0.055	0.43
	Euglena	2.5	0.060	0.23

[a] The rates were measured at −49 °C in 7 mM phosphate buffer, pH 7.5, 30% ethylene glycol, and 600 μM oxygen. The optical pathlength was 2 mm, cytochrome oxidase was 1.0 μM, and the scattering enhancement factor was 7 at 550–540 nm.

brated for 5 min. Oxygenated buffer at −22 °C (1 mL) containing 7 mM phosphate (pH 7.5) and 30% ethylene glycol was then layered on top of the sample and mixed with a stirring rod for 15 s before the cuvette was transferred to a −78 °C bath. Stirring was continued until freezing began and the sample remained at −78 °C until required.

Optical Measurements. The kinetics of oxidation of cytochrome *c* and cytochrome oxidase were measured using a time-sharing multichannel spectrophotometer (Chance et al., 1975a) and recording transmittance differences at two wavelength pairs, 550 nm minus 540 nm, and 608 nm minus 630 nm. The sample cuvette was placed in the cold chamber of the instrument and allowed to equilibrate for 5 to 15 min with the light source off, until a stable temperature of −49 °C was reached. The measuring light was turned on, the two tracings positioned on the oscilloscope and strip-chart recorder, and photolysis of the CO compound of cytochrome oxidase accomplished by a microsecond flash from a 585-nm liquid dye laser or a 10-ms xenon flash. Corning filter 9788 was used to remove infrared light in both cases. The resulting reactions of cytochrome aa_3 with oxygen and cytochrome *c* with cytochrome aa_3 were recorded until a plateau was reached 10 to 20 min later.

Results

A comparison of the kinetics obtained with different cytochromes *c* is given in Figure 1. The tracings show the decrease in absorbance at 550 nm minus 540 nm, corresponding to oxidation of cytochrome *c* after photolysis of the CO compound of cytochrome aa_3. Of primary interest is the initial rapid rate of oxidation 5 s after the flash, since this rate appears to represent the transfer of an electron from the cytochrome *c* molecule already associated with oxidase at the time of the flash. The second phase of the kinetics that predominates after about 2 min may represent the slow diffusion at −49 °C of other molecules of cytochrome *c* into a position where electron transfer can occur. As can be seen, the initial rates are very fast and similar for mitochondrial particles repleted with various cytochromes *c*, including horse, human, yeast iso-2, and CDNP-lysine-60. These rates of oxidation are equal or better than those with whole beef heart mitochondria utilizing endogenous cytochrome *c* (Figure 1). The measured rates at 5 s after the flash are given in Table I.

However, much lower initial rates are observed with *Euglena* cytochrome *c*, as well as with CDNP-cytochromes *c* modified at lysyl residues 13 and 72. These are the proteins that were previously found to have much lower affinity for the oxidase in steady-state kinetic and binding studies (Ferguson-Miller et al., 1976, 1978b). The rates of electron transfer observed are only about a tenth of those given by native horse cytochrome *c* or intact beef mitochondria as shown in Figure 1 and Table I.

The main question arising with regard to these results is whether the less active cytochromes *c* were associated with the oxidase at the time of the flash so that the initial rate of oxidation was a measure of the actual rate of electron transfer, or whether these cytochromes *c* were predominantly at other sites because of their weakened binding to the oxidase, so that diffusion to the enzyme was rate limiting. If the latter situation were the only cause of the slow rates, then increasing the cytochrome *c* concentration should cause a discernible initial fast rate since more of the protein would be associated with the oxidase. However, varying the concentrations of modified cytochromes *c* from 2 to 12 μM (up to tenfold excess over the oxidase) caused no change in the rates of oxidation (Figure 2), indicating that the reaction measured was independent of cytochrome *c* concentration. This was the case whether the initial rate was similar to that of the native protein (e.g., CDNP-lysine-60 cytochrome *c*) or was strongly inhibited (CDNP-lysine-13 and -72 cytochromes *c*). These results support the conclusion that the initial rate measured was that of electron transfer within a complex of cytochrome *c* and cytochrome oxidase.

Another related possibility is that the particle preparation respiring in the presence of the cytochromes *c* of low activity never reached the anaerobic state required for CO inhibition of the oxidase. To ensure that full reduction was achieved,

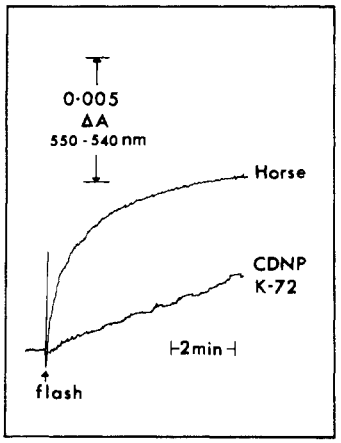

FIGURE 2: Oxidation of cytochrome *c* by mitochondrial particles in the presence of ascorbate and diaminodurene at −49 °C. Keilin-Hartree particles in 75 mM phosphate-borate buffer (pH 7.8) containing 50% glycerol and 20 mg protein per mL were diluted with an equal volume of 7 mM phosphate-borate buffer containing 30% ethylene glycol, 0.12 mM diaminodurene, 0.5 mM ascorbate, and 12.4 mM succinate. Further treatment of the sample was as described in Experimental Procedure. Final concentrations were: particle protein, 4 mg per mL; oxygen, 600 μM; cytochrome aa_3, 1.5 μM; CDNP-lysine-72 horse cytochrome *c* (CDNP K-72), 12.3 μM; and native horse cytochrome *c* (horse), 3.8 μM. The optical pathlength was 2 mm and the scattering enhancement factor was 7.

ascorbate and diaminodurene were included in the reaction mixture since they are able to rapidly reduce both native and inhibited cytochromes *c*.[2] The results obtained in the presence of these reducing agents with native and CDNP-lysine-72 horse cytochromes *c* are shown in Figure 2. Both gave initial oxidation rates equal to those observed when succinate was the only substrate present (compare with Figure 1). The initial changes in the 608–630 nM tracings (not shown) were very similar for all the cytochromes *c* tested, a further indication that the oxidase was reduced and CO inhibited to the same extent in all cases.

Discussion

The initial rate of oxidation of cytochrome *c* measured at −49 °C in the experiments described above appears to represent the rapid transfer of an electron to heme a or a_3 from the cytochrome *c* molecule that is in the appropriate close association with the oxidase at the time the reaction is started. The fact that changing the concentration of cytochrome *c* does not change the initial rates as measured supports this interpretation. If movement of the cytochrome *c* (diffusion or orientation) were required, concentration dependence and much slower rates would be expected at this low temperature. Such processes are identified with the later phase of the reaction, occurring between 2 and 10 min after initiation.

The results obtained with cytochromes *c* of different species and with those having different lysines modified demonstrate that the same residues that have been shown to affect the electrostatic association of cytochrome *c* and oxidase under steady state kinetic conditions (Ferguson-Miller et al., 1976, 1978b) also affect the rate at which an electron is transferred between cytochrome *c* and the enzyme in the present system where it appears they are already associated. Horse, yeast iso-2, human, and CDNP-lysine-60 cytochromes *c* show normal or stronger than normal binding to purified cytochrome oxidase and also give rates of electron transfer to the oxidase in beef mitochondrial particles at −49 °C that are equal or greater than the rates observed with whole beef heart mitochondria oxidizing endogenous cytochrome *c*. However, *Euglena*, CDNP-lysine-72, and CDNP-lysine-13 horse cytochromes *c*, all of which show lower affinity binding to purified oxidase, are also inhibited in their rates of electron transfer at −49 °C. These results verify that the front surface of the cytochrome *c* molecule in the vicinity of the top half of the exposed heme edge is the area most essential to the electron transfer process with the oxidase. Alteration of charged residues in this region affects not only the formation of a competent electron transfer complex, but also the electron transfer process within it. Indeed, it is easy to imagine that placing a bulky anionic carboxydinitrophenyl group in the area that would normally be positively charged and in contact with a negatively charged binding site on the oxidase would cause a less effective interaction of the two proteins and possibly change the alignment of the hemes, leading to longer distances for the electron to traverse.

A further corollary is that the rate of physiological electron transfer is strongly dependent on the distance between the heme centers involved and/or the geometry of their arrangement. Both long distance tunnelling (Devault & Chance, 1966) and an outer sphere type of electron transfer or short distance tunnelling (Marcus, 1964; Hopfield, 1974; Potasek & Hopfield, 1977) would be expected to be strongly, though differently dependent on the distance. It is difficult to make any quantitative estimate of what increase in distance between the redox centers would be expected as a result of the presence of the CDNP group in the interaction domain. Chemical considerations suggest that the carboxydinitrophenyl moiety when attached to lysines 13 or 72 may lie flat across the hydrophobic patch at the center of the contact area, with its carboxyl group hydrogen bonded to the opposite lysine (Brautigan et al., 1978a). This might cause steric hindrance as well as lowered electrostatic forces, resulting in a quite different orientation of the cytochrome *c* molecule and an increased distance between heme c and heme a. The best currently available estimate of the normal distance between heme a and heme c has been obtained by measuring the degree of quenching by cytochrome oxidase of the fluorescence of a derivative of cytochrome *c* where the iron has been replaced by zinc (Vanderkooi et al., 1978). On this basis, a distance between the two hemes greater than 30 Å is calculated from Förster theory of electronic excitation energy transfer (Förster, 1959). The implication of the present experiments is that relatively small increases in distance caused by the CDNP moiety may produce as much as a factor of ten difference in rate. If so, this could provide important evidence concerning the actual electron transfer mechanism. Unfortunately, the current technology and theory involved in measuring distances between heme centers are not sufficiently developed to allow quantitation of these postulated distance changes.

[2] S. Ferguson-Miller, unpublished observations.

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Fluorescent Labeling of Mitoplast Membrane. Effect of Oxidative Phosphorylation Uncouplers†

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ABSTRACT: Mitoplasts isolated from rat liver mitochondria were treated with fluorescamine at ratios ranging from 10 to 140 nmol per mg of protein. The labeled mitoplasts were separated into membrane and soluble fractions by osmotic lysis. The labels were found mainly in the membrane fraction. The soluble fraction had a negligible amount of the fluorescent labels even though the isolated soluble fraction could be labeled by fluorescamine to about half of the extent as could the membrane fraction. Thus, fluorescamine can only label exposed primary amino groups on the outside surface of mitoplasts. In the presence of succinate or β-hydroxybutyrate the extent of labeling of the membrane increases about 30%. This substrate-induced enhancement is prevented by oxidative phosphorylation uncouplers at uncoupling concentrations. Further analysis of labeled membrane showed that about 50% of the labels were associated with proteins and the remainder with lipids. This distribution remains unchanged as the labeling conditions vary, e.g., in the presence or absence of substrates or uncouplers. The protein labeling patterns showed that there are four major labeled polypeptides with apparent molecular weights of 49 000, 34 000, 24 000, and 14 000, respectively. These results suggest that energization of the mitoplast membrane exposes more positively charged primary amino groups on the outside surface and that this is achieved by rearranging the membrane proteins and lipids.

Fluorescamine, which reacts specifically with primary amines (Udenfriend, et al., 1972; Weigele et al., 1972), has been shown to label the surface of erythrocytes (Nakaya et al., 1975) and chick embryo fibroblasts (Hawkes et al., 1976). Studies on fluorescamine labeling of protein in $NaDodSO_4$[1] complexes suggest that a highly charged membrane surface could prevent the reagent from passing through the biomembranes (Tu & Grosso, 1976). Fluorescamine reacts nearly quantitatively with primary amines in aqueous media (the "labeling efficiency", defined by Weigele et al. (1972), is 85–90%). Thus, it can be used to titrate exposed primary amines.

Mitochondrial inner membrane can be separated by digitonin fractionation as described by Schnaitman & Greenawalt (1968), Chan et al. (1970), and Greenawalt (1974). The isolated inner membrane fraction or mitoplasts contain matrix protein enclosed in the membrane vesicles. Those authors also showed that mitoplasts can undergo respiration-dependent configurational changes, including changes from orthodox (expanded) to condensed (contracted) configurations, concomitant with respiratory transitions from state 4 to state 3. Uncouplers of oxidative phosphorylation can also induce changes of mitoplasts from orthodox to condensed configurations (Greenawalt, 1974) like those seen in intact mitochondria (Hackenbrock, 1966; Harris et al., 1968; Blair & Munn, 1972). The configurational changes induced by the different energy states could be associated with organizational changes of either the membrane proteins, as emphasized by Weinbach & Garbus (1969), Wilson & Azzi (1968), Wilson (1969), Wilson & Brooks (1970), and Hanstein & Hatefi (1974), or the lipids, as suggested by Chen & Hsia (1974), Bakker et al. (1975), Terada (1975), and Zimmer et al. (1972). Using lipid and protein spin labels, Zimmer (1977) observed that both protein and the lipid participate in structural changes induced in the mitochondrial membrane by FCCP.

In the present report, the structural changes of mitoplast membranes under different metabolic conditions were exam-

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[1] Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione; MAO, monoamine oxidase.

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