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Articles

Electron Transfer in Monomeric Forms of Beef and Shark Heart Cytochrome *c* Oxidase†

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ABSTRACT: Beef heart cytochrome *c* oxidase is dimeric in reconstituted membranes and in nonionic detergents at physiological pH [Henderson, R., Capaldi, R. A., & Leigh, J. (1977) *J. Mol. Biol. 112*, 631; Robinson, N. C., & Capaldi, R. A. (1977) *Biochemistry 16*, 375], raising the possibility that this aggregation state is a prerequisite for enzymatic activity. A procedure for dissociating the enzyme into monomers is presented. This involves treating the protein with high concentrations of Triton X-100 at pH 8.5. The electron transfer activity of the monomer is comparable to that of the dimer under identical assay conditions. The beef heart cytochrome *c* oxidase monomer was found to be heterogeneous in hydrodynamic studies, probably due to dissociation of associated polypeptides, including subunit III. Monomer molecular weights in the range 129 000–160 000 were obtained. Previous studies have indicated that shark heart cytochrome *c* oxidase is monomeric under physiological conditions. Sedimentation equilibrium studies reported here confirm this. The elasmobranch enzyme, with a similar polypeptide composition to that of the beef enzyme, was determined to have a molecular weight of 158 000.

Cytochrome *c* oxidase (EC 1.9.3.1) catalyzes a four-electron reduction of molecular oxygen to water and couples this reaction to the net movement of protons across the mitochondrial inner membrane [for a review see Azzi (1980)]. It is generally agreed that cytochrome *c* oxidase from fungal sources is a complex of seven or probably eight different polypeptides (Sebald et al., 1973; Poyton & Schatz, 1975). Cytochrome *c* oxidase isolated from mammalian sources contains at least 12 different polypeptides (Steffens & Buse, 1976; Wilson et al., 1980; Darley-Usmar et al., 1981; Merle & Kadenbach, 1980).

There is now a controversy about how many of the polypeptides in cytochrome *c* oxidase preparations are true subunits of the enzyme. Our approach is to define the subunit composition of cytochrome *c* oxidase in terms of the minimal structure able to transfer electrons from cytochrome *c* to molecular oxygen and to couple this reaction to the generation of a proton gradient across the inner mitochondrial membrane. It appears that a complex of at most eight different polypeptides is fully functional (Ludwig et al., 1979). This does not rule out the possibility that the additional polypeptides have a regulatory role.

Three of the subunits (I–III) are coded for and synthesized in the mitochondria of all eukaryotes (Fox, 1979; Anderson et al., 1981; Bibb et al., 1981, Fox & Leaver, 1981). The smaller subunits, IV, V, VI, VII-Ile, and VII-Ser (terminology for the beef enzyme), are made in the cytoplasm (Anderson et al., 1982). The amino acid sequences of all of the subunits of beef heart cytochrome *c* oxidase have been obtained either from the DNA or directly by protein sequencing [Anderson et al., 1982; see Azzi for review (1980)]. Thus the minimum molecular weight of the two heme, two copper complex containing one copy of each of the eight subunits can be calculated. This value is 160 000 daltons. A complex containing one copy each of the 12 polypeptides that are considered to be subunits by Steffens & Buse (1976), Verheul et al. (1981), and Merle & Kadenbach (1980) would have a molecular weight of 200 000.

Preparations which are missing subunit III have been described recently (Saraste et al., 1981). Such preparations catalyze electron transfer efficiently but do not show coupled proton translocation. The calculated molecular weight of a complex missing subunit III, but containing the other seven subunits, would be 130 000.

Determinations of the molecular weight of beef heart cytochrome *c* oxidase in nonionic detergents give values in the range 300 000–400 000 (Robinson & Capaldi, 1977; Rosevear et al., 1980; Saraste et al., 1981), indicating that the stable species from this source is a dimer. Enzyme in vesicular

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membranes is also dimeric (Henderson et al., 1977). The only condition in which a monomeric form of the beef enzyme was obtained required deoxycholate, a detergent in which cytochrome *c* oxidase is inactive (Robinson & Capaldi, 1977; Fuller et al., 1979). There is, therefore, no direct evidence for a functional beef enzyme monomer. In fact, when lysine-13 arylazidocytochrome *c* was used to modify cytochrome *c* oxidase, it was found to inhibit enzyme activity in a molar ratio of one cytochrome *c* bound per dimer (Bisson et al., 1980). This result has been interpreted in terms of an important role for the dimer in cytochrome *c* oxidase function (Wickstrom et al., 1981).

The studies of beef heart cytochrome *c* oxidase contrast with those on several other eukaryotes including chicken, camel (Darley-Usmar et al., 1981), elasmobranch fish (Wilson et al., 1980), and rat liver (Thompson et al., 1982). It was reported that the enzyme from each of these species is monomeric under physiological conditions although in no case was a careful measurement of molecular weight made.

Studies described here clarify the significance of the aggregation state in cytochrome *c* oxidase activity. Monomeric cytochrome *c* oxidase from beef heart has been obtained which is functional in electron transfer. The physical characteristics, including the molecular weight of the enzyme from shark heart, have been examined. It is confirmed that cytochrome *c* oxidase from this species is a functional monomer.

Materials and Methods

Cytochrome c Oxidase Preparations. Beef heart cytochrome *c* oxidase was prepared by the method of Capaldi & Hayashi (1972) and typically had a heme *a* to protein ratio of 9–10 nmol of heme *a*/mg of protein. Hammerhead shark heart cytochrome *c* oxidase (Sphyrna lewini) was prepared by the method of Yonetani (1960) and was the generous gift of Drs. D. Bickar and J. Bonaventura, Duke University Marine Laboratory, Durham, NC. The shark oxidase was further purified either by gel filtration on Sephacryl S-200 or by ion-exchange chromatography using DEAE-agarose gel. The shark oxidase had a heme to protein ratio of 8–9 nmol of heme *a*/mg of protein. Protein concentrations were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Extinction coefficients used for determining the concentration of both the beef heart and shark cytochrome *c* oxidase were those reported by Yonetani (1961). The extinction coefficient for cytochrome *c* oxidase at 280 nm was determined independently for each preparation and was found to be 210 mM^{-1} cm^{-1} per heme *a* for the beef heart enzyme and 269 mM^{-1} cm^{-1} per heme *a* for the shark enzyme.

Gel Filtration and Triton X-100 Binding. A column (1 × 24 cm) containing Sephacryl S-200 was used to measure the amount of Triton X-100 bound to cytochrome *c* oxidase samples: samples of cytochrome *c* oxidase (1–2 mg) were loaded onto the column equilibrated with 0.1% Triton X-100, 200 mM NaCl, and 10 mM Tris-HCl[1] buffer at pH 8.5 or pH 7.4. Samples were collected from the column and their absorbance measured at 280 and 420 nm on a Beckman DU7 spectrophotometer.

The amount of Triton X-100 bound to a given sample of enzyme was calculated by taking the sum of the absorbance due to the 0.1% Triton X-100 buffer and absorbance due to protein at 280 nm and subtracting this from the measured absorbance at this wavelength. The number of Triton X-100 molecules bound was calculated by using the published extinction coefficient for this detergent (Makino et al., 1973; Tanford et al., 1974).

DEAE-agarose Chromatography. Samples treated with 5% Triton X-100 at high salt concentration were diluted 10-fold with water and bound to a DEAE-agarose column (0.7 × 1 cm). Excess Triton X-100 was washed from the protein with buffers of the desired pH containing 0.1% Triton X-100. The samples were then eluted from the column with a buffer containing 0.1% Triton X-100, 10 mM Tris-HCl, and 200 mM NaCl at either pH 7.4 or pH 8.5. Samples prepared in this way were then ready to be loaded on the Sephacryl S-200 column.

The DEAE-agarose column was also used to exchange detergents. After binding the protein to the column, it was washed with 20–25 times the bed volume of the desired detergent. In the case of lysophosphatidylcholine, it was necessary to increase the salt concentration to 1 M to wash the cytochrome *c* oxidase from the column. These samples were then dialyzed against the desired buffers and detergent at an appropriate salt concentration before use.

Reaction with Lysine-13 Modified Arylazidocytochrome c. Arylazidocytochrome *c* specifically modified at lysine-13 was prepared as described by Bisson et al. (1980). Shark cytochrome *c* oxidase was diluted to a concentration of 3.3 μM in a buffer containing 0.2% Tween 80 and 25 mM Tris–acetate, pH 7.8. Lysine-13 modified arylazidocytochrome *c* was mixed with cytochrome *c* oxidase over a range of concentrations. Samples were illuminated under UV light for 30 min on ice. After illumination, an aliquot of each sample was taken for $NaDodSO_4$–polyacrylamide gel electrophoresis (20–30 μg) and for activity measurements.

$NaDodSO_4$–polyacrylamide gels were prepared as described by Fuller et al. (1981): 20–30 μg of cytochrome *c* oxidase was denatured in a solution containing 8 M urea, 5% $NaDodSO_4$, 1% 2-mercaptoethanol, and 0.25 M Tris-HCl, pH 6.2, for 30 min at room temperature. Samples were electrophoresed on slab gels (10 × 14 cm), stained, and destained as described by Downer et al. (1976). Channels were scanned at 560 nm on a Gilford spectrophotometer with a linear scanning attachment.

The amount of subunit II reacted with arylazidocytochrome *c* was estimated by difference from the amount of unreacted subunit (measured from the gel scan) and expressed as a percentage of the area of subunit II in a control sample (no cytochrome *c*). Subunit IV was used to normalize the area of subunit II between control and experimental samples as the area under this peak showed little variation.

Antibody blotting experiments were conducted as described by Millett et al. (1982) by using the procedure of Towbin et al. (1979).

Native Gel Electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out on 5% acrylamide gels by using system 1 of Maurer (1971) supplemented with 0.1% Triton X-100. Typically, protein samples were diluted to a final concentration of 5 mg/mL in 5% Triton X-100 and 320 mM Tris-HCl, pH 8.5, and incubated on ice for 2 h. A 10-μL aliquot was then applied to 0.2-cm cylindrical gels and electrophoresed at 6 °C for 1–3 days at 20–50 V.

The cylindrical gel was incubated in 5% $NaDodSO_4$ and 50 mM Tris-HCl, pH 6.2, for 45 min at room temperature and fused onto a 15% polyacrylamide–urea gel for the second

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; lyso-PC, lysophosphatidylcholine; TMPD, *N,N,N',N'*-tetramethylphenylenediamine dihydrochloride; DEAE, diethylaminoethyl.

dimension electrophoresis in $NaDodSO_4$ as discussed above.

Activity Measurements. Activity was measured polarographically by using an oxygen electrode. The arylazidocytochrome *c*–cytochrome *c* oxidase complex was assayed in a buffer containing 50 mM sodium phosphate, pH 7.4, 1% Tween 80, 10 mM sodium ascorbate, and 1 mM TMPD. The concentration of cytochrome *c* in the assay was 30 μM and that of cytochrome *c* oxidase, 100–125 nM.

Fractions collected from the Sephacryl S-200 column were assayed in 50 mM sodium phosphate, pH 7.4, containing 0.1% lysophosphatidylcholine or 0.5% Tween 80.

Analytical Ultracentrifugation. Sedimentation velocity and sedimentation equilibrium analyses were performed on the Beckman–Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. Double-sector cells were used with an An-D rotor, and the sample was scanned at 420 nm. Protein molecular weights were determined by the method of Tanford et al. (1974).

A partial specific volume of 0.743 was used for the beef heart enzyme (Robinson & Capaldi, 1977). The same value was used for the shark heart enzyme since the amino acid composition was similar to the beef heart enzyme (V. Darley-Usmar, unpublished results). Calculations of the molecular weight were corrected for the effect of bound detergent by using 0.908 as the partial specific volume for Triton X-100 (Tanford et al., 1974).

Results

Conditions in Which Beef Heart Cytochrome c Oxidase Is a Dimer. Previous studies have shown that beef heart cytochrome *c* oxidase is a dimer at physiological pH (pH 7.4) in 0.1% Triton X-100 (I = 0.2; T = 22 °C) (Robinson & Capaldi, 1977). This was confirmed in the present study. The enzyme was dissolved in 5% Triton X-100 (pH 7.4), bound to a DEAE-agarose column, washed with 0.1% Triton X-100 to lower the overall detergent concentration, and then eluted in the same buffer with 0.2 M NaCl added. The number of molecules of detergent bound to the protein after passage through a Sephacryl S-200 column was determined spectrophotometrically. This value of 150 ± 44 per heme aa_3 (three determinations) is the number of molecules in a Triton X-100 micelle (Helenius & Simons, 1975). Robinson & Capaldi (1977) found 180 ± 10 Triton X-100 molecules bound per heme aa_3. The difference in these values is easily accounted for by small differences in lipid composition (samples analyzed by Robinson and Capaldi retained more bound phospholipid than preparations used here) and by differences in buffer conditions which will affect the composition of the detergent–lipid–protein mixed micelles (Helenius & Simons, 1975).

Samples eluting from the Sephacryl S-200 column were used directly for sedimentation equilibrium analysis. The plot of ln c/ω^2 vs. r^2 gave a straight line through most of the cell with a slope equivalent to a molecular weight of 326 000 after correction for bound detergent (Figure 1). The slope of the line near the bottom of the cell indicates the presence of a small amount of material with a higher molecular weight.

Aliquots of cytochrome *c* oxidase after the DEAE-agarose column or gel filtration on Sephacryl S-200 were also examined by sedimentation velocity analysis. Values for the sedimentation coefficient of between 10 and 13 S were obtained (Table I). The activity of the enzyme assayed in lysophosphatidylcholine was 160 mol of cyt *c* oxidized s^{-1} (mol of aa_3)$^{-1}$.

The state of aggregation of the beef heart enzyme was measured in several other detergents at pH 7.4 by sedimentation velocity. Values of 13 S and higher were obtained in Tween 80, indicating the presence of large aggregates in this detergent. Values in the range 11–13 S were obtained in lysophosphatidylcholine, indicating that the enzyme is dimeric in this detergent at physiological pH.

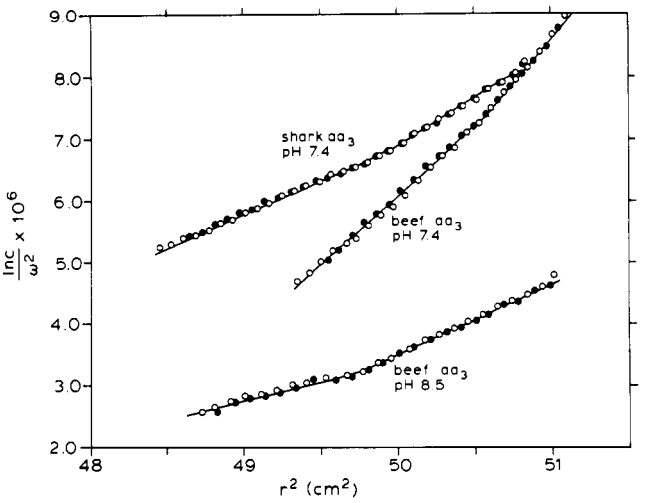

FIGURE 1: Sedimentation equilibrium of shark and beef heart cytochrome *c* oxidase. Samples were centrifuged at 6400 or 9000 rpm at 20 °C in 10 mM Tris-HCl, 200 mM NaCl, and 0.1% Triton X-100 at the indicated pH. Plots of ln *c* vs. r^2 after 24 and 36 h were superimposable, indicating that the samples had come to equilibrium. Protein concentrations were in the range 0.2–0.5 mg/mL. Shark aa_3, pH 7.4: shark oxidase after gel filtration on Sephacryl S-200 at pH 7.4. Beef aa_3, pH 7.4: the beef enzyme treated with 5% Triton X-100 (pH 7.4) and fractions eluted from the Sephacryl S-200 column analyzed. Beef aa_3, pH 8.5: the beef enzyme pretreated with high pH (8.5) and 5% Triton X-100 and fractions eluted from the Sephacryl column (pH 8.5) analyzed. Open and closed circles represent data taken at 24 and 36 h, respectively.

Table I: Aggregation State and Activity of Cytochrome *c* Oxidase[a]

sample	sed velocity (S) T X-100	sed velocity (S) lyso-PC	sed velocity (S) Tween 80	sed equil	activity (e s^{-1} per aa_3)
beef, pH 7.4	10–13 (85%)			3.26×10^5	160
beef, pH 8.5	6.5–7.5 (90%)	6.5–7.5 (90%)	6.5–7.5 (90%)	1.29×10^5	210
beef, pH 8.5–7.4	6.5–7.5 (90%)	6.5–7.5 (90%)			
shark, pH 7.4	6.5–7.5 (90%)		6.5–7.5 (90%)	1.58×10^5	250

[a] Samples of cytochrome *c* oxidase were analyzed by sedimentation velocity, sedimentation equilibrium (in Triton X-100), and assayed for activity. The sedimentation coefficient is given for the major sedimenting species together with its proportion relative to the total amount of protein applied to the ultracentrifuge column. Values given for molecular weights after sedimentation equilibrium are corrected for bound Triton X-100. Activities were measured polarographically. Beef, pH 7.4: beef oxidase after pretreatment with 5% Triton X-100 at pH 7.4. Beef, pH 8.5: beef oxidase after treatment with 5% Triton at pH 8.5 followed by exchange of detergents by DEAE-agarose chromatography (lyso-PC, lysophosphatidylcholine). Beef, pH 8.5–7.4: beef oxidase treated at pH 8.5 in 5% Triton X-100 and then brought to pH 7.4 for analysis by DEAE-agarose column chromatography in the detergents indicated. Shark, pH 7.4: shark oxidase after gel filtration in Sephacryl S-200 at pH 7.4. T X-100, Triton X-100.

Monomeric Beef Heart Cytochrome c Oxidase. A range of conditions for dissociating the beef heart cytochrome *c* oxidase dimer into monomers was explored. The approach

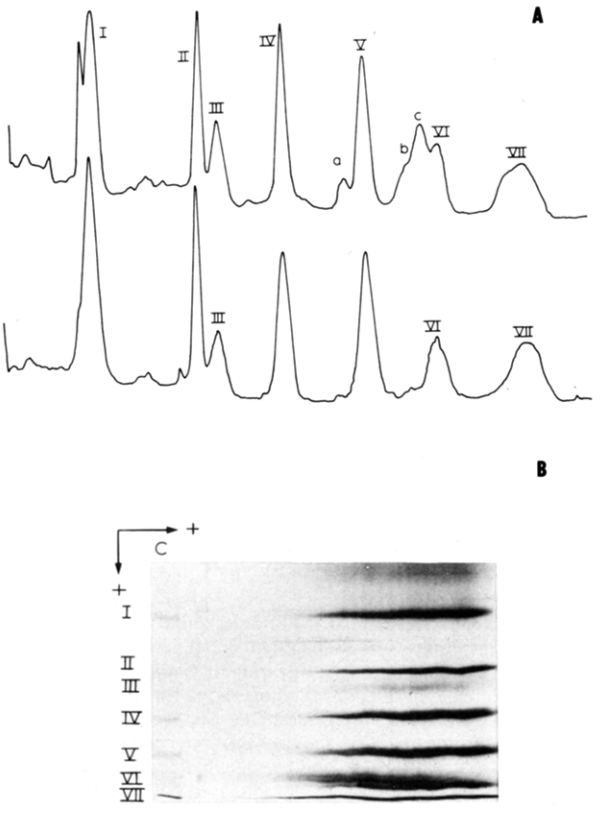

FIGURE 2: Gel electrophoresis of cytochrome *c* oxidase. (A) Polypeptide composition of the stock beef heart cytochrome *c* oxidase (upper trace) and the monomeric enzyme following treatments with high pH and high Triton X-100 concentrations and DEAE-agarose chromatography (lower trace). Polypeptides were resolved on a polyacrylamide gel containing $NaDodSO_4$ and urea as described under Materials and Methods. (B) Beef heart cytochrome *c* oxidase was dissociated into monomers and electrophoresed in the first dimension on a native rod gel (not shown). After dissociation in $NaDodSO_4$ the rod gel was fused to a second-dimension $NaDodSO_4$–polyacrylamide slab gel. A control channel (C) of untreated oxidase was included in the second dimension. Direction of migration is indicated, and the subunits are numbered according to Ludwig et al. (1979).

adopted was as follows: Beef heart cytochrome *c* oxidase (10 mg/mL) was incubated in 5% Triton X-100 and 320 mM Tris-HCl, pH 8.5, for 2 h on ice (as in native gel electrophoresis) and then bound to the DEAE-agarose column. It was eluted from the column in 0.1% Triton X-100, 0.1% Tween 80, 0.1% lysophosphatidylcholine, or 0.1% lauryl maltoside in NaCl and 10 mM Tris-HCl buffer at pH 8.5 or pH 7.4.

Figure 2A shows the polypeptide composition of the enzyme eluted from the DEAE-agarose column in 0.1% Triton X-100, 200 mM NaCl, and 10 mM Tris-HCl, pH 8.5. There was a removal of impurities such as the polypeptide migrating close to subunit I. Also the concentrations of polypeptides a–c were significantly reduced and the amount of subunit III present was lower. This last observation is not surprising because the conditions needed to make monomers are similar to those used by Ludwig et al. (1979) and Saraste et al. (1981) to obtain an enzyme preparation missing subunit III.

Enzyme eluted from the column with Tween 80, lysophosphatidylcholine, or lauryl maltoside at pH 7.4 or pH 8.5 showed the same altered polypeptide composition. This partial removal of polypeptides generates a preparation which is heterogeneous as evident in native gel electrophoresis (Figure 2B). Several discrete species were separated by this method. The composition of each was identified by a second-dimensional electrophoresis in $NaDodSO_4$. The major species resolved contains subunits I, II, IV, V, VI, and VII's. This would

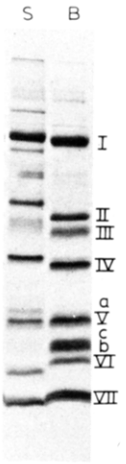

FIGURE 3: Polyacrylamide gel electrophoresis of shark and beef heart cytochrome *c* oxidase. Shark (S) and Beef heart (B) cytochrome *c* oxidases (30 μg of protein) were dissociated in $NaDodSO_4$ and urea. After electrophoresis the gels were stained with Coomassie blue. Beef heart subunits are numbered in order of decreasing apparent molecular weight as described by Ludwig et al. (1979).

have a monomer molecular weight of 130 000. A species containing subunits I–VII (160 000 daltons) and species in which a–c are present were also separated.

Sedimentation equilibrium data are shown in Figure 1 for cytochrome *c* oxidase treated with high concentrations of Triton X-100 at pH 8.5 which has then been eluted from the DEAE-agarose column in low concentrations of Triton X-100 (pH 8.5). The plot of $\ln c/\omega^2$ vs. r^2 for this sample shows two phases with the slope nearest the top of the cell equivalent to a molecular weight of 129 000 after correction of bound detergent (190 ± 19 molecules; five determinations) and the slope nearest the bottom of the cell equivalent to a molecular weight of 160 000. These two phases must be a result of the subunit heterogeneity discussed above. The sedimentation equilibrium data establish that the enzyme is monomeric after treatment with high concentrations of Triton X-100 at pH 8.5. Sedimentation velocity studies of aliquots of the same preparations used for sedimentation equilibrium gave *s* values in the range 6.5–7.5 S (Table I). This is therefore the range of values for the monomeric species.

The results of sedimentation velocity studies of the enzyme eluted from the DEAE-agarose column in a variety of different detergent conditions is summarized in Table I. At pH 8.5 at least 90% of the beef heart enzyme sedimented with an *s* value of between 6.5 and 7.5 S, i.e., as a monomer in all of the detergents tested. Enzyme dissociated at pH 8.5 and then washed from the DEAE-agarose column at pH 7.4 also sedimented as the monomer in 0.1% Triton X-100 and 0.1% lysophosphatidylcholine or in Tween 80.

Assay of the enzyme eluted from the column in 0.1% lysophosphatidylcholine, at pH 7.4, gave an activity of 210 mol of cytochrome *c* s^{-1} (mol of $aa_3)^{-1}$. It is very unlikely that the enzyme reaggregates upon dilution from a protein concentration of 0.5 mg/mL (for sedimentation studies) to 0.002–0.005 mg/mL (for assay) in the same detergent concentration. The beef heart cytochrome *c* oxidase monomer, therefore, appears to be as active as a monomer as it is as a dimer.

Molecular Weight of Shark Heart Cytochrome c Oxidase. Cytochrome *c* oxidase isolated from shark heart was bound to DEAE-agarose and eluted from the column in 0.1% Triton X-100 or 0.1% Tween 80 (pH 7.4) with 0.2 M NaCl. The polypeptide composition of the enzyme eluting from this column is remarkably similar to that of the beef heart enzyme

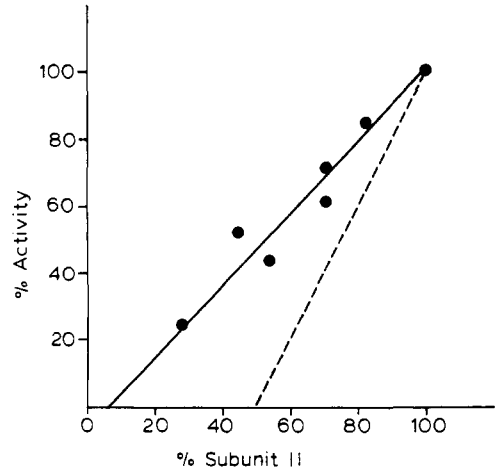

FIGURE 4: Reaction of shark heart cytochrome *c* oxidase with lysine-13 arylazidocytochrome *c*. Shark cytochrome *c* oxidase was reacted with varying amounts of lysine-13 arylazidocytochrome *c*. After reaction, samples were taken for $NaDodSO_4$–polyacrylamide gels and activity measurements. The percentage activity of each sample is shown vs. the percentage of subunit II unmodified. The dotted line indicates the theoretical curve for the "half of sites" effect described by Bisson et al. (1980).

(Figure 3), the major difference being in the apparent increased molecular weights of subunits II and IV. [The identification of subunits I–IV in the shark enzyme with the analogous polypeptides from the beef heart enzyme was made by the gel electrophoretic blotting technique of Towbin et al. (1979) using antibodies made against purified subunits of the beef heart enzyme.]

Sedimentation velocity experiments on samples eluting from the DEAE-agarose column gave values of 6–7 S for the enzyme in Tween 80 or Triton X-100 at pH 7.4 in agreement with Wilson et al. (1980). This is in marked contrast to the results for the beef heart enzyme which sedimented as species of 13 S or greater under the same conditions. Sedimentation equilibrium of the shark enzyme in 0.1% Triton X-100 (pH 7.4) gave a mainly straight line plot of ln c/ω^2 vs. r^2 with a slope equivalent to a molecular weight of 158 000 (Figure 1) after correction for bound Triton X-100 (131 ± 24 molecules per aa_3). The plots of ln c/ω^2 vs. r^2 for beef and shark (five determinations) in Figure 1 were obtained under identical conditions, emphasizing the different sizes of the two species in the same detergent. A molecular weight of 158 000 for the shark enzyme compares with a value of 160 000 for the sum of the subunits of a beef heart cytochrome *c* oxidase monomer. Unlike beef heart cytochrome *c* oxidase, then, the shark enzyme is monomeric in Tween 80 and lysophosphatidylcholine at physiological pH without prior treatment with the strongly dissociating detergents such as Triton X-100. The molecular activity of the shark enzyme (monomer) measured polarographically was 250 mol of cytochrome *c* oxidized s^{-1} (mol of $aa_3)^{-1}$, a value comparable to the beef heart enzyme (monomer) under the same conditions.

Reaction of Shark Heart Cytochrome c Oxidase with Cytochrome c. Samples of shark cytochrome *c* oxidase were mixed with different amounts of lysine-13 arylazidocytochrome *c* and photoactivated on ice as described by Bisson et al. (1980). Aliquots were taken for activity measurements and $NaDodSO_4$ gel electrophoresis. The modified cytochrome *c* formed a covalent linkage with subunit II. The amount of complex formed was estimated by difference from the amount of unmodified subunit II remaining and is shown plotted against activity in Figure 4. Inhibition of electron transfer activity is directly proportional (1/1 ratio) with the amount of derivative covalently bound to the oxidase. Shark heart cytochrome *c* oxidase does not show the half of site effect given by reaction of arylazidocytochrome *c* with the beef heart enzyme dimer (Bisson et al., 1980).

Discussion

The importance of the dimer in cytochrome *c* oxidase activity has been suggested indirectly by several studies and has been speculated upon in terms of a control mechanism of electron transfer (Bisson et al., 1980; Wikstrom et al., 1981).

A majority of studies of cytochrome *c* oxidase involve the enzyme isolated from beef heart or from yeast. The isolated enzymes from both species are stable as dimers (Robinson & Capaldi, 1977). Beef heart cytochrome *c* oxidase has been obtained in a monomeric form by treatment with deoxycholate but in a crystalline array unsuitable for activity measurements (Fuller et al., 1979). There had been no direct measurements of the activity of monomer beef heart cytochrome *c* oxidase before the present study. Cytochrome *c* oxidase has been isolated from avian and other mammalian species and has been reported to be active as a monomer (Wilson et al., 1980; Darley-Usmar et al., 1981; Thompson et al., 1981). However, the molecular weight determinations in these studies were not conclusive.

The experiments reported here describe a procedure for dissociating the beef heart cytochrome *c* oxidase dimer into monomers with high Triton X-100 concentration and at relatively high pH (pH 8.5). This procedure results in a heterogeneous mixture with varying polypeptide compositions. A major fraction had a molecular weight of 129 000 corresponding to a species containing subunits I, II, IV, V, VI, VII-Ile, and VII-Ser (cf. with a theoretical molecular weight calculated from a summation of subunit molecular weights of approximately 130 000). A preparation with a similar subunit composition has been studied by Saraste et al. (1981) and estimated to have a molecular weight of 210 000. They interpret their data in terms of a dimer missing subunit III. Their interpretation was based primarily on the summation of molecular weights of subunits estimated from $NaDodSO_4$–polyacrylamide gel electrophoresis. This procedure underestimates the molecular weight of subunit I by as much as 20 000 (Darley-Usmar & Fuller, 1981). The molecular weight of a III-less dimer calculated by using the correct molecular weights for individual subunits is 260 000 or greater, depending on whether components a–c are present. Indeed, component a is present in the III-less enzyme prepared by these authors (Wikstrom et al., 1981) bringing the theoretical molecular weight to more than 280 000. The value of 210 000 assigned by Saraste et al. (1981) to a III-less dimer does not conform to the expected size of III-less monomer or a III-less dimer, making interpretation of these results difficult.

We have been able to obtain beef heart enzyme in monomeric or dimeric form as verified by sedimentation equilibrium. This allows the correlation of the monomeric species with sedimentation coefficients in the range 6.5–7.5 S and the dimer with the sedimentation coefficients in the range 10–13 S.

Love et al. (1970) claimed to have dissociated the enzyme into a one heme *a* complex at pH 10 or above. This species had a sedimentation coefficient of 6 S and would thus conform to our definition of a monomer containing two hemes and two coppers.

Once dissociated into monomers, as described above, the beef heart enzyme remained monomeric when Triton X-100 (pH 8.5) was exchanged for lysophosphatidylcholine (pH 7.4) by chromatography on DEAE-agarose. The monomer could

thus be assayed for activity. Studies are in progress to identify the factors important for dissociating dimers into monomers and for stabilizing the monomeric form at pH 7.4. Preliminary results suggest that removal of subunit III after its dissociation from the complex by the high concentrations of Triton X-100 at pH 8.5 is critical for maintaining the monomer. There is reaggregation of the monomeric enzyme into dimers at low salt concentrations and at pH 7.4 when subunit III is not removed by a prior step such as DEAE chromatography (G. Georgevich, unpublished results).

Molecular weight determinations were also made on shark cytochrome *c* oxidase. $NaDodSO_4$–polyacrylamide gels resolved this preparation into nine different polypeptides. The four largest of these were shown to be analogous to subunits I–IV of beef heart cytochrome *c* oxidase. Only small differences in molecular weight between the subunits of the two species were apparent (Figure 3). There is no sequence data for subunits of the shark enzyme, and therefore an accurate theoretical molecular weight for the shark oxidase monomer cannot be calculated. Given the almost identical polypeptide compositions of the shark and beef enzymes, the monomer molecular weights must be similar (Figure 3). The molecular weight of the shark enzyme at neutral pH in Triton X-100 was determined to be 158 000 in sedimentation equilibrium experiments. The sedimentation coefficient was 6.5–7.5 S. Thus, shark cytochrome *c* oxidase is a monomer under conditions in which beef heart cytochrome *c* oxidase is a dimer.

The monomeric form of both beef heart and shark heart cytochrome *c* oxidase was active in electron transfer. Values in the range 150–250 mol of cytochrome *c* (mol of aa_3)$^{-1}$ were obtained for both species, and these are comparable to those obtained for the beef dimer under the same conditions. This is direct evidence that the cytochrome oxidase monomer is active. There is supportive indirect evidence for the same conclusion. 1-Ethyl-3-[3-(trimethylamino)propyl]carbodiimide was found to inhibit cytochrome oxidase activity. Protection from this inactivation was achieved at a molar ratio of one cytochrome *c* per oxidase monomer (Millett et al., 1982). Thionitrobenzoate-modified yeast cytochrome *c* inhibited cytochrome *c* oxidase in the ratio of one mole derivative per mole of cytochrome *c* oxidase (Fuller et al., 1981). There was no "half of site" effect with these inhibitors in contrast to the results with lysine-13 arylazidocytochrome *c* (Bisson et al., 1980). Results presented here show that there was no half of site effect when lysine-13 arylazidocytochrome *c* is bound to monomeric shark cytochrome *c* oxidase.

Our results do not rule out a function for the cytochrome *c* oxidase dimer but limit any role to a control function and/or some as yet undefined role in coupling electron transfer with proton pumping.

Registry No. Cytochrome *c* oxidase, 9001-16-5.

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