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Definition of cytochrome *c* binding domains by chemical modification: Kinetics of reaction with beef mitochondrial reductase and functional organization of the respiratory chain*

[mono(carboxydinitrophenyl) cytochromes *c*/cytochrome bc_1 complex/2,3-dimethoxy-5-methyl-6-decylhydroquinone/ionic strength effects/steady-state kinetics]

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ABSTRACT An assay has been developed to study the steady-state kinetics of the reduction of cytochrome *c* by purified beef heart mitochondrial cytochrome *c* reductase (cytochrome bc_1 complex, complex III). An analogue of coenzyme Q_2 (2,3-dimethoxy-5-methyl-6-decylhydroquinone) was employed as an antimycin-sensitive reductant. The kinetics of reaction of ten different mono(4-carboxy-2,6-dinitrophenyl) derivatives of horse cytochrome *c* were determined. The modified proteins showed higher apparent K_m values than the native protein and greater sensitivity to ionic strength, defining an interaction domain on cytochrome *c* for purified cytochrome *c* reductase. This interaction site is located on the front surface of the molecule (which contains the exposed heme edge) and surrounds the point at which the positive end of the dipole axis crosses the surface of the protein. The site is similar to that previously determined for mitochondrial cytochrome *c* oxidase and yeast cytochrome *c* peroxidase, suggesting that the primary interaction with redox partners is directed by the dipolar charge distribution on cytochrome *c*. The extensive overlapping of the interaction domains for the mitochondrial cytochrome *c* oxidase and reductase indicates that cytochrome *c* must be mobile in order to transfer electrons between them, depending on their relative positions in the membrane. Whether such mobility is necessary in intact mitochondria depends on whether the interactions with the complete membrane-bound system are the same as with the purified components.

Describing the mode of electron transfer between cytochrome *c* and its mitochondrial reaction partners requires a determination of the area (or areas) on the surface of the molecule involved in the interaction and therefore presumably containing the site of electron transfer. To this end, three different approaches have been employed: (*i*) study of the enzymic reactions of cytochrome *c* preparations singly modified at a number of surface residues (see reviews in refs. 1 and 2, also refs. 3–20); (*ii*) examination of the relative chemical reactivity of ϵ-amino groups of the lysines on cytochrome *c* free in solution, as compared to those on cytochrome *c* complexed to purified preparations of its electron transfer partners (21–23); (*iii*) comparison of the kinetics of reaction of cytochromes *c* of various species (24, 25).

The major advantage of using chemically modified cytochromes *c* is that in analyzing enzyme kinetics one deals directly with the electron transfer activity without any assumptions concerning the enzymic competency of the stable complexes formed. However, the necessity of obtaining highly purified singly modified cytochromes *c*, required for unambiguous results, is time consuming and difficult. Nevertheless, the chemical modification approach has been used to definitively identify the lysine residues involved in the reaction of cytochrome *c* with cytochrome oxidase (3, 4, 6, 9, 14, 15, 17) and with cytochrome b_5 (16, 18). More recently, it has been used to identify the interaction domains on cytochrome *c* for yeast cytochrome *c* peroxidase (5, 11, 12) and, as described here, for mitochondrial cytochrome *c* reductase (10, 19, 20).

The main attraction of identifying interaction domains by comparing the relative chemical reactivity of surface residues upon complex formation with an enzyme is the ease with which this is carried out. This approach is subject to the following shortcomings: (*i*) no measure of electron transfer activity is involved, so it is impossible to ascertain whether the stable complexes formed correlate with the productive electron transfer complex; (*ii*) only high-affinity binding can be observed, and any low-affinity binding sites are either unnoticed or readily misidentified.

Active site delineation by comparison of the kinetics of reaction of native cytochromes *c* from different species suffers from the fact that it is difficult to reach definite conclusions from comparisons of proteins that vary by numerous residues. Such analyses are particularly tenuous when the differences in amino acid sequences and reactivities are as large as they are between the cytochromes *c* of prokaryotic and eukaryotic organisms (24, 25). In the best of cases, one has to assume that the immediate vicinity of a particular amino acid sequence change is the only area of the molecule at which the alteration has an effect on the enzymic activity. This has been shown to be incorrect for the primate cytochromes *c*, even in cases in which there are few amino acid substitutions (1, 4, 6, 26–28). Such evolutionary transformations often result in structural changes affecting the entire molecule (27, 28), in contrast to artificial external chemical modifications, which have only local influences (1, 8).

Definition of the interaction domain on cytochrome *c* for mitochondrial cytochrome *c* reductase (reduced ubiquinone-cytochrome *c* reductase, cytochrome bc_1 complex, complex III) by using singly modified 4-carboxy-2,6-dinitrophenyl- (CDNP) lysine cytochromes *c* is reported below. Our results indicate that the sites of interaction for cytochrome *c* reductase and oxidase on cytochrome *c* are nearly identical. A preliminary account of this work has appeared (10).

MATERIALS AND METHODS

Bovine serum albumin ("albumin") (Sigma; fraction V, fatty acid-poor), Tween 20 (Sigma), Tris (Sigma), and EDTA

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Abbreviations: CDNP-, 4-carboxy-2,6-dinitrophenyl; DQH_2, 2,3-dimethoxy-5-methyl-6-decylhydroquinone.

* This is paper no. 5 in a series. Paper no. 4 is ref. 12.

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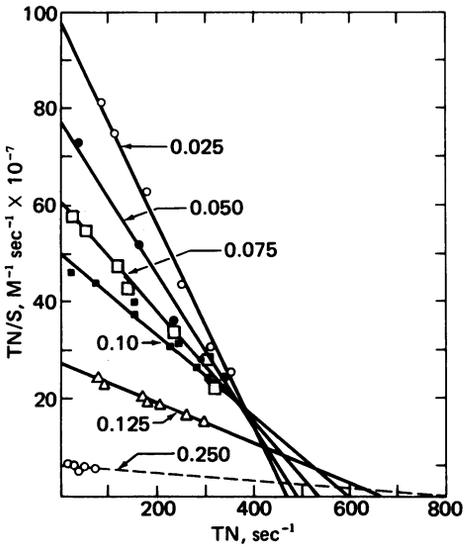

FIG. 1. Ionic strength dependence of the reaction of horse cytochrome *c* with purified beef cytochrome bc_1 complex. The data is presented as an Eadie–Hofstee single reciprocal plot in which the velocities have been converted to turnover numbers (TN) by dividing by the enzyme concentration. S, substrate concentration. The buffer was Tris/acetate, pH 7.5, containing 0.5 mM EDTA and 0.01% albumin, at the ionic strengths (M) indicated on the plot.

(Schwarz/Mann) were from the indicated sources. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone was synthesized from vanillin as reported by Mayer and Isler (29), and the method of Wan *et al.* (30) was used to synthesize the 6-decyl analog of coenzyme Q_2. The decylquinone was reduced with sodium borohydride (the reduced compound is abbreviated DQH_2).

Horse cytochrome *c* was prepared by the procedure of Margoliash and Walasek (31) as modified by Brautigan *et al.* (2). CDNP-cytochromes *c* modified at lysines 8, 13, 27, 60, 72, and 87 were prepared as described (7, 8). The CDNP derivatives at lysyl residues 7, 25, 73, and 86 were prepared by N. Osheroff, D. L. Brautigan, and E. Margoliash (unpublished method). Every one of these preparations was shown to be better than 99% pure. Prior to enzymic assays all cytochromes *c* were fully reduced with minimal dithionite, or ascorbate in the case of the CDNP-cytochromes *c* (7, 9), and chromatographed on a column (0.7 × 30 cm) of Sephadex G-50 superfine (Pharmacia) in 50 mM Tris/acetate, pH 7.5, to separate any polymeric material. The cytochromes *c* were then fully oxidized with minimal ferricyanide, collected on a small CM-cellulose column (0.5 ml), washed with about 50 ml of 50 mM Tris/acetate, pH 7.5, and eluted with 100 mM Tris/acetate, pH 7.5, containing 200 mM sodium chloride. This concentrated solution was appropriately diluted for enzymic assay.

Beef mitochondrial complex III was prepared by the method of Rieske (32). The concentration of cytochrome c_1 was estimated from the difference spectrum of ascorbate-reduced minus ferricyanide-oxidized forms, using a millimolar extinction coefficient of 17.5 for $(A_{552.5} - A_{540})$ (33). Under these conditions only cytochrome c_1 is reduced. The enzyme was prepared for the assay by dilution with 50% (vol/vol) ethylene glycol in 100 mM Tris/acetate pH 8.9 buffer containing 0.5 mM EDTA, 1.0% albumin, and 0.1% Tween 20, and stored at −20°C.

The rate of reduction of cytochrome *c* was measured spectrophotometrically with an Aminco DW-2 dual wavelength recording spectrophotometer at 416 − 410 nm ($\Delta\epsilon_{mM} = 40.3$). Tris/acetate pH 7.5 buffer (2.75 ml) at 25°C, containing 0.5 mM EDTA and 0.01% albumin, was pipetted into a 1-cm quartz cuvette. Cytochrome *c* was added to a final concentration of 0.04–2.0 μM and the absorbances were balanced such that the 416 − 410 nm reading was zero. DQH_2 was added (final concentration of 8.0–16.5 μM) by using a plastic mixing plunger (Savant), and the nonenzymic rate of reduction of cytochrome *c* was monitored for 1.5–3 min. Beef complex III was placed on a plastic mixing plunger and rapidly mixed into the cuvette (0.033 nM final concentration). The reaction was followed to completion and the linear initial portions of the time course allowed accurate measurement of initial steady-state rates of reduction of cytochrome *c*.

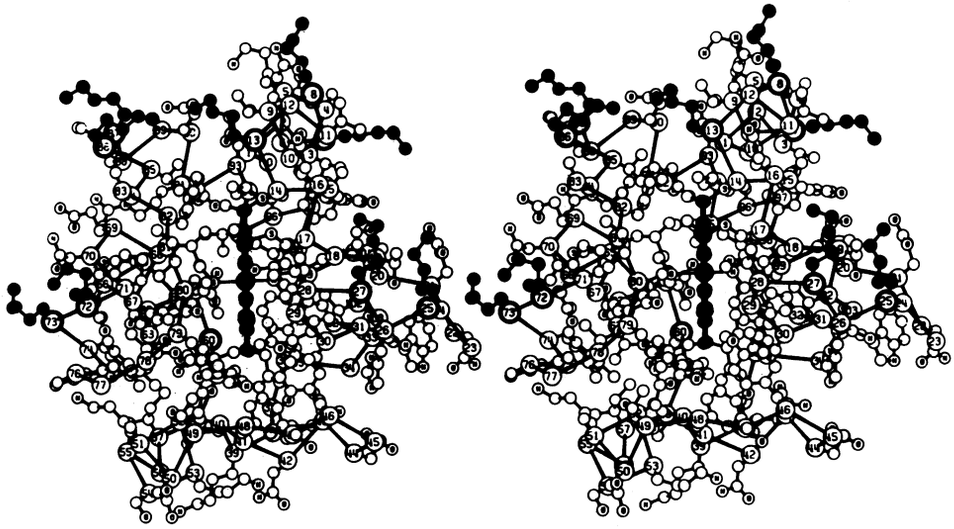

FIG. 2. Stereoscopic diagram of cytochrome *c* as viewed from the front, from an electron density map of tuna ferricytochrome *c* at a resolution of 2.0 Å (35). The heme is viewed on edge in the center of the diagram and is marked in black. The larger circles are the α-carbon atoms with the residue numbers written in them and the smaller circles represent side-chain atoms. The heavier circles and filled circles mark the lysines for which CDNP derivatives of horse cytochrome *c* have been examined (lysines 13, 72, 86, and 27 on the front surface; lysines 87, 7, and 8 on the top front; lysines 73 and 25 on the left and right sides, respectively; and lysine 60 on the back). Among these the only residue that differs in the horse and tuna proteins is at position 60, lysine in horse and asparagine in tuna (see ref. 1).

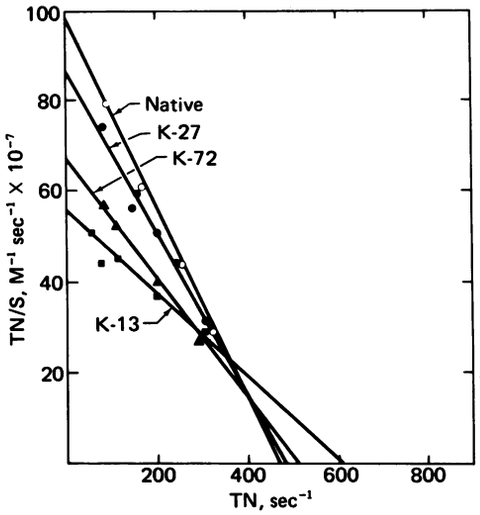

FIG. 3. Activities of several CDNP-cytochromes *c* with cytochrome bc_1 complex at low ionic strength. The buffer was 25 mM Tris/acetate, pH 7.5, containing 0.5 mM EDTA and 0.01% albumin. The CDNP-cytochromes *c* are those indicated in the figure (K = Lys). Other conditions as given for Fig. 1.

RESULTS

Activity of Native Horse Cytochrome *c*. The reaction of horse cytochrome *c* with purified beef complex III was assayed with DQH_2 as a specific reductant of the complex. The effect of ionic strength on the kinetics is shown in Fig. 1. There is only a 3-fold increase in the apparent K_m over the ionic strength range from 0.025 to 0.10 M, a range over which the apparent dissociation constant (K_S), determined by gel filtration for the binding of oxidized cytochrome *c* to the complex III preparation, increased by approximately 2 orders of magnitude (unpublished data). The maximal turnover numbers range from 480 sec^{-1} at an ionic strength of 0.025 M to approximately 800 sec^{-1} at 0.250 M, values that are consistent with a first-order rate constant for the cytochrome c_1 to cytochrome *c* electron transfer step of 1000–5000 sec^{-1} calculated by Nicholls (34) from spectrophotometric data obtained under steady-state conditions.

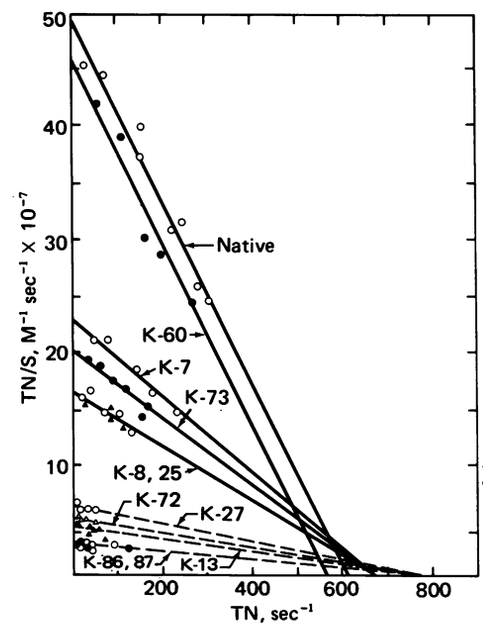

FIG. 4. Activities of CDNP-cytochromes *c* with cytochrome bc_1 at high ionic strength. The buffer was 100 mM Tris/acetate, pH 7.5, containing 0.5 mM EDTA and 0.01% albumin. The CDNP-cytochromes *c* are those indicated on the figure. Other conditions as given for Fig. 1.

Activity of CDNP-Cytochromes *c*. A stereoscopic diagram of cytochrome *c* as viewed from the front, the surface containing the exposed heme edge, is shown in Fig. 2. The lysyl residues that have been modified and the heme are indicated in black. Modification of the lysines at positions 8, 13, 27, 72, 86, and 87—all on the front surface of the molecule—has been shown to interfere with the interaction of cytochrome *c* with cytochrome oxidase (3, 9, 14, 15, 17). However, in the reaction with the reductase at low ionic strength, the CDNP-cytochromes *c* modified at lysyl residues 13, 27, and 72 exhibit kinetic behavior not very different from that of native horse cytochrome *c*, as illustrated in Fig. 3. At an ionic strength of 0.025 M, the cytochrome *c* modified at residue 13 exhibits the most altered kinetics, the reaction parameters being close to those obtained with the native protein at an ionic strength of 0.10 M. The CDNP-lysine-27 and -72 cytochromes *c* yield intermediate activities. By contrast, binding measurements of various CDNP-cytochromes *c* with purified beef cytochrome oxidase (9) and purified *Neurospora crassa* oxidase and reductase (36) indicate that at low ionic strength these modified cytochromes *c* bind with much lower affinity to these enzymes than does the native protein.

When the ionic strength is raised to 0.10 M, the CDNP-cytochromes *c* display a much wider range of activities (Fig. 4). Modification at the following lysyl residues results in the greatest perturbations of the kinetics, with the order of decreasing apparent K_m values being 86 = 87 > 13 > 72 > 27. A second group of modified cytochromes *c* display an intermediate range of activities. Their K_m values decrease in the following order: 8 = 25 > 73 > 7. CDNP-lysine-60 cytochrome *c* exhibits kinetics that differ only slightly from those of the native protein at all ionic strengths.

Fig. 5 illustrates the similarity between the interaction domains observed for cytochrome *c* reductase (Fig. 5*A*) and oxidase (Fig. 5*B*) on diagrams of a planar projection of the front hemisphere of cytochrome *c*. It can be seen that even those lysines directly involved in the two interaction domains are not equally important for the two enzyme systems. Notwithstanding such differences, it is clear that the interaction domains on cytochrome *c* for cytochrome *c* reductase and cytochrome *c* oxidase are very similar. The site of interaction for yeast cytochrome *c* peroxidase has also been found to be very similar (5, 11, 12, 21), though not quite identical to either the reductase or the oxidase sites. In all three cases the interaction domains are roughly centered on the point at which the dipole axis of cytochrome *c* crosses the surface of the protein near the β-carbon of phenylalanine-82 (37, 38). This commonality suggests that the primary interaction of cytochrome *c* with its enzymic reaction partners is directed by the dipolar charge distribution on the protein, but the orientations in the final complexes are undoubtedly governed by more complicated close-range interactions.

DISCUSSION

The advantage of the spectrophotometric steady-state assay employing purified complex III is the elimination of the intermediate steps involved in the NADH or succinate dehydrogenase complexes, which under many conditions may be rate limiting (25, 39). At the same time it overcomes the problem encountered with purified cytochromes c_1 and *c* by introducing the minimal number of steps needed to obtain se-

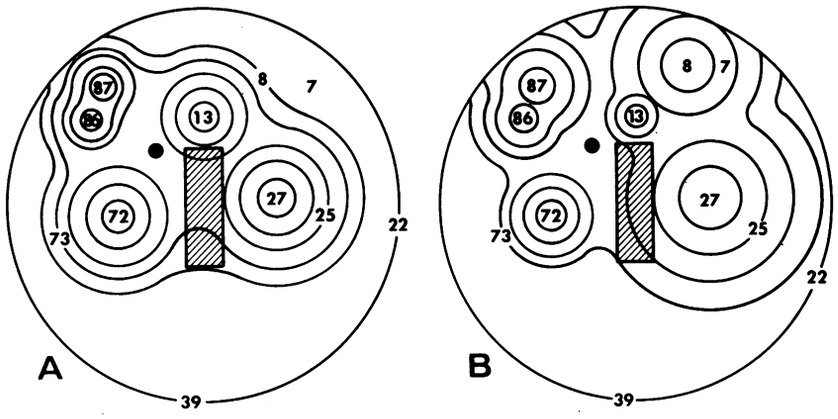

FIG. 5. Interaction domains on cytochrome *c* for mitochondrial cytochrome *c* reductase (*A*) and mitochondrial cytochrome *c* oxidase (*B*) outlined on diagrams of planar projections of the front hemisphere of the molecule. The shaded rectangle represents the exposed heme edge; the black dot represents the point at which the positive end of the dipole axis of cytochrome *c* crosses the surface of the protein; and the numbers are centered on the positions of the β-carbons of the lysyl residues that have been chemically modified to determine their influence on the functional activity of cytochrome *c*. The contours represent circles drawn around the β-carbons of those CDNP-modified lysyl residues for which the corresponding activities with the reductase (*A*) and the oxidase (*B*) were no more than 15% of that of the native protein. The activities, as defined by the apparent K_m values, were normalized to the activity of the most inactive derivative, in each case taken as 1.0. The contours were drawn with radii of integral multiples of 1.125 Å times the relative activity of each derivative. Prior to normalization, the activities of the modified cytochromes *c* with the oxidase were adjusted by the addition of a constant factor equal to the difference between the relative activities of the most inhibited derivatives for the two enzyme systems. In this way the ranges of activity covered by the CDNP-cytochromes *c* were roughly the same for both systems. This scaling was necessary because the K_m values measured in the assays for the two activities are not equivalent kinetic parameters. The denser the pattern of contours, the more important the contribution of that area to the interaction.

lective reduction of cytochrome c_1 by DQH_2 via cytochrome *b*. The use of this analogue of coenzyme Q_2, which has intermediate hydrophobic character, avoids the problems of direct reduction of cytochrome *c* encountered with the more water-soluble durohydroquinone and the insolubility of the longer chain quinones in the aqueous assay system.

The following is a simple scheme that describes the steps involved in the reaction monitored spectrophotometrically:

$$E^{2+} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E^{3+} + P.$$

E represents cytochrome c_1 in its reduced and oxidized states (2+ and 3+); S is the substrate, ferricytochrome *c*; and P is the product, ferrocytochrome *c*. The oxidized enzyme is maintained reduced by excess DQH_2 via cytochrome *b*. The steady-state kinetic parameters for the forward reaction in the above scheme are (40):

$$K_m = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{k_1(k_2 + k_{-2} + k_3)} \text{ and } V_{max} = \frac{k_2k_3E_T}{k_2 + k_{-2} + k_3}$$

in which E_T is the total enzyme concentration. As shown by the present study, the apparent K_m values for the reaction of cytochrome *c* with the reductase do not correspond to measured K_S values (k_{-1}/k_1) under low ionic strength conditions. From the expression of the K_m above, it is apparent that when k_{-1} is equal to k_3, then the K_m is the K_S. This suggests that, for the reaction of native horse cytochrome *c* with the reductase, k_3 may be much greater than k_{-1} under low ionic strength conditions, and that k_{-1} may approach k_3 as the ionic strength is increased. This would explain the relatively small changes in the apparent K_m with increasing ionic strength as compared to the large changes observed in K_S. The effect of derivatization of cytochrome *c* on the individual rate constants cannot be determined from the data presented; however, it is likely that derivatization affects the catalytic rate constants, (k_2 and k_{-2}) (41), as well as the "on" (k_1) and "off" (k_{-1} and k_3) rate constants. Whether derivatization predominantly affects the rate of electron transfer in the ES complex or significantly alters K_S and K_P, the observed changes in the apparent K_m provide a valid measure of the proximity of a given residue to the site of electron transfer.

On this basis the site of interaction on cytochrome *c* for the reductase was defined as shown in Fig. 5. The nearly complete overlap of the enzymic interaction surfaces would appear to require cytochrome *c* to move in order to shuttle electrons between the mitochondrial reductase and oxidase. In contrast to such a view of the mechanics of electron transfer, there is some data to indicate that different areas on cytochrome *c* may be involved in the interaction with oxidase and reductase. The three major lines of evidence are: (*i*) differences in kinetics of reaction of cytochrome *c* with the oxidase and reductase (24, 39, 42, 43); (*ii*) differential inhibition of the two reactions by site-specific anti-cytochrome *c* antibodies (44); and (*iii*) retention of succinate oxidase activity in mitochondrial membranes to which cytochrome *c* has been covalently crosslinked (45, 46).

However, the observed kinetic differences may be due to the individual characteristics of the enzymes rather than different domains on cytochrome *c* (42, 43) and to difficulties in interpreting succinate- or NADH-cytochrome *c* reductase kinetics for which the initial rates are zero order (39). Similarly, the steric hindrance imposed by anti-cytochrome *c* antibodies may differentially affect the reductase and the oxidase. Studies in which cytochrome *c* has been covalently bound to mitochondrial membranes are more difficult to reconcile with a single interaction domain model. To consider them definitive evidence that cytochrome *c* does not require mobility for its physiological electron transfer function, more rigorous demonstrations are required to show that all the cytochrome *c* present is bound to the oxidase, that it is fully immobilized, and that no more than one molecule of cytochrome *c* is linked per molecule of oxidase.

It should be emphasized that the determination of the interaction domain on cytochrome *c* for the reductase, presented in this paper, was carried out with a purified enzyme preparation that does not necessarily reflect the physiological situation in the mitochondrial membrane, in which the reductase may be complexed with the oxidase (47). Our findings are in good

agreement with those of Ahmed *et al.* (20), obtained with trifluoroacetyl and trifluoromethylphenylcarbamoyl derivatives of cytochrome *c*, and those of Rieder and Bosshard (23), who identified the reductase interaction domain from the change in the chemical reactivity of lysyl side chains of cytochrome *c* on complexation with the enzyme. However, because both these studies were also carried out with purified preparations of cytochrome *c* reductase they do not settle the question of possible differences with the intact mitochondrial membrane system.

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