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Cross-linking of Ubiquinone Cytochrome *c* Reductase (Complex III) with Periodate-Cleavable Bifunctional Reagents†

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ABSTRACT: Two novel cross-linkers, disuccinimidyl tartarate (DST) and *N,N'*-bis(3-succinimidyloxycarbonylpropyl)tartaramide (SPT), have been synthesized. These reagents span 6 and 18 Å, respectively, between functional groups and contain a *vic*-glycol bond which can be cleaved with periodate under mild reaction conditions. Both DST and SPT have been used to examine the near-neighbor relationships of polypeptides in ubiquinone cytochrome *c* reductase (complex III) from beef heart mitochondria. Among the cross-linked products resolved were pairs containing I + II, II + VI, I + V, and VI + VII. Polypeptides III and IV, a cytochrome *b* aproprotein, and the cytochrome c_1 hemoprotein, respectively, were also resolved in several cross-linked products.

Ubiquinone cytochrome *c* reductase or complex III is an oligomeric protein which spans the mitochondrial inner membrane and is involved in electron transport and in the coupling of this reaction to ATP synthesis or ion transport. The complex contains b heme, c_1 heme, and non-heme-iron centers in the molar ratio 2:1:1 (for review, see Rieske, 1976). The polypeptide composition of complex III has been studied in several laboratories (Das Gupta and Rieske, 1973; Yu et al., 1974; Hare and Crane, 1974; Gellerfors and Nelson, 1975; Bell and Capaldi, 1976). By using highly resolving $NaDodSO_4$–polyacrylamide gel systems, we have been able to identify nine different polypeptides in complex III with molecular weights ranging from 50 000 to 4400 (Bell and Capaldi, 1976; Capaldi et al., 1977). The smallest component, polypeptide IX, represents 1–2% by weight of the protein and is only clearly seen when large amounts of protein are electrophoresed.

As one approach to studying the arrangement of polypeptides in complex III, we have used DSP and DTBP, two cleavable bifunctional reagents to cross-link neighboring polypeptides through their available lysine residues (Smith and Capaldi, 1977). With low levels of either reagent, several pairs of polypeptides were covalently linked together, including I + II, II + VI, I + V, and VI + VII. With higher levels of DSP, an aggregate containing all of the polypeptides was obtained and this is, presumably, the complex III monomer with an apparent molecular weight of 310 000.

Unfortunately, the amount of information that could be obtained using either DSP or DTBP was limited by technical problems. Both reagents are bridged by a disulfide bond and thus separation of cross-linked products for analysis must be done in the absence of reducing agents. Several components of complex III behave anomalously when β-mercaptoethanol

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or DTE is not used in dissociating the enzyme (Capaldi et al., 1977), and as a result the two-dimensional $NaDodSO_4$ gel electropherograms used to identify the components in various cross-linked products are difficult to interpret.

Another limitation of using DSP and DTBP is a result of the size of these molecules. Both reagents can only bridge lysines which are around 11-Å apart and they would not cross-link apposed polypeptides in which these reactive groups are not appropriately positioned.

To circumvent the above-mentioned problems, we have synthesized two new cross-linking reagents, disuccimidyl tartarate (DST) and *N,N'*-bis(3-succinimidyloxycarbonylpropyl)tartaramide (SPT), both of which are bridged by a *vic*-glycol bond. Glycol residues are stable under most conditions but can be specifically cleaved by periodate treatment (Lutter et al., 1974; Coggins et al., 1976). Both reagents react predominantly with the lysine residues, but DST spans a distance of around 6 Å while SPT spans a distance of around 18 Å. Taken together then, DST and SPT allow for the covalent linking of closely apposed polypeptides with widely different placement of lysine groups on their surfaces.

Experimental Section

Complex III was prepared by the method of Rieske (1967). Small aliquots of the enzyme (10–40 mg/mL) were stored at −20 °C. Protein concentrations were determined by the method of Lowry et al. (1951). Heme b and c_1 concentrations were estimated as described by Williams (1964). Ubiquinol cytochrome *c* reductase activity was measured as described previously (Smith and Capaldi, 1977) using a CoQ_2 analogue with a ten-carbon side chain as substrate (kindly provided by Dr. Bernard Trumpower, Dartmouth Medical School). Triton X-100 was obtained from Sigma.

Synthesis of Cross-linker. Disuccinimidyl tartarate (DST) was prepared using the general method of Anderson et al. (1964). A solution of dry tartaric acid and 2.1 molar equiv of *N*-hydroxysuccinimide in tetrahydrofuran was treated at 0 °C with a solution containing 2 equiv of dicyclohexylcarbodiimide in the same solvent. After 16 h of stirring, the dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo to yield the product as a white powder: NMR (Me_2SO-d_6) δ 6.84 (d, J = 7 Hz, 1 H, O*H*), 5.05 (d, J = 7 Hz, 1 H, C*H*OH), 2.97 (s, 4 H, CH_2CH_2) (succinimidyl *ester*). The NMR spectrum of this substance showed a small amount of the free alcohol as the only detectable impurity.

N,N'-Bis(3-succinimidyloxycarbonylpropyl)tartaramide (SPT). A mixture of disuccinimidyl tartarate and 2 molar equiv of finely powdered methyl 4-aminobutyrate hydrochloride was treated with an excess of triethylamine in tetrahydrofuran. After 2–5 h of stirring, the reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was hydrolyzed at a pH of 10.1–10.8, and the aqueous mixture was washed with several volumes of ether. The pH was adjusted to pH 2.1 with 1 M HCl and the solution was lyophilized. The solid residue was taken up in tetrahydrofuran, concentrated, and then triturated with a mixture of ether–acetone (2:1) to yield white crystals (mp 130–135 °C): NMR (Me_2SO-d_6) δ 12.1 (br, 1 H, -C(=O)O*H*), 7.8 (br, 1 H, C(=O)N*H*, 5.6 (br, 1 H, CHO*H*), 4.3 (br, 1 H, C*H*OH), 3.37 (apparent t, J = 6 Hz, 2 H, $NHCH_2CH_2$), 2.31 (t, J = 6 Hz, 2 H, $C(=O)CH_2CH_2$), 1.79 (quint, J = 6 Hz, 2 H, $CH_2CH_2CH_2$). The diacid was then converted to the succinimidyl ester as described before but using dimethylformamide as a solvent.

Enzyme was prepared for cross-linking by diluting to 2 mg/mL in 0.05 M triethanolamine hydrochloride buffer (pH 8.0) containing 0.1% Triton X-100 or 0.33% DOC. The protein solution was dialyzed against several changes of buffer to remove residual ammonium sulfate which would react with the cross-linkers. DST and SPT were dissolved at 20 mg/mL in Me_2SO. The cross-linking reaction was started by adding the bifunctional reagent to give a final concentration between 0.2 and 2.0 mg/mL. Reaction times were varied between 5 and 30 min. All experiments were done at room temperature. The reaction was quenched with 50 μL of 1 M ammonium acetate per mL of protein solution. $NaDodSO_4$- polyacrylamide gel electrophoresis in "tube" gels was performed by the method of Weber and Osborn (1969) and by the method of Swank and Munkres (1971). The approach taken in dissociating complex III for electrophoresis depended on the gel system to be used. For 7.5 or 10% acrylamide gels run in the Weber–Osborn system, samples were dissolved in 3 mg of $NaDodSO_4$/mg of protein and 7–8% β-mercaptoethanol and heated to 37 °C for 2 h. Samples to be run on Swank–Munkres type gels were dissociated in 3 mg of $NaDodSO_4$/mg of protein, 8 M urea, and 7–8 β-mercaptoethanol by heating to 100 °C for 1 min.

Two-dimensional gel electrophoresis was performed by a modification of the procedure of Wang and Richards (1974). The first-dimension tube gels containing cross-linked products were soaked for 2 h in several changes of a solution containing 0.015 M sodium periodate, 0.1% $NaDodSO_4$, 0.02 M sodium phosphate, pH 7.0 (Lutter et al., 1974). Gels run in the Swank-Munkres system were soaked in 0.1% $NaDodSO_4$, 0.02 M sodium phosphate, pH 7.0, to remove urea before the periodate treatment. After this cleavage of cross-linked products, gels were washed once in distilled water and then cemented to a slab gel by a thin layer of acrylamide. Electrophoresis in the second dimension of the slab was conducted as described previously (Smith and Capaldi, 1977).

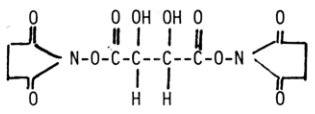
Samples of complex III were subjected to gel filtration on a Sepharose 4B column using the conditions described by Robinson and Capaldi (1977). Protein in the trailing edge of the peak was pooled, dialyzed against a large volume of distilled water for 4–8 h to remove some of the Triton X-100, and then concentrated with solid sucrose. Concentrations of 10–15 mg/mL of protein were achieved. Above this there were difficulties due to residual detergent being concentrated, and gels of these samples ran anomolously.

Results

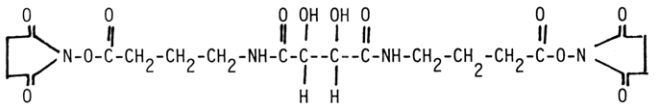
Structure of the Periodate-Cleavable Cross-linkers. The chemical structures of DST and SPT are shown in Figure 1. The distance between the functional moieties was estimated to be approximately 6 Å for DST and 18 Å for SPT. Both reagents were only marginally soluble in aqueous buffer and were dissolved in $(CH_3)_2SO$ for use. Their rate of hydrolysis was slow, DST having a half-life of 60 min in water–Me_2SO (1:1), while SPT was stable for a period of 4–5 h in aqueous buffers.

Reaction of DST and SPT with Complex III. Complex III was dissolved at between 0.5 and 2 mg/mL in 0.5% Triton X-100 or 0.33% DOC for cross-linking experiments. Sedimentation equilibrium studies indicate that the complex is predominantly a monomer under such conditions but with small amounts of higher aggregates present (R.J. Smith, unpublished studies). This is consistant with the results of our previously published cross-linking experiments (Smith and Capaldi, 1977). When high levels of DSP were used to cross-link the complex, the major product was a monomer (apparent mol wt 310 000) with small amounts of larger aggregates also stabilized.

The detergent-solubilized complex was treated with DST



Disuccinimidyl tartarate (6 Å)



N,N′-Bis(3-succinimidyloxycarbonylpropyl) tartaramide (18 Å)

FIGURE 1: Structure of the novel *vic*-glycol-bridged cross-linkers.

or SPT at concentrations in the range 0.1–1.0 mg/mg protein and for variable lengths of time. The extent of reaction was then examined by $NaDodSO_4$--polyacrylamide gel electrophoresis. Cross-linked samples were analyzed directly or in some experiments were first subjected to gel filtration on a Sepharose 4B column in 0.5% Triton X-100 before examination (i.e., the same conditions as used in cross-linking). The cross-linked enzyme eluted in the same volume (K_d = 0.53) as untreated complex III and with a smaller Stokes radius than cytochrome *c* oxidate (mol wt 325 000; Robinson and Capaldi, 1977). Fractions were collected from the trailing edge of the protein peak for analysis in order to reduce further the amount of aggregated material in samples being examined.

Enzyme cross-linked with 0.5 mg of DST/mg of protein or with SPT at the same levels and for the same length of time was found to contain several products of molecular weight below 150 000. The ubiquinol cytochrome *c* reductase activity of the complex was unchanged by treatment with these levels of DST, and only 30% of the activity was destroyed after reaction with SPT. The above conditions were therefore chosen for two-dimensional analysis.

Identification of Polypeptides in Various Cross-linked Aggregates. The cross-linked products generated by DST or SPT were cleaved with periodate as described under Experimental Procedures, and the component polypeptides in each aggregate could then be identified in two-dimensional electropherograms. When larger aggregates were to be analyzed, a 7.5% Weber–Osborn type gel (Weber and Osborn, 1969) was used, while for smaller sized products 10% Weber–Osborn type gels or 6% Swank–Munkres gels (Swank and Munkres, 1971) were preferred for the first-dimensional electrophoresis. In all cases, a 10% Swank–Munkres type gel was used for second-dimensional analysis.

Figure 2 shows a two-dimensional electropherogram of complex III which has been reacted with DST, the 6-Å reagent. The most prominent cross-linked product seen was a dimer containing polypeptides I + II. This aggregate was the major product of cross-linking with DSP and DTBP. Dimers containing polypeptides II + VI, I + V, and VI + VII, all of which had been seen in DSP- or DTBP-treated complex III, were also obtained with DST as the cross-linking agent. Among the cross-linked products not previously resolved were pairs containing polypeptides III or IV. Such products would have been

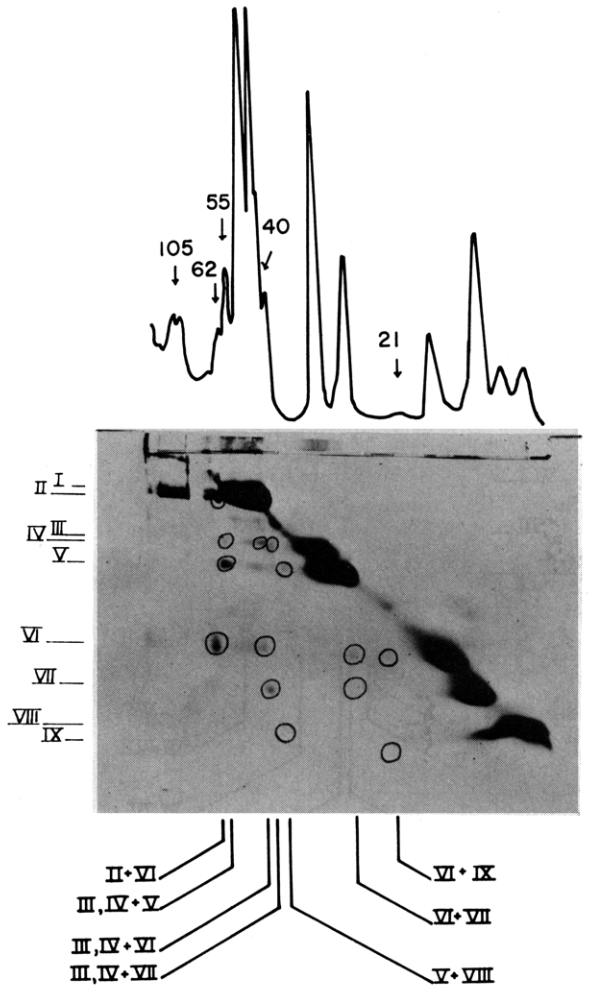


FIGURE 2: A two-dimensional electropherogram of complex III cross-linked with 0.5 mg of DST/mg of protein for 30 min. The spots on a diagonal are uncross-linked polypeptides. Components of various cross-linked products are circled and listed below the electropherogram. The upper trace is the polypeptide profile of cross-linked complex III of a 6% Swank–Munkres type gel. Molecular weights of some cross-linked species are shown.

obscured in studies with DSP or DTBP done without reducing agents and in which III and IV were smeared out over a significant portion of the gels. Unfortunately, our gel systems did not completely resolve III from IV at the high levels of protein needed to identify cross-linked products, and therefore it was impossible to determine unambiguously which of these two components are in aggregates. Cross-linked products with apparent molecular weights of 55 000, 42 000, and 40 000 could contain III and/or IV with V, VI, and VII, respectively.

All of the products just described were also seen in cross-linked complex III taken from the trailing edge of the column. However, the spots on these two-dimensional gels were not as well resolved because less protein could be applied to the gel for reasons discussed under the Experimental Section.

Two cross-linked products, V + VIII and VI + IX, were seen in gels of complex III examined before gel filtration but these were missing in samples off the column. Both were only very minor products in any of the gels examined. They could be intercomplex products or more likely they were not seen because of the smaller amount of protein applied in these experiments.

SPT which can react with lysines up to 18 Å apart gave the cross-linking pattern shown in Figure 3. As listed in Table I, the near neighbors identified with this reagent were the same

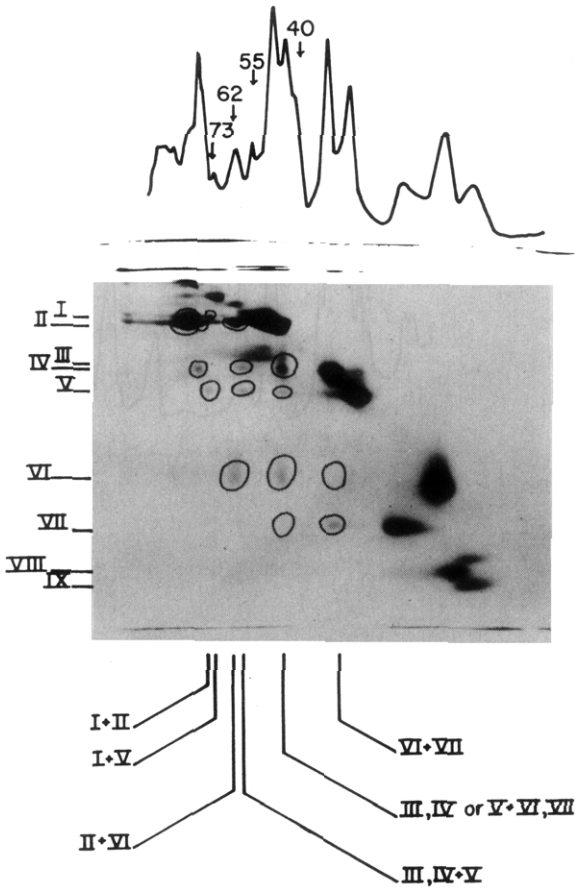


FIGURE 3: A two-dimensional electropherogram of complex III cross-linked with SPT (0.5 mg/mg for 30 min). The cross-linked polypeptides are circled and the cross-linked polypeptide pairs are indicated below. The upper trace is a polypeptide profile of the cross-linked complex III electrophoresed in the first dimension in a 7.5% Weber–Osborn type gel.

ones seen in studies with the 6- and 11-Å bridging cross-linkers, although the relative proportions of the different products were not the same.

Discussion

Cross-linking studies provide a useful approach to studying the arrangement of components in multipeptide aggregates. Several bifunctional agents bridged by a disulfide bond are available and have been used successfully in studies of the topology of ribosomes (Wittmann, 1976), red cell membranes (Wang and Richards, 1974), and mitochondrial membrane systems (Bragg and Hou, 1974; Birchmeier et al., 1976; Baird and Hammes, 1976; Briggs and Capaldi, 1977; Smith and Capaldi, 1977). Such cross-linking agents have the major disadvantage that analysis of cross-linked products must be done in the absence of reducing agents in order not to cleave the bridging disulfide bond. The omission of β-mercaptoethanol or DTE from media used to dissociate samples for gel electrophoresis can in turn lead to anomalous migration of components on gels, and this makes interpretation of the cross-linking data more difficult.

Bifunctional reagents containing *vic*-glycol as the cleavable bond are stable under reducing conditions and have been used in recent studies of near-neighbor interactions in ribosomes (Lutter et al., 1974) and in the pyruvate dehydrogenase complex (Coggens et al., 1976). Here we describe the synthesis of two new *vic*-glycol bridged bifunctional reagents, DST and SPT, which differ in chain length from each other in that they span maximally 6 and 18 Å, respectively, between functional

TABLE I: A Summary of the Pairs of Polypeptides in Complex III Cross-linked with Different Bifunctional Reagents.

pairs resolved	obsd mol wt	calcd mol wt	cross-linker used: DST	DTBP/DSP	SPT
I + II	92 000	88 000	+	+	+
I + V	77 000	73 000	+	+	+
II + VI	60 000	60 000	+	+	+
III or IV + V	55 000	53 000	+		+
		51 000			
III or IV + VI	42 000	42 000	+		+
		40 000			
III or IV + VII	40 000	38 000	+		+
		36 000			
VI + VII	24 000	24 000	+	+	+

ends. Both reagents are relatively stable in aqueous solution and reactive enough to give cross-linking of complex III at levels of reagent which do not greatly inhibit the electron-transfer activity of the protein complex.

The various cross-linked products generated by DST and SPT are summarized in Table I. Importantly, all of the pairs seen with DSP and DTBP were confirmed by the experiments with DST and SPT, including aggregates of polypeptides I + II, a dimer of the two core proteins; II + VI, a dimer involving core protein and the cytochrome c_1 associated polypeptide; I + V, a dimer of core protein and the non-heme-iron proteins; and VI + VII, the cytochrome c_1 associated polypeptide and a cytochrome *b* associated polypeptide.

Studies with DST and SPT have revealed several near neighbors in complex III which were not seen with DSP or DTBP. Most of these contained polypeptide III, a cytochrome *b* apoprotein or polypeptide IV, the cytochrome c_1 hemoprotein. These aggregates were not resolved in studies with DSP or DTBP because reducing agents could not be used in the first-dimensional separation of cross-linked products, and, as a result, both polypeptides smeared out over the gels. Unfortunately, III and IV are not completely separated from each other in the second dimension of the slab and it is not certain which of these two polypeptides are present in the various cross-linked products resolved. From the cross-linking data then, either III or IV or both components may be near neighbors of polypeptides V, VI, and VII. However, the proximities of some of these pairs are more likely than others based on fractionation data. Polypeptide IV is isolated along with VI as a tightly bound aggregate in any of the purification procedures for cytochrome c_1 and so it seems likely that they are near neighbors in the complex. Similarly, the polypeptide III is isolated along with component VII in all fractionation schemes for separating cytochrome *b*, and these two components are, therefore, likely to be interacting neighbors in complex III.

Under the conditions of cross-linking, complex III is predominantly a monomer but there are smaller amounts of dimer and perhaps other aggregates in the preparation. It is possible then that some of the products seen were generated by intercomplex rather than intracomplex bridging. However, this is unlikely at least for the pairs listed in Table I because they were all generated in large amount relative to the amount of aggregated material present and were seen in samples from which aggregated material had been further depleted by gel filtration prior to analysis.

In summary, the many near neighbors seen in our cross-linking studies provide the first clues to the organization of polypeptides in complex III. In conjunction with these studies,

we have been conducting labeling experiments to determine which of the components in complex III are at the matrix side and which are exposed at the intracistral side of the mitochondrial inner membrane. The cross-linking and labeling data taken together can be used to develop a model of the arrangement of polypeptides in complex III. This model will be presented along with the labeling experiments in a forthcoming paper.

Acknowledgments

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Cytochrome b_{562} from *Escherichia coli*: Conformational, Configurational, and Spin-State Characterization†

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ABSTRACT: The protein conformation, heme configuration, and the spin state of heme iron of cytochrome b_{562} from *Escherichia coli* have been investigated using circular dichroism (CD), optical, and resonance Raman (RR) spectroscopy as the probes. Studies are reported on the effect of ionic strength on the CD spectra of the ferric and ferrous forms, of temperature variation on the CD spectrum of the ferric form, and of pH variation in the range 3–11 on optical, circular dichroism, and resonance Raman spectra of the ferric form of the protein. Differences in conformational sensitivity to increasing ionic strength of the medium are seen between ferri- and ferrocytochrome b_{562}. The thermal denaturation of ferricytochrome b_{562} at neutral pH is found to occur in two distinct steps centered at about 35 and 67 °C, with $\Delta H°$ and $\Delta S°$ of about 74 eu and 23 kcal, and 254 eu and 74 kcal, respectively. The effect of pH on the optical spectrum of ferricytochrome b_{562} is seen in the presence of three distinct pH forms in the range 3–11 with apparent pKs of about 6 and 8.7. The acidic transition is accompanied by minimal perturbation of the optical spectrum. The basic transition, pK_a of 8.7, is accompanied by a red shift of the Soret peak and the visible spectrum and the generation of a new band at about 635 nm. The CD spectrum does not indicate any significant variation of the protein secondary structure in any of the pH transitions, but the heme symmetry is altered during the alkaline transition from a less symmetric heme to a more symmetric heme. The resonance Raman spectra of the three pH forms are found to be typical of low-spin heme iron systems. A heme configuration of methionine/histidine ligation at the two axial positions of heme iron for the acidic and neutral forms and transformation to a form with lower heme symmetry, i.e., with lysine/histidine or histidine/histidine ligation, have been concluded.

Cytochrome b_{562} from *E. coli* is thought to be a soluble electron carrier for a system located in the membrane, although the protein itself is not bound to the membrane (Lemberg & Barrett, 1973; Hager & Itagaki, 1967). In mammalian systems this physiological function is attributed to cytochrome *c*, while cytochromes of type *b* are membrane-localized (Dickerson & Timkovich, 1975; Hagihara et al., 1975; Ferfuson-Miller et al., 1978). The similarity in function of the two quite different proteins has attracted our attention, and, in order to understand this phenomenon, we have undertaken an extensive physico-

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