Near-Neighbor Relationships of the Subunits of Cytochrome *c* Oxidase†

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ABSTRACT: Cytochrome *c* oxidase in detergent dispersion has been cross-linked with two reversible cross-linking agents, dithiobissuccinimidylpropionate (DSP) and dimethyl-3,3′-dithiobispropionimidate (DTBP), and the cross-linked products formed have been analyzed by two-dimensional gel electrophoresis. Under mild reaction conditions, several subunit pairs were seen including II and V, V and VII, IV and VI. With higher levels of DSP, larger aggregates were seen until a cross-linked product with an apparent molecular weight of 140 000 was the predominant band on gels. This is the smallest molecular weight aggregate to contain all seven subunits of the enzyme and most likely represents the "unit" or two heme and two copper containing complex of cytochrome *c* oxidase.

Cytochrome *c* oxidase is the terminal member of the electron transport chain, an integral part of coupling site III and an intrinsic component of the mitochondrial inner membrane.

As a first step in defining its structure we have examined the number of different subunits in the beef heart enzyme complex. Seven different polypeptides were identified and each was isolated in a denatured form (Downer et al., 1976; Briggs et al., 1975). The molecular weights of these subunits, averaged from values obtained by several different methods of sodium dodecyl sulfate gel electrophoresis as well as by gel filtration in sodium dodecyl sulfate and in 6 M Gdn·HCl,[1] are as follows: I, 35 400; II, 24 100; III, 21 000; IV, 16 800; V, 12 400; VI, 8200; VII, 4400. Subunits I and III are hydrophobic in character and have a polarity (Capaldi and Vanderkooi, 1972) of 35.5 and 39.9, respectively; subunit II has a polarity of 44.7; while the four smallest subunits have an average polarity of 48.2 (Downer et al., 1976; Briggs et al., 1975), which is in the range of values found for water-soluble proteins. In terms of the number and size of subunits, the beef heart enzyme is very similar to yeast and *Neurospora* cytochrome *c* oxidase (Poyton and Schatz, 1975; Sebald et al., 1973; Rubin and Tzagoloff, 1973).

Our efforts are now directed toward obtaining a three-dimensional picture of cytochrome *c* oxidase at the level of the disposition of individual subunits. One approach to this is to chemically cross-link neighboring subunits with bifunctional reagents. These types of studies have already provided valuable information about the oligomeric structure of a number of soluble proteins (Davies and Stark, 1970) as well as about the topography of ribosomes (Bickle et al., 1972; Lutter et al., 1974; Acharya et al., 1974), chromatin (Thomas and Kornberg, 1975), and the protein–protein interactions involved in stabilizing the structure of the red cell membrane (steck, 1972; Capaldi, 1973; Hulla and Gratzer, 1972).

A recent advance in cross-linking experiments has been the introduction of reversible reagents such as dithiobissuccinimidylpropionate (DSP) (Bragg and Hou, 1975) and dimethyl-3,3′-dithiobispropionimidate dihydrochloride (DTBP) (Wang and Richards, 1974a), which are cleaved in the presence of sulfhydryl reagents to regenerate the component polypeptides in any aggregate. Interacting components can then be identified directly on gels rather than being assessed from the molecular weight of the aggregate.

We have used these cleavable cross-linking agents to establish some of the near-neighbor interactions among the subunits of cytochrome *c* oxidase. Both DTBP and DSP give a number of cross-linked products including subunit dimers and larger aggregates.

Experimental Procedure

Beef heart mitochondria were isolated as described by Crane et al. (1956). Cytochrome *c* oxidase was prepared as described by Capaldi and Hayashi (1972). Small aliquots of the enzyme (20–50 mg/ml) were stored at −20 °C.

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Heme a concentrations were estimated by the pyridine hemochromogen difference spectral method of Williams (1964) using $\Delta\epsilon_{587-620}$ = 21.7 mM^{-1} cm^{-1} or from the reduced spectrum of cytochrome *c* oxidase in 1% Triton X-100–0.1 M phosphate (pH 7.0) using $\Delta\epsilon_{603-630}$ = 16.5 mM^{-1} cm^{-1}. Both methods gave equivalent results. Cytochrome *c* oxidase activity was measured spectrophotometrically by following the oxidation of ferrocytochrome *c* at 550 nm. The assay mixture contained cytochrome *c* (25 μM) in 0.05 M phosphate buffer (pH 7.0) containing 0.5% Tween 80 at 25 °C (Smith, 1955; Vanneste et al., 1974).

Enzyme was prepared for cross-linking by gel filtration in Triton X-100 or Tween 80. Cytochrome *c* oxidase (10 mg) was dissolved with 20 mg of Triton X-100 or Tween 80 in 1 ml of 0.09 M NaCl–0.02 M Tris-HCl (pH 8.0, I = 0.10). Chromatography was carried out at 4 °C in a column (1.0 × 60 cm) of Sepharose 4B equilibrated in the same buffer containing either 1 mM Triton X-100 or 0.05% Tween 80.

Fractions containing the cytochrome *c* oxidase were pooled and concentrated. The enzyme was suspended at between 1 and

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[1] Abbreviations used: ETP, beef heart submitochondrial particles; Gdn·HCl, guanidine hydrochloride; DSP, dithiobissuccinimidylpropionate; DTBP, dimethyl-3,3′-dithiobispropionimidate dihydrochloride; mol wt, molecular weight; Tris, tris(hydroxymethyl)aminomethane.

4 mg/ml in 25 mM triethanolamine hydrochloride (pH 8.0) containing either 1 mM Triton X-100 or 0.05% Tween 80 and cross-linked with DTBP or DSP at room temperature. Immediately before use, DTBP was dissolved at 20 mg/ml in the triethanolamine hydrochloride buffer. DSP was dissolved at the same concentration in Me_2SO. The cross-linking reaction was started by adding the cross-linking agent to give final concentrations between 0.1 and 2.0 mg/ml. Reaction times were varied between 5 min and 20 h. The reaction was stopped by quenching with 50 μl of 1 M ammonium acetate per ml of reaction mixture. Samples concentrated to 8–10 mg/ml with dry sucrose were made 3% in sodium dodecyl sulfate and incubated at 37 °C for 30 min. These conditions for dissociating the complex were used because, at the higher temperatures employed before, several of the subunits of cytochrome *c* oxidase aggregate irreversibly (Yamomoto and Orii, 1974; Downer et al., 1976) and also because disulfide bonds may be broken by heating (Wang and Richards, 1974b).

Gel Electrophoresis. Several different methods of gel electrophoresis were employed. Some samples were run on Biophore preformed gels containing 4 or 12% acrylamide monomer in a buffer system containing 0.205 M Tris, 0.205 M acetic acid, and 0.1% (w/v) sodium dodecyl sulfate at pH 6.4 (system I).

For high resolution of smaller molecular weight components, samples were run on 12.5% polyacrylamide gels in a buffer containing 8 M urea, 0.1% sodium dodecyl sulfate, and 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris base. This is system II in the study of Downer et al. (1976).

A two-dimensional gel electrophoresis system adapted from that described by Wang and Richards (1974a,b) was used. The slab gel (0.5-cm thick and 9.0-cm high) was cast from 15% acrylamide monomer and 0.5% bisacrylamide. This was overlayed with a 3.0-cm layer of 1% agarose containing 10% β-mercaptoethanol. The buffer systems used and conditions for running the gel were those described in system II of Downer et al (1976). Gels were fixed and stained as described by Downer et al. (1976). Gels were destained in a Bio-Rad diffusion destainer. Densitometric traces of the gels were made at 550 nm with a Gilford linear transport attachment to a Beckman DU spectrometer. Gels were calibrated for molecular-weight determination using the following standard proteins: thyroglobulin, β-galactosidase, γ-globulin, bovine serum albumin, catalase, ovalbumin, aldolase, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, and cytochrome *c*. Cross-linked bovine serum albumin and aldolase were also used as standards. Standards gels were run both with and without dithioerythritol: protein mobilities were essentially the same with both methods.

Results

Characterization of Enzyme Used in Cross-Linking Reactions. Cytochrome *c* oxidase was obtained by the method of Capaldi and Hayashi (1972). Preparations contained between 9.0 and 11.3 nmol of heme a/mg of protein and had molecular activities of 120–160 μmol of cytochrome *c* reduced s^{-1} (μmol of heme a_3)$^{-1}$ (at 25 °C and 25 μM cytochrome *c*). For most experiments, samples were solubilized in Triton X-100 and eluted through a column of Sepharose 4B containing Triton X-100 in the elution buffer. This step removed any residual ammonium sulfate which would quench the reaction between protein and cross-linking agent. It also accomplished a further purification of the enzyme by removing the high-molecular-weight impurities (such as the polypeptides of F_1) present in small amounts in the original preparation. Small and variable amounts of two impurities are still present after column chromatography, one appearing on gels just above subunit V (13 100) and the other between subunits V and VI (8500).

The column procedure also provided conditions for cross-linking under which the enzyme has been well characterized. Robinson and Capaldi (in preparation) have shown that cytochrome *c* oxidase dispersed in 1 mM Triton X-100 is monodisperse as judged by sedimentation equilibrium studies and is a dimer (a four heme complex of molecular weight 345 000), containing 50–60 μg of phospholipid per mg of protein, most of which is cardiolipin. The amount of phosphatidylethanolamine, which could react with the cross-linker, is less than 2 molecules per 2 heme complex.

Additional experiments were done with enzyme solubilized in Tween 80, conditions in which the composition of the complex being reacted has not been defined, but in which the enzyme is optimally active. Similar results were obtained in both Triton X-100 and Tween 80.

Resolution of Cross-Linked Products by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Cytochrome *c* oxidase was cross-linked with DTBP or DSP at various concentrations of cross-linker and for different times of reaction. The two reagents affected the activity of the enzyme differently. With DTBP, which retained the net charge on the protein, the activity was not significantly diminished at levels of reagent needed to generate the aggregates shown in Figures 1 and 3. With DSP, which increased the net negative change of the enzyme by converting the lysines into uncharged groups, the activity was altered more dramatically. Under the conditions used to generate the gel patterns in Figures 1 and 4, the activity was 80% lost. However, after extensive cross-linking with either DTBP or DSP, the visible spectrum of the reduced form of the enzyme was not altered, indicating that there had been no major disruption of the complex such as might change the juxtaposition of the subunits of the cytochrome *c* oxidase complex. The disappearance of individual subunits and appearance of cross-linked products obtained by reaction with DTBP and DSP were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Cross-linked samples were dissociated for electrophoresis with high levels of sodium dodecyl sulfate, but without β-mercaptoethanol or other reducing agents present as these reagents would cleave the disulfide bond in the center of the cross-linker molecule. Gels of unreacted cytochrome *c* oxidase dissociated without disulfide reagents showed all seven different polypeptides migrating with molecular weights similar to those obtained when β-mercaptoethanol or dithioerythritol was included in the dissociating medium. It appears, therefore, that disulfide bonds are not involved in the interactions between subunits of cytochrome *c* oxidase.

All polypeptides were cross-linked with DTBP or DSP and they disappeared from the gel pattern in the order: IV > III, V, VI, VII > II $\gg$ I. Distinct new bands were generated in the molecular weight range 20 000 to 40 000 by relatively low levels of either DTBP (1.0 mg/mg of protein) or DSP (0.13 mg/mg of protein) (Figure 1 and Table I). Bands of higher molecular weight were not seen after cross-linking with higher levels of DTBP but were generated by incubating cytochrome *c* oxidase with increased concentrations of DSP. Enzyme incubated with 0.2 mg of DSP/mg of protein for 30 min and then run on 4% polyacrylamide gels in sodium dodecyl sulfate showed broad bands centered at 45 000, 68 000, and 102 000 daltons along with a significant amount of unreacted subunit I (Figure 2). At higher levels of cross-linker (0.5 mg/mg of

TABLE I: Subunit Dimers Resolved after Cross-Linking with the Cleavable Bifunctional Reagents.

Mol Wt of Product	Subunits Involved	Sum of mol wt of Subunits	Cross-Linker	Conditions (mg/mg)	Conditions Time (min)	Gel System (see Methods
17.2	V, VII	16.8	DTBP	1.0	90	I; 12% gel
19.8	V, VII	16.8	DTBP	1.0	10	II
23.0	IV, VI	25.0	DSP	0.13	60	I; 12% gel
29.0	IV, VI	25.0	DSP	0.10	5	II
29.1	IV, VI	25.0	DTBP	1.0	10	II
30.1	III, V	33.4	DTBP	1.0	10	II
34.0	II, V	36.5	DSP	0.13	60	I; 12% gel
33.0	II, V	36.5	DSP	0.10	5	II
35.0	II, V	36.5	DSP	0.50	30	I; 4% gel

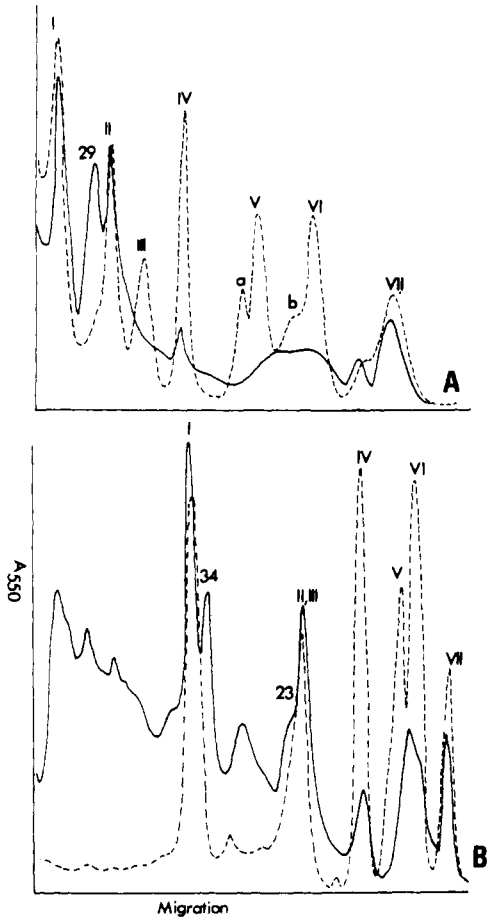

FIGURE 1: Densitometric trace of sodium dodecyl sulfate gels showing effects of cross-linking. The dotted line represents unreacted enzyme; the solid line, cross-linked enzyme. (A) A 12.5% sodium dodecyl sulfate–urea gel of a sample of enzyme dissolved at 1.0 mg/ml and reacted with 1.0 mg/mg of DTBP for 10 min; (B) a 12% sodium dodecyl sulfate gel of a sample of enzyme at the same protein concentration and reacted with 0.13 mg/mg of DSP for 60 min. In both cases, a considerable decrease in the amounts of individual subunits (especially III–VII) occurs. New cross-linked aggregates arise: at 29 000 in gel A and at 23 000 and 34 000 in gel B.

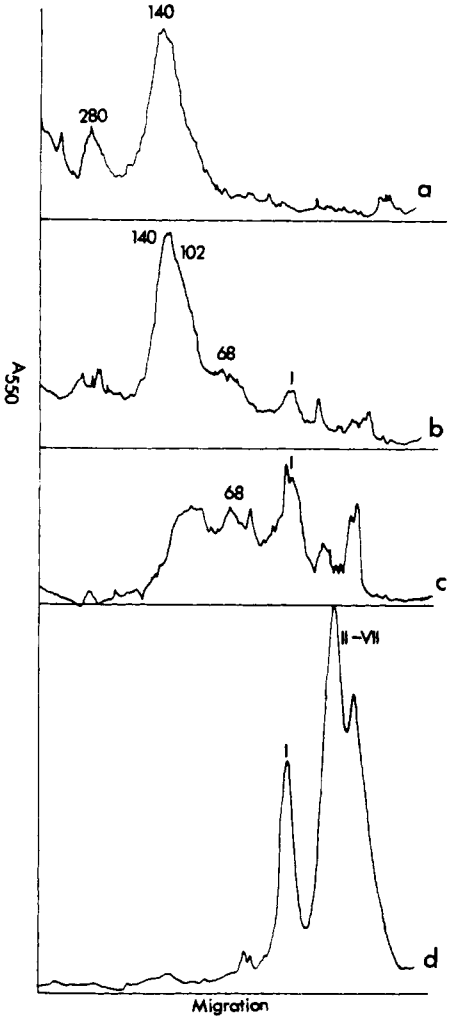

FIGURE 2: Densitometric traces of 4% sodium dodecyl sulfate gels of cytochrome *c* oxidase dissolved at 1.0 mg/ml and cross-linked with DSP: (a) at 0.5 mg/ml for 20 h; (b) at 0.5 mg/ml for 30 min; and (c) at 0.2 mg/ml for 30 min. Trace d shows unreacted enzyme for comparison. Peaks corresponding to cross-linked aggregates are labeled with their molecular weights in kilodaltons. Each of the gels contained 100 μg of enzyme.

protein for 30 min), the lower molecular weight aggregates and free subunit I were diminished in amount and an aggregate with a molecular weight of 140 000 was generated. Under still more rigorous conditions of cross-linking (0.5 mg of DSP/mg of protein for 4–20 h), the band at 140 000 dominated the gel scan, a new band at 280 000 appeared, and the low-molecular-weight bands disappeared almost completely. Still present, however, as un-cross-linked species were the impurities of 13 100 and 8500 daltons. These polypeptides were not seen in any of the aggregates resolved and this is taken as further evidence that they are indeed contaminants of cytochrome *c* oxidase preparations.

Identification of the Subunits Contributing to the Various Cross-Linked Aggregates. The two bifunctional reagents used

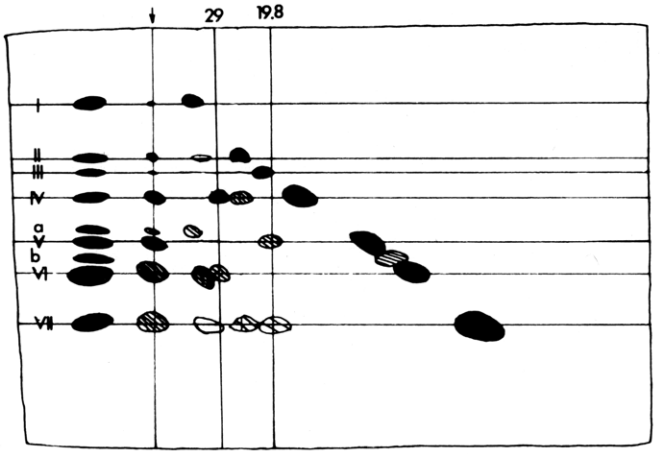

FIGURE 3: Two-dimensional gel analysis of cross-linked aggregates. Unreacted cytochrome *c* oxidase appears on the left side. The gel used in the first dimension is equivalent to the one shown in Figure 1A. Horizontal lines indicate the position of each subunit in the second dimension, and vertical lines indicate the components of cross-linked aggregates.

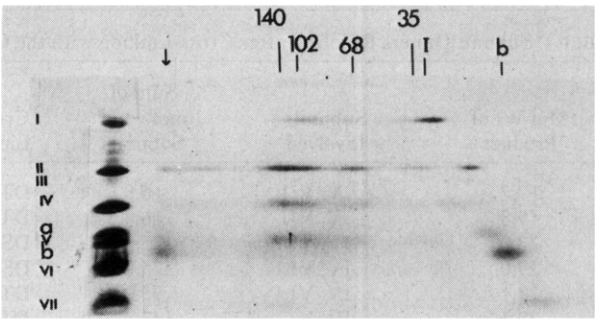

FIGURE 4: Two-dimensional gel analysis of high-molecular-weight aggregates. The gel used in the first dimension is equivalent to the one shown in Figure 2b. Several of the cross-linked products are identified on the gel by molecular weight. A standard of unreacted cytochrome *c* oxidase (100 μg) is shown down the left-hand side of the gel. The arrow marks the top of the tube gel; 300 μg of cross-linked enzyme were electrophoresed into this gel.

in this study could be cleaved by sulfhydryl reagents to regenerate the individual polypeptides contributing to the various cross-linked products. For optimal resolution, cross-linked aggregates were separated in the horizontal dimension of a two-dimensional gel and each aggregate was analyzed for the subunits it contained in the vertical dimension. A cylindrical gel containing cross-linked products was embedded in a layer of agarose containing 10% β-mercaptoethanol which overlayed a polyacrylamide slab gel. Protein was electrophoresed out of the cylindrical gel through the agarose and into the polyacrylamide. Unreacted subunits appeared on a diagonal across the slab except for subunit III, which could be seen off and slightly below the diagonal. Cross-linked products were cleaved by β-mercaptoethanol as they migrated through the agarose and the component subunits in each aggregate then ran as a function of their individual molecular weight to positions well below the diagonal. The expected position of individual subunits was determined for each slab by electrophoresing a sample of untreated enzyme along one side of the gel.

An analysis of some of the cross-linked products obtained with relatively low levels of DTBP is shown in Figure 3. The band at 29 000 contained subunits IV and VI (aggregate mol wt 25 000). Other dimers were seen, including V and VII. This pair ran with the same mobility as subunit IV on 12% sodium dodecyl sulfate gels and therefore was not recognized as a cross-linked product in cylindrical gels. The apparent molecular weight of the V and VII dimer was 17 200 on 12% sodium dodecyl sulfate gels and 19 800 on 12.5% sodium dodecyl sulfate–8 M urea gels (cf. aggregate mol wt 16 800). A band at 33 000 was routinely seen in samples of enzyme cross-linked with low levels of DSP. This aggregate contained subunits II and V (aggregate mol wt 36 500). For all dimers resolved, the apparent molecular weights obtained on gels were close to those calculated by summing the molecular weights of the individual subunits (Table I).

Several spots are seen on gels as part of a cross-linked product but the partner or partners are not resolved. These are usually higher molecular weight subunits (II or IV) which are probably associated with smaller subunits (V, VI, or VII). If only a small number of copies of an aggregate are formed, the mass of low-molecular-weight subunit(s) present may be too small to detect with Coomassie brilliant blue stain. After cross-linking with DSP, this is a particular problem because the cross-linker changes the positively charged amino acids into uncharged groups which no longer bind the dye.

An analysis of some of the larger aggregates obtained with DSP is shown in Figure 4. A band at 35 000 is resolved and shown to be the dimer of subunits II and V. The band at 68 000 is seen to contain subunits I, II, IV, V, VI, and VII, presumably associated into a variety of different trimers, tetramers, etc. The smallest aggregate with a molecular weight large enough to contain all seven subunits (as estimated by summing the molecular weights of the individual subunits) is the band at 140 000. The only aggregate seen with a molecular weight above 140 000 is a band at 280 000, which is generated under extreme conditions of cross-linking. There are no bands visible between the 140 000 and 280 000 bands, suggesting that the larger of these two bands is generated by cross-linking two of the smaller aggregates.

Discussion

Two cleavable cross-linking agents have been used to probe the structure of the cytochrome *c* oxidase complex. These reagents react predominantly with lysine residues (Hunter and Ludwig, 1962) and serve to covalently link neighboring polypeptides in which these reactive groups are within 11 Å of each other. For cytochrome *c* oxidase, DSP is more reactive than DTBP, possibly in part because it is uncharged and can approach the protein more readily than the charged imidate (DTBP), and also because it is not as rapidly hydrolyzed in aqueous buffer as DTBP.

Cytochrome *c* oxidase is cross-linked in several discrete steps. By reaction with low levels of cross-linker, a number of dimers are generated. These have been identified as II and V, III and V, V and VII, IV and VI. Some part of each of these sets of polypeptides must be within 11 Å of each other. The close proximity of the first three of these pairs but not of IV and VI would be predicted from the model of cytochrome *c* oxidase proposed recently by Eytan et al. (1975). This model is based on the accessibility of the various subunits in intact mitochondria and in ETP (inside-out inner membrane particles). However, correlations between our cross-linking study and the labeling experiments of Eytan et al. (1975) must be accepted with caution until it has been demonstrated that the order of migration of the polypeptides is the same in the two very different gel procedures used in the two studies.

In addition to the pairs listed, many other cross-linked products with molecular weight below 100 000 were seen, but these could not be analyzed definitively and so were not in-

cluded in Table I. The many different cross-links formed suggest that the polypeptides in cytochrome *c* oxidase are present as extended chains rather than as discrete globular units in the complex. A similar extended arrangement of polypeptides has been suggested for ribosomal proteins based on both cross-linking studies and fluorescence energy-transfer measurements (Wittmann, 1976; Huang et al., 1975; Lake et al., 1974).

After reaction with high levels of DSP, the major cross-linked product has a molecular weight of 140 000 and contains all of the subunits of the enzyme. This is most likely the "unit complex" containing the two hemes and two copper atoms. The molecular weight of the complex obtained on gels is somewhat higher than the value of 125 000 calculated by summing the molecular weights of one copy of each of the seven different subunits, leaving open the possibility that there are multiple copies of one or more of the subunits of the beef heart cytochrome *c* oxidase (see, for example, Phan and Mahler, 1976).

Under the most extreme conditions of cross-linking used, a band with an apparent molecular weight of 280 000 is resolved. This is presumably the cytochrome *c* oxidase dimer or four-heme complex. The molecular weight determined on gels is somewhat lower than the value of 345 000 obtained from sedimentation equilibrium studies of cytochrome *c* oxidase dissolved at the same concentration and in the same buffer conditions that were used in cross-linking experiments (Robinson and Capaldi, in preparation).

In summary then, cytochrome *c* oxidase can be cross-linked in a series of discrete steps into the unit complex and then into a four-heme complex or enzyme dimer. The sequence in which the cross-linked aggregates appear is evidence that intracomplex cross-links are formed much faster than intercomplex bridges are generated. It will be interesting to see if intracomplex cross-links are formed preferentially when cytochrome *c* oxidase is surrounded by different electron transport components as in the mitochondrial inner membrane and this work is in progress.

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