The Functional and Physical Form of Mammalian Cytochrome c Oxidase Determined by Gel Filtration, Radiation Inactivation, and Sedimentation Equilibrium Analysis*

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When solubilized in laurylmaltoside, cytochrome oxidases from beef heart and rat liver mitochondria exist as monodisperse populations that are stable, highly active, and have apparent molecular weights of 300,000 to 350,000, as measured by gel filtration. To determine whether these are monomeric (2 heme A, 2 Cu) or dimeric forms of the enzyme, we performed radiation inactivation and sedimentation equilibrium analyses. From radiation inactivation experiments under two different sets of conditions, we obtained estimates for the functional molecular weight of beef heart cytochrome oxidase of 114,000 and 99,000, much less than a dimer and significantly smaller than a 200,000 molecular weight monomer containing one copy of each of the 12 subunits normally present in the complex. The same functional size is obtained for a rat liver oxidase preparation depleted of subunit III. The physical molecular weight of cytochrome oxidase was determined by sedimentation equilibrium measurements in solvents of different densities using mixtures of H2O and D218O. Estimates of $M_r = 194,000 \pm 9,000$ for the beef heart oxidase and $M_r = 152,000 \pm 6,000$ for the rat liver enzyme were obtained, consistent with the size predicted for monomers of their subunit composition. From these results we conclude that mammalian cytochrome oxidases from beef heart and rat liver exist in laurylmaltoside as monomers capable of high rates of electron transfer and normal substrate binding. Further, these functions appear to be associated with a subset of the peptides present in the monomer, mainly composed of subunits I and II.

Mammalian cytochrome oxidase (EC 1.9.3.1) is isolated from mitochondrial membranes as a complex containing two hemes, two coppers, and a variable number of polypeptides and phospholipids, depending on the source and preparation technique. The number of subunits required for catalytic activity and their specific roles in cytochrome c oxidation, electron transfer to oxygen, and the controversial proton-pumping function (Wikstrom and Penttila, 1982; Mitchell and Moyle, 1983) of the enzyme are not known. Solubilization of the membrane and purification of the enzyme have profound effects on the kinetic parameters of cytochrome oxidase, making it difficult to establish criteria to define the "native state" (Thompson et al., 1982). Physical studies on the purified enzyme in various detergents (Robinson and Capaldi, 1977; Georgevich et al., 1983) and on membranous crystalline forms (Henderson et al., 1977) indicate that the protein can exist as a dimeric complex (four hemes, four copers, and two copies of each of 7 to 12 polypeptides). It remains unclear whether the dimeric form is necessary for any function or whether it is an artifact of purification reflecting the physical properties of the detergents used for dispersion or a tendency of oxidase to aggregate with other electron transfer chain components in the native membrane (Hochman et al., 1982). Further confusion arises because of the variety of molecular weights reported for forms of the enzyme designated as monomers and the relatively drastic conditions required to produce them (Love et al., 1970; Saraste et al., 1981; Georgevich et al., 1983; Nalecz et al., 1983).

Laurylmaltoside has been used for the study of cytochrome oxidase because of its ability to maintain the enzyme in a homogeneously dispersed active form capable of electron transfer rates that approach those of the physiological state (Thompson et al., 1982). Gel filtration of beef heart and rat liver oxidase in the presence of laurylmaltoside results in elution of a single monodisperse species with an apparent molecular weight of 300,000–350,000. In order to identify this species as a monomer ($M_r \sim 200,000$) or a dimer, an accurate determination of the amount of bound detergent is essential. Estimates obtained from UV absorption or radioactive labeling often are not sufficiently precise, and even when apparently satisfactory values can be obtained, the determination of molecular weight by gel filtration is unreliable because large asymmetric membrane proteins frequently behave anomalously (Le Maire et al., 1980; Nozaki et al., 1976).

To clarify the structural form of cytochrome oxidase responsible for the high rates of electron transfer observed in laurylmaltoside, we have analyzed the protein-detergent complex of cytochrome oxidase from beef heart and rat liver by sedimentation equilibrium and radiation inactivation tech-

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1 The molecular weights of the 12 subunits calculated from the amino acid sequence of beef heart cytochrome oxidase (Bush et al., 1982) are: Subunit I, 56,593; Subunit II, 26,049; Subunit III, 29,918; Subunit IV, 17,153; Subunit V, 12,436; Subunit VIa, 10,670; Subunit VIb, 8,419; Subunit Vlc, 8,406; Subunit VII, 10,688; Subunit VIIIa, 5,541; Subunit VIIb, 4,982; Subunit VIIIc, 6,244.
EXPERIMENTAL PROCEDURES

Materials—Ascorbic acid (Mallinckrodt, Inc.), N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (Eastman), Tris (Calbiochem, Ulltral, 2-NAD (Grade III, Sigma), ATP (Grade I, Sigma), asolectin (Associated Concentrates, Long Island, NY). D2O (Merck, Sharp and Dohme, Canada Limited), and D2O (Stohler Isotope Chemicals, Waltham, MA) were obtained from the indicated sources. Laurylmaltoside was synthesized according to Rosevear et al. (1980).

Cytochrome c (Sigma Type VI) was repurified according to Brautigan et al. (1979). Cholic acid (Sigma) was recrystallized 3 times from 10% ethanol. Rat brain hexokinase prepared as in Chou and Wilson (1983) was a generous gift of Dr. John Wilson. Rat liver cytochrome oxidase was prepared by the method of Thompson and Ferguson-Miller (1983).

Purification of Beef Heart Cytochrome Oxidase—This enzyme was purified by a modification of the method of Hartzell and Beinert (1974). Father particles (20 mg biuret protein/ml) in 2% cholate, 100 mM potassium phosphate, pH 7.5, are brought to 22% ammonium sulfate and centrifuged for 30 min at 40,000 × g. The supernatant is dialyzed against a 6-fold excess of 5 mM potassium phosphate, pH 7.4, for 1 h and allowed to stand on ice overnight and then centrifuged at 40,000 × g for 30 min. The pellet contains 90% of the oxidase activity. It is dispersed by homogenization in 1% histidine, 10 mM EDTA, 20 mM Tris, pH 7.4 (HEP buffer), diluted to 40 mg/ml, and made 10% in ethanol. After a hour of incubation on ice, the enzyme is removed from the alcohol solution by centrifugation for 30 min at 40,000 × g. The pellet is washed with remaining alcohol by resuspension in the same volume of HEP buffer and recentrifugation. The second pellet is homogenized and is diluted to 40 mg biuret protein/ml, and 1.5 mg of sodium cholate/mg protein are added. Solid ammonium sulfate is added to achieve 10% saturation. The mixture is stirred overnight and then centrifuged at 40,000 × g for 30 min. The supernatant contains 75% of the oxidase activity. It is subjected to ammonium sulfate cut at 25, 35, and 37% saturation. At 40–42% ammonium sulfate, oxidase is precipitated as an oily pellet, taken up in HEP buffer, and frozen in liquid nitrogen.

Assay Methods—Steady-state kinetic measurements of cytochrome oxidase were performed in 50 mM potassium phosphate, pH 6.5, 0.5 mM N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride, 2.5 mM ascorbate using laurylmaltoside in concentrations that support maximal activity, 1 mM for the beef heart and 0.25 mM for the rat liver enzyme, over a cytochrome c concentration range of 0.02–50 μM. The lipid-depleted rat liver oxidase (Thompson and Ferguson-Miller, 1985) was assayed in the presence of 0.25 mg/ml of asolectin vesicles to stabilize the enzyme when diluted in the assay mixture. Asolectin vesicles (40 mg/ml) were prepared in 2% cholate, 100 mM Hepes, pH 7.2, by sonication to clarity. Rates of oxygen consumption were measured polarographically as described by Ferguson-Miller et al. (1976, 1978). Turnover rates are expressed as moles of cytochrome c oxidized/mol of cytochrome oxidase/s. Rat brain hexokinase activity was measured using a coupled enzyme assay as described by Chou and Wilson (1972) with at least a 50-fold excess of the coupling enzyme, glucose-6-phosphate dehydrogenase.

Lipid Content of the Cytochrome Oxidase Preparations—Phospholipid analysis of purified cytochrome oxidase was performed as described by Thompson and Ferguson-Miller (1983). Total phosphorus content of the beef heart oxidase was 14 mol of phosphorus/mol of cytochrome oxidase. The rat liver enzyme preparation used has been shown to be very lipid depleted with 3 mol of phosphorous/mol of cytochrome aa3 (Thompson and Ferguson-Miller, 1983).

Get Filtration—Molecular sieve chromatography of purified beef heart cytochrome oxidase was performed on a column (2.6 × 47 cm) of Fractogel TSK HW-55 (S) with a 2.5-cm top layer of Sephadex G-25 (Pharmacia). The column was equilibrated in 10 mM Tris-C1, 1 mM KCl, 1 mM EDTA, 0.1% laurylmaltoside at pH 7.4, 7.8, or 8.4 in the same buffer, and at pH 7.9, containing 10 mM potassium borate for Tris-C1. In other experiments, the column was equilibrated in 10 mM Tris-C1, 50 mM KCl, 1 mM EDTA, pH 7.5, or in 50 mM potassium phosphate, pH 6.5. Beef heart oxidase (1.5 ml, 27 μM) was chromatographed at a flow rate of 90 ml/h. The rat liver enzyme (0.1 ml, 7 μM) was chromatographed on a column (0.75 × 43 cm) of Sephacryl S-300 (Pharmacia) equilibrated in 10 mM Tris-C1, 100 mM KCl, 1 mM EDTA, 0.04% laurylmaltoside, pH 8.0, at a flow rate of 5 ml/h.

Radiation Inactivation Analysis—Irradiation of enzymes with high-energy electrons results in primary ionization events within the protein structure which cause random destruction of covalent bonds. In general it appears that a single ionizing event produces damage sufficient to inactivate the peptide that is hit. If that peptide is essential for the activity of the enzyme, complete loss of enzyme function will be observed. The principles of target analysis predict that a biological activity, A, decays exponentially with increasing radiation dose D,

\[ A / A_0 = e^{-D / K} \]

where A0 is the native activity, A is the surviving activity after dose D, and K is proportional to the mass of the functional unit responsible for the biological activity. The value of K is equal to the reciprocal of D0, the dose at which 37% of the activity survives. Taking into account the energy involved in a primary ionization (66 eV) (Kemper and Schlegel, 1979), the following expression for the molecular weight can be derived when D0 is measured in rads, \( M_0 = 6.4 \times 10^3 / D_0 \). Secondary radiation effects, such as free radical formation in the solvent can produce more damage than expected from the direct interaction of the high-energy electron with the peptide, but these secondary effects can be minimized by irradiation at low temperatures. However, at low temperatures enzymes exhibit less sensitivity to radiation, as reflected by higher values of D0. Analysis of the temperature dependence of inactivation of a number of enzymes (Kemper and Haigler, 1982) reveals an exponential increase in D0 with decreasing temperature. From the relationship shown in Fig. 2 of Kemper and Haigler (1982), we determine the factors,

\[ D_{01} = 93 \text{°C} \]
\[ D_{02} = 30 \text{°C} \]
\[ D_{03} = 93 \text{°C} \]
\[ D_{04} = 30 \text{°C} \]

These factors were used to correct the data obtained by irradiation at low temperatures, in order to calculate the molecular weight of the functional unit of the enzyme.

Irradiation at −72 °C was performed using 2 MeV electrons produced by a van der Graaff accelerator (Dow Chemical Co., Midland, MI). Beef heart cytochrome oxidase was diluted to 18 μM in 10 mM Tris-C1, 100 mM KCl, 1 mM EDTA, pH 7.8, containing 0.3% methanol, 0.017% butylated hydroxytoluene, and 1.66% laurylmaltoside. Samples of 200 μl were frozen in open plastic vials and stored in liquid nitrogen. The temperature during irradiation was maintained by positioning the vials in a metal block submerged in a dry ice-methanol bath.

Irradiation at −135 °C was performed at the Armed Forces Radiobiology Research Institute, Bethesda, MD, using the experimental setup described by Kemper and Haigler (1982). The samples were prepared by placing a 200-μl aliquot in a glass ampule, freezing in liquid nitrogen, purging with nitrogen gas, and rapidly sealing with an oxygen torch. Beef heart oxidase was diluted to 30 μM in 10 mM Tris-C1, 100 mM KCl, 1 mM EDTA, pH 7.9, 1% sucrose, 0.02-50%, or in 50 mM potassium phosphate, pH 6.5. Beef heart oxidase (1.5 ml, 27 μM) was chromatographed at a flow rate of 90 ml/h. The rat liver enzyme (0.1 ml, 7 μM) was chromato-graphed on a column (0.75 × 43 cm) of Sephacryl S-300 (Pharmacia) equilibrated in 10 mM Tris-C1, 100 mM KCl, 1 mM EDTA, 0.04% laurylmaltoside, pH 8.0, at a flow rate of 5 ml/h.

Sedimentation Equilibrium—The sedimentation equilibrium distribution of a particle sedimenting at a given angular velocity depends on its mass, its volume in solution, and the density of the solution.
and can be described by the equation,

$$M^*(1 - \beta \rho_s) = \frac{2RT}{\omega^2} \left( \frac{d\ln C}{d\tau^2} \right)$$

(1)

where $M^*$ is the molecular weight of the sedimenting particle, $\beta$ is its partial specific volume, $\rho_s$ is the density of the solvent, $R$ is the gas constant, $T$ is the absolute temperature, $\omega$ is the angular velocity, $C$ is the concentration of the particle, and $r$ is the distance from the axis of rotation. When the sedimentating particle is a protein-detergent complex, the contributions of its individual components to the sedimentation equilibrium distribution can be separated (Reynolds and Tanford, 1976), and Equation 1 becomes,

$$M_p(1 - \beta \rho_s^p) + M_D(1 - \beta \rho_s^d) = \frac{2RT}{\omega^2} \left( \frac{d\ln C}{d\tau^2} \right)$$

(2)

where $M_p$ is the molecular weight and $\rho_s^p$ is the partial specific volume of the protein moiety, $M_D$ is the sum of the molecular weights of the detergent molecules associated with the protein, and $\rho_s^d$ is the partial specific volume of the associated detergent.

If the solvent has the same density as the detergent, the detergent no longer affects the sedimentation equilibrium of the protein. Thus, when $\rho_s = \rho_s^d = 1/\rho_D$, Equation 2 becomes

$$M_p(1 - \beta \rho_s^p) = \frac{2RT}{\omega^2} \left( \frac{d\ln C}{d\tau^2} \right)$$

(3)

Therefore, $M_p$ can be determined if the density of the detergent is known and if the sedimentation equilibrium distribution of the detergent-protein complex can be obtained for that density. Normally, this objective is achieved by measuring the sedimentation distribution in solutions of different densities using mixtures of H$_2$O and D$_2$O. Once the dependence of the distribution on density is determined, extrapolation to the density of the detergent (Tanford and Reynolds, 1976) yields a value of $M_p(1 - \beta \rho_s^p)$ from which the molecular weight of the protein moiety can be calculated. Because we found that laurylmaltoside has a relatively high density compared to other nonionic detergents, we increased the accuracy of the extrapolation by using D$_2$O to vary the solvent density.

A complementary way of analyzing the data obtained from sedimentation equilibrium measurements in solvents of different densities is that described by Edelstein and Schachman (1967). Their procedure provides additional information in that the partial specific volume and the molecular weight of the sedimenting particle and deuterium in the solvent. This exchange results in an increase in the molecular weight of the sedimenting particle and a decrease in its partial specific volume. Using $k$, the ratio of the molecular weight of the sedimenting particle at a density $\rho_s$ to the molecular weight in water, we can correct Equation 1 for proton-deuterium exchange in the following way (Edelstein and Schachman, 1967),

$$kM^*(1 - \beta \rho_s) = \frac{2RT}{\omega^2} \left( \frac{d\ln C}{d\tau^2} \right) = M^*(k - \beta \rho_s)$$

(4)

A value of $k = 1.0155$ has been determined for proteins in 100% D$_2$O by direct measurements of deuterium exchange and increase in molecular weight (Edelstein and Schachman, 1967). Since the exchange is relatively fast and directly proportional to the concentration of deuterium, $k$ values at other concentrations of deuterium can be calculated readily (e.g. $k = 1.0078$ at 50% D$_2$O). To correct for proton-deuterium exchange by laurylmaltoside, it was assumed that all the hydroxyl hydrogens exchange freely with the solvent, giving a $k$ value of 1.0137.

By solving simultaneous equations describing the sedimentation equilibrium distributions at two different densities (Equation 4) the partial specific volume and the molecular weight of the sedimenting particle can be calculated. We analyzed the data for 7 or more experiments at different densities to obtain average values for the partial specific volumes of the protein-detergent complexes and the detergent micelle; these average values were used to calculate the molecular weights.

**RESULTS**

**Gel Filtration Analysis**—Chromatography of purified beef heart cytochrome oxidase on Fractogel TSK-HW-55 (superfine) with a 2.5-cm top layer of Sephadex G-25 (Pharmacia). The column was equilibrated in 10 mM Tris-Cl, 100 mM KCl, 1 mM EDTA, 0.1% laurylmaltoside, pH 7.8. The vertical bar indicates the range of molecular weights obtained from the elution volume of the enzyme (7 experiments). The inset shows the elution profile of the cytochrome oxidase (1.5 ml, 27 μM) chromatographed at a flow rate of 90 ml/h. The column was calibrated with the following molecular weight standards: blue dextran, 2,000,000; ferritin, 440,000; 1-glutamic dehydrogenase, 320,000; catalase, 240,000; aldolase, 158,000; yeast alcohol dehydrogenase, 140,000; bovine serum albumin, 68,000; α-chymotrypsinogen, 25,000; cytochrome c, 12,500.
absorbing species with the same apparent molecular weight (data not shown). The ratio of 280- to 420-nm absorbance was constant throughout the peak, further indicating the presence of a single species. The absorbance at 280 nm revealed a small peak eluting after the enzyme which was identified as laurylmaltoside micelles by independent chromatography of the detergent alone. When gel filtration was performed at pH 7.4, 8.4, or 9.7 or at lower ionic strength (10 mM Tris-Cl, 50 mM KCl, 1 mM EDTA, pH 7.8), there was no change in the elution profile (data not shown). Under the conditions used for determining maximal turnover of the enzyme (50 mM potassium phosphate, pH 6.5) the enzyme also eluted as a homogeneous species with the same apparent molecular weight of 350,000. Some preparations of beef heart oxidase contain, in addition to a major peak at 350,000, minor amounts of larger and occasionally smaller species. In some cases these are active forms, corresponding to a dimer and a fragment of a monomer (Thompson et al., 1982), while in others the larger species are inactive polymers. Appearance of the latter seems to correlate with less pure enzyme, higher lipid content, and previous exposure to denaturing conditions (such as storage at 4°C). Beef heart cytochrome oxidase of high purity (≥9 nmol of heme a/mg of protein) and low lipid content (≤14 mol of lipid phosphorous/mol of aa) can be stored in liquid nitrogen for over 2 years and continue to exhibit monomeric behavior in laurylmaltoside.

Purified rat liver cytochrome oxidase chromatographed on Sephacryl S-300 gives an apparent molecular weight of 310,000 ± 30,000 (Fig. 2) with no correction made for the contribution of bound laurylmaltoside.

**Radiation Inactivation Analysis**—Irradiation of purified beef heart cytochrome c oxidase at −72 and −135°C with high energy electrons resulted in a decrease in activity of the enzyme as measured by oxygen consumption under optimal conditions for the determination of maximal turnover. As shown in Fig. 3, beef heart cytochrome c oxidase activity exhibits an exponential decay with increasing radiation dose at −135°C up to 96 megareads when the remaining activity is only 0.4%. The radiation inactivation of rat liver cytochrome oxidase is described by the same exponential decay as the beef heart enzyme (Fig. 3). After correction for the temperature of irradiation, we obtain a molecular weight estimate for the functional unit of the beef heart and the subunit III-depleted rat liver cytochrome oxidase of 114,000. At −72°C, the decay in activity with increasing dose is exponential as predicted from the principles of target analysis, but the sensitivity to radiation damage is higher (Fig. 3, inset). The molecular weight estimate for beef heart cytochrome oxidase from these data after correction for irradiation temperature is 99,000. Despite differences in the experimental setup and conditions, the estimates from the data at −72 and −135°C are in very good agreement.

Steady-state kinetic analysis of the irradiated samples was performed to analyze for possible radiation-induced alterations in the normal interactions of cytochrome c with oxidase. Fig. 4 shows a comparison of the Eadie-Hofstee plot for native rat liver cytochrome oxidase with a sample irradiated with 12 megareads. No major changes in the biphasic interactions of cytochrome c with irradiated enzyme were observed, indicating that the activity remaining is due only to fully active units.

As a separate control for the method, rat brain hexokinase was irradiated at −135°C under the same experimental conditions as cytochrome oxidase. Fig. 5 shows the decay in rat brain hexokinase activity with increasing dose. Each point represents the maximum velocity derived from a Lineweaver-Burk plot of the kinetic data for the sample. No changes in $K_m$ values for ATP or glucose were observed, even in highly irradiated samples. From these data we obtain an estimate of 106,000 for the molecular weight of the functional unit of rat brain hexokinase, which is in good agreement with the $M_1$ = 98,000 determined from sedimentation equilibrium analysis, sucrose gradient centrifugation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Chou and Wilson, 1972).

**Sedimentation Equilibrium Analysis in Solutions of Different Densities**—Fig. 6A shows the sedimentation equilibrium distribution of laurylmaltoside micelles at three different solvent densities. The concentration distribution of laurylmaltoside during centrifugation was monitored using the hydrophobic dye Cibacron blue F3GA (Pharmacia). We followed absorption at 326 nm, the wavelength at which the largest change in absorption occurs when the dye is incorporated into laurylmaltoside micelles. Incorporation of the dye at the low concentrations used (5 μM) does not appear to affect micelle composition since the critical micelle concentration is found to be the same when Cibacron blue F3GA or 2-β-toluidinyl-naphthalene 6-sulfonate (Chiang and Lukton, 1975) are used to measure micelle formation. To assure that the distribution observed in the ultracentrifuge cell corresponds to that of dye

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6 T. Tinetti and S. Ferguson-Miller, unpublished results.
incorporated into micelles, the ratio of dye molecules to laurylmaltoside micelles was lower than unity in all experiments.

All individual runs yielded linear plots of the logarithm of concentration against the square of radial displacement ($\ln C$ versus $r^2$) (Fig. 6A), indicating a single sedimenting species, as expected from gel filtration studies revealing a monodisperse population of laurylmaltoside micelles (Rosevear et al., 1980). The values of $rac{2RT}{\omega^2} \left( \frac{d\ln C}{dr^2} \right)$ for laurylmaltoside micelles at different densities are plotted against $\rho_r$ in Fig. 7. By extrapolation to the $x$ intercept and correction for protiodeuterium exchange, a value of 1.22 g/ml for the density of laurylmaltoside is obtained. Determination of the partial specific volume of the detergent using the method of Edelstein and Schachman (1967) gives the same value ($1/\rho_D = \rho_D = 1.22$ g/ml, average of 8 determinations). This value was used to calculate the molecular weight of the micelle at each solvent density, using Equation 4. The average of 13 determinations is 76,000 ± 3,000. The size of the micelle is larger than previously estimated by gel filtration ($M_c = 50,000$; Rosevear et al., 1980). The number of molecules in a laurylmaltoside micelle (149) is very similar to the number in a micelle of Triton X-100 (143) (Kushner and Hubbard, 1954).

Sedimentation equilibrium studies of beef heart and rat liver cytochrome oxidases were performed under the same ionic and detergent conditions used for determining the molecular weight by gel filtration. The plots of $\ln C$ versus $r^2$ were linear for all runs, as shown in Fig. 6B for three rat liver oxidase experiments and in Fig. 6C for three beef heart oxidase experiments, indicating the presence of homogenous cytochrome oxidase species. Measurements made before and after centrifugation revealed that oxidase activity was unaffected during the experiments.

Fig. 7 summarizes the results of sedimentation of beef heart and rat liver cytochrome oxidases in laurylmaltoside at different densities. The dotted line indicates the density of laurylmaltoside in $D_2^{18}O$, from which point the molecular weight of the protein moiety can be calculated directly using Equation 3. To do this it is necessary to know the partial specific volume of the protein. Using various published data on the amino acid composition of beef heart cytochrome oxidase, partial specific volumes are calculated by the method of Cohn and Edsall (1943). These range from 0.738 (using the data of Buse and Steffens (1976) corrected by inclusion of tryptophan as estimated by Kuboyama et al. (1972)) to 0.740 (using the amino acid composition of Kuboyama et al., 1972). An estimate of 0.739 was made from the data of Briggs (1977) as cited by Capaldi (1979). Robinson and Capaldi (1977) also quote a value of 0.743. These numbers do not differ greatly but result in molecular weight estimates that range from 150,000 to 161,000 for the rat liver oxidase and 191,000 to 206,000 for the beef heart enzyme, amounting to a <10% uncertainty. The molecular weight estimates for the protein moiety of beef heart and rat liver cytochrome oxidase shown in Table I were made using the average of the calculated

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**Fig. 3.** Inactivation of beef heart (●—●) and rat liver (■—■) cytochrome oxidase with high energy electrons. Irradiation was performed on frozen samples kept at −135 °C as described under "Experimental Procedures." Maximal activity was determined from polarographic measurements of oxygen consumption over a cytochrome c concentration range of 0.02-50 μM by extrapolation to the maximal turnover. For assay conditions see "Experimental Procedures." The line drawn represents data analyzed by the method of least squares constrained to 1.0 at zero dose. At zero dose, the maximal turnovers of the rat liver and beef heart enzymes were 300 s⁻¹ and 363 s⁻¹, respectively. The inset shows the inactivation of beef heart cytochrome oxidase by irradiation at −72 °C. The conditions for irradiation are described under "Experimental Procedures." This preparation had a maximal turnover of 635 s⁻¹ prior to irradiation.

**Fig. 4.** Eadie-Hofstee plots of the kinetics of oxidation of cytochrome c by rat liver cytochrome oxidase before (○—○) and after (■—■) a dose of 12 megarads at −135 °C. Activity was measured polarographically in 50 mM potassium phosphate, pH 6.5, as described under "Experimental Procedures." T/N, turnover.
partial specific volumes (0.739). An independent calculation of the \( \tilde{\rho} \) for the rat liver enzyme was not possible because the complete amino acid composition is not available. However, the error introduced by using the value obtained for the beef heart oxidase is unlikely to be significant because of the close homology between these two proteins (Grosskopf and Feldman, 1981; Anderson et al., 1982). To assess whether the absence of subunit III could have an effect on the partial specific volume, we calculated \( \tilde{\nu} \) from the data of Capaldi (1979) after subtracting the contribution of subunit III, assuming that peptides V, VIa, and VIc were also absent. We found the same \( \tilde{\nu} \) values as that calculated from the amino acid composition of the complete beef heart enzyme.

The influence of the prosthetic groups on the partial specific volume calculation must also be considered, since the method of Cohn and Edsall (1943) takes into account only the contribution of amino acids. In the case of two other heme-containing enzymes, cytochrome c with 1 heme/12,500 molecular weight and hemoglobin with 4 hemes/68,000 molecular weight, the partial specific volumes calculated from amino acid composition (cytochrome c, \( \tilde{\rho} = 0.736 \) (Margoliash and Schechter, 1966); hemoglobin, \( \tilde{\rho} = 0.742 \) (Tristan and Smith, 1983)) are in good agreement with empirically determined values (cytochrome c, \( \tilde{\nu} = 0.728 \) (Ehrenberg, 1957); hemoglobin, \( \tilde{\nu} = 0.749 \) (Lamm and Polson, 1936)). Therefore, it is unlikely that the two hemes and two copper atoms per 200,000 molecular weight in cytochrome oxidase will influence the partial specific volume to a significant extent.

The sedimentation data were further analyzed as described under "Experimental Procedures" to give the partial specific volumes and molecular weights of the entire protein-detergent complexes. These results are shown in Table I. The difference between the molecular weight of the complex and the protein moiety gives the amount of detergent bound. A significantly different amount of detergent is associated with the two enzymes. The bound detergent does not appear to correspond to a single micelle in either case, consistent with the findings of Le Maire et al. (1983) for other protein-detergent complexes.

**DISCUSSION**

**Gel Filtration**—Apparent molecular weights of 300,000 to 350,000 for rat liver and beef heart oxidases, respectively, were found by gel filtration. These values did not change over a pH range from 6.5–9.7, where monomer-dimer interconversions have been reported by others (Love et al., 1970; Georgevich et al., 1983). A molecular weight of this magnitude could be interpreted as that of a dimer if the detergent makes little or no contribution to the Stokes radius (Nalecz et al., 1983), or as that of a monomer, if the amount of bound detergent is sizable and makes a proportional contribution to the Stokes radius. Given the small apparent molecular weight of the laurylmaltoside micelle (Rosevear et al., 1980) and earlier low estimates (±125,000) of the molecular weight expected for a monomer (Downer et al., 1976; Wilson et al., 1980), it was initially proposed that the predominant enzyme species in laurylmaltoside was a dimer and that the dimer form might be important for the high electron transfer activity observed (Rosevear et al., 1986; Ferguson-Miller et al., 1982). Further studies were, therefore, undertaken to assess the functional significance of the dimer, utilizing the technique of radiation inactivation analysis.

**Radiation Inactivation**—When initially applied to cytochrome oxidase, the technique of radiation inactivation analysis gave estimates for the functional size of the enzyme which indicated that less than the complete set of subunits present in the complex was necessary for electron transfer activity (Kagawa, 1967; Thompson et al., 1982). In light of the controversy regarding the number and functional roles of the subunits in cytochrome oxidase (Kadenbach and Merle, 1981) and questions concerning theoretical aspects of the technique (Simon et al., 1982), further experimentation was required to clarify the significance of these findings. The accuracy of the molecular weight determinations was improved by using a wide range of dose and two experimental arrangements differing in particle energy, rate of energy deposition, geometry, and temperature. As a further control, the molecular weight of the single subunit enzyme, rat brain hexokinase, was determined, since it provides a less complex system than cytochrome oxidase with which to examine whether a single hit to any part of a polypeptide chain, even a very large one, results in loss of enzyme activity. The excellent agreement between the known (Chou and Wilson, 1972) and the measured molecular weight of hexokinase is a convincing validation of the method.

In the case of a multisubunit complex, the basic premise of target analysis predicts that the decay in activity with dose would be exponential and proportional to the sum of the molecular weights of the subunits that are required for the activity being measured. The electron transfer activity of both the beef heart and rat liver enzymes decayed from 100 to 0.5%...
of the original activity as a single exponential function of dose, giving molecular weight estimates of 99,000 and 114,000, in the two independently calibrated systems. Although higher than our previous estimate of 70,000 based on much fewer data, these values are consistent with the original conclusion (Thompson et al., 1982) that neither a dimer nor a complete set of subunits is necessary for high rates of electron transfer and normal interactions with cytochrome c. But this molecular weight estimate suggests that more than just two of the largest subunits, I and II (total molecular weight = 83,000) (Thompson et al., 1982), may be involved. Considering the possibility that subunit III might also be required, we irradiated a subunit III-depleted rat liver enzyme and found a molecular weight of 114,000, the same as that obtained for the beef heart oxidase that contains subunit III. The probable direction of any systematic experimental error in these estimates is toward a larger rather than a smaller molecular weight (Suarez and Ferguson-Miller, 1984), and, therefore, it can be concluded with some confidence that the electron transfer and cytochrome c binding functions of the oxidase are independent of subunit III and a number of the smaller subunits.

**Sedimentation Equilibrium**—Although radiation inactivation indicates that a dimer of oxidase is not required for the enzyme to catalyze high rates of electron transfer, it does not address the question of whether the physical form of the enzyme is, in fact, a dimer. Nor does it eliminate the possibility that the peptides making up the active unit are derived from two monomers in a functional dimer. However, analysis of the sedimentation equilibrium behavior of cytochrome oxidase in solutions of different densities revealed that the enzyme was present as a monomer. The sedimentation equilibrium plots (lnC versus r²) showed little evidence of heterogeneity, thus confirming the gel filtration results indicating monodisperse populations. The molecular weight obtained for the beef heart protein, 194,000, is in good agreement with the value of 200,000 predicted from the sequence of the 12 subunits present in the cytochrome oxidase complex (Base et al., 1982) and the value of 220,000 predicted from the same protein ratio of this preparation (Table II). Electrophoresis in a Kadenbach gel system (Merle and Kadenbach, 1980) revealed 12 subunits (Thompson, 1984).

The molecular weight estimated for rat liver cytochrome oxidase, 152,000, agrees with the 155,000 value predicted from the heme to protein ratio of this preparation and is less than that expected for a monomer with a complete set of subunits. These results are consistent with the absence of subunit III in the preparation, as revealed by polyacrylamide gel electrophoresis and N,N'-dicyclohexylcarbodiimide-binding experiments (Thompson and Ferguson-Miller, 1983), as well as the absence of peptides V, VIa, and Vlc (Thompson, 1984).

In essence, the method used to obtain these molecular weights is a combination of the analysis developed by Edelstein and Schachman (1967) and Tanford and Reynolds (1976) for sedimentation equilibrium in solutions of different densities. The use of D₂O solutions permitted a more accurate estimate of the density of the detergent and the protein molecular weight. The major advantage of the method is that no correction for detergent binding is required, in contrast to classical sedimentation equilibrium analysis where the accuracy of the estimate of protein molecular weight is dependent on the determination of the amount of detergent bound, which entails rigorous experimental procedures to obtain reliable estimates (Hackenberg and Klingenberg, 1980).

To calculate the molecular weight of the protein-laurymaltoside complexes, partial specific volumes were determined directly from sedimentation data using the method of Edelstein and Schachman (1967). The accuracy of this technique is demonstrated in the thorough studies of Hackenberg and
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Klingenberg (1980) on the ADP/ATP carrier protein, which show excellent agreement between the \( \bar{v} \) determined using this method and the \( \bar{v} \) calculated from direct measurements of the contribution of each of the components to the complex. Our molecular weight estimates for the complexes are lower than those obtained from gel filtration data (Table 1), consistent with the findings of Le Maire et al. (1980) that gel filtration can give overestimates of the molecular weight of protein-detergent complexes. The difference between the molecular weight of the complex and that of the protein moiety provides an estimate of the amount of associated detergent. The amount of detergent

\[
\rho = \frac{\bar{v} \cdot M_c}{M_p},
\]

where \( \bar{v} \) is the partial specific volume of the protein and \( M_c \) and \( M_p \) are the molecular weights of the complex and the protein, respectively.

The amount of associated detergent can be determined experimentally using the method described by Edelstein and Schachman (1967). The partial specific volumes of the laurylmaltoside-protein complexes were determined experimentally using the method described by Edelstein and Schachman (1967): \( \bar{v} = 0.739 \) (rat liver complex); \( \bar{v} = 0.770 \) (beef heart complex).

**Table 1**

| Protein moiety | Protein-detergent complex | Bound detergent |
|----------------|---------------------------|----------------|
| **Beef heart cytochrome oxidase** (9 nmol heme \( \alpha \)/mg protein) | 194,000 ± 9,000 | 300,000 ± 11,000 | 106,000 ± 20,000 |
| **Rat liver cytochrome oxidase** (12.5 nmol heme \( \alpha \)/mg protein) | 152,000 ± 6,000 | 210,000 ± 11,000 | 58,000 ± 17,000 |

**Figure 7.** Plot of \( \frac{2RT}{\omega^2} \) versus solvent density for beef heart cytochrome oxidase (O), rat liver cytochrome oxidase (■), and laurylmaltoside micelles ( ). The dotted line indicates the density of laurylmaltoside. The sedimentation of laurylmaltoside was performed as described in Fig. 6 except for the experiments at densities 1.052 g/ml and 1.089 g/ml where D2O was used instead of D2H2O. The density of D2H2O at 20 °C (1.215 g/ml) was determined using the method described by Edelstein and Schachman (1967). The conditions for the rat liver and beef heart cytochrome oxidase sedimentation experiments are given in the legend of Fig. 6, except that in the experiments indicated by , the concentration of beef heart cytochrome oxidase was 1.21 mM.

**Conclusions**—The studies described in this paper show that beef heart and subunit III-depleted rat liver cytochrome oxidase can exist as homogeneous monomeric complexes that are stable, catalyze high rates of electron transfer, and exhibit both high- and low-affinity interactions with cytochrome c, as determined by polarographic kinetics. Active monomeric forms of cytochrome oxidase from various species have been reported previously (Brunori et al., 1979; Darley-Umar et al., 1981), but the beef heart enzyme is usually found as a dimer, except when exposed to high pH and high detergent concentrations, conditions which also cause heterogeneity and loss of subunits (Love et al., 1970; Wilson and Greenwood, 1970; Wikstrom et al., 1981; Saraste et al., 1981; Georgevich et al., 1983). The ability of laurylmaltoside to stabilize an active monomeric form of beef heart oxidase under relatively mild conditions illustrates the unusual effectiveness of this detergent as a dispersing agent (Rosevear et al., 1980; Thompson and Ferguson-Miller, 1983). Nuclese et al. (1983) have observed a similar species of beef heart oxidase in laurylmaltoside under comparable conditions, with an apparent molecular weight from gel filtration analysis of 350,000. Although they interpret this to be a dimer, our sedimentation equilibrium studies indicate that an oxidase-lauylmaltoside complex of this apparent size corresponds to a monomer.

Radiation inactivation gives functional molecular weights for both beef heart and rat liver cytochrome oxidase that are smaller than their physical molecular weights, suggesting that the electron transfer and cytochrome c-binding activities of mammalian cytochrome oxidase are associated with subunits I and II, but not III. This conclusion is consistent with evidence that these two mitochondrially coded subunits contain the heme and copper groups involved in the oxidation-reduction reactions of the enzyme (Tanaka et al., 1975; Steffens and Buse, 1979; Winter et al., 1980) and with studies showing that cytochrome c can be covalently cross-linked to subunit II under conditions favoring active complex formation (Briggs and Capaldi, 1977; Millett et al., 1982, 1983). Although subunit III is not required for rapid electron transfer or respiratory control (Thompson and Ferguson-Miller, 1983), it may be important for coupling of electron transfer to proton...
extraction in the eukaryotic enzyme. However, several bacterial cytochrome oxidases have been isolated which contain only two subunits; the peptides are immunologically cross-reactive with mammalian subunits I and II (Ludwig, 1980), yet these enzymes are apparently competent in proton extrusion (Solioz et al., 1982; Yoshida et al., 1984). The reason for the increase in structural complexity in cytochrome c oxidase during evolution from two subunits in bacteria, to 7–12 in mammalian sources (Ludwig et al., 1979; Merle and Kadenbach, 1980) and possibly to tissue-specific subunit composition (Kadenbach et al., 1985), remains unclear. Since the mechanism of electron transfer appears to be highly conserved, the added structural features may play a role in nuclear or cellular regulation of mitochondrial energy production.

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REFERENCES

Andersen, S., de Brujin, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, J. G. (1982) J. Mol. Biol. 158, 683-717
Brewster, D. D., and Ferguson-Miller, S., and Margoliash, E. (1979) Methods Enzymol. 53, 128-164
Briggs, M. M. (1977) Ph.D. thesis, University of Oregon
Briggs, M. M., and Capaldi, R. A. (1977) Biochemistry 16, 73-77
Brunori, M., Colosimo, A., Rainoni, G., Milt, W. M., and Antonini, E. (1979) J. Biol. Chem. 254, 10768-10775
Buse, G., and Steffen, G. J. (1976) in Genetics and Biochemistry of Chloroplasts and Mitochondria (Bucher, T., Neupert, W., Sehgal, W., and Werner, S., eds) pp. 189-194, Elsevier/North-Holland Biomedical Press, Amsterdam
Buse, G., Steffen, G. G., Steffen, G. J., Meinecke, R., Bleidir, R., and Erdweg, M. (1982) in European Bioenergetics Conference Reports (LTBM-CNRS, ed) Vol. 2, pp. 163-164, Lyon
Capaldi, R. A. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R. A., ed) pp. 201-231, Marcel Dekker, Inc., New York
Capaldi, R. A. (1985) Biochim. Biophys. Acta 884, 291-306
Chang, H. C., and Lutkin, A. (1976) J. Phys. Chem. 79, 1935
Chou, A. C., and Wilson, J. E. (1972) Arch. Biochem. Biophys. 15, 48-55
Cohn, E. J., and Edsall, J. T. (1943) Proteins, Amino Acids and Peptides, pp. 375-377, Reinhold Publishing Corp., Washington, D. C.
Darley-Usmar, V., Allani, N., Ayashi, H., Jones, G. D., Sharpe, A., and Wilson, M. T. (1981) Biochem. Biophys. Res. Commun. 105, 443-452
Downer, N. W., Robinson, N. C., and Capaldi, R. A. (1976) Biochemistry 15, 2593-2596
Edelstein, S. J., and Schachman, H. K. (1967) J. Biol. Chem. 242, 306-311
Ehrenberg, A. (1967) Acta Chem. Scand. 11, 1257
Ferguson-Miller, S., Breuntgen, D. L., and Margoliash, E. (1978) J. Biol. Chem. 253, 149-159
Ferguson-Miller, S., Van Asten, T., and Rosevear, P. (1982) in Electron Transport and Oxygen Utilisation (Ho, C., ed) pp. 287-303, Elsevier/North-Holland, NY
Georgievich, G., Darley-Usmar, V. M., Malatesta, P., and Capaldi, R. A. (1983) Biochim. Biophys. Acta 722, 1317-1322
Grosskopf, K., and Feldman, H. (1981) Curr. Genet. 4, 151-158
Hackett, K. H., and Klingsberg, M. (1980) Biochemistry 19, 548-555
Hartwell, C. R., and Beinert, H. (1974) Biochim. Biophys. Acta 368, 318-338
Henderson, R., Capaldi, R. A., and Leigh, J. S. (1977).  J. Mol. Biol. 112, 631-648
Hochman, J., Schindler, M. L., Lee, J. G., and Ferguson-Miller, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 8888-8890
Kadenbach, B., and Merle, K. (1981) FEBS Lett. 135, 1-11
Kadenbach, B., Jarausch, J., Hartmann, R., and Merle, P. (1983) Anal. Biochem. 129, 517-521
Kawagoe, Y. (1987) Biochem. Biophys. Acta 131, 586-588
Kemper, E. S., and Haigler, H. T. (1982) J. Biol. Chem. 257, 12979-12999
Kemper, E. S., and Schacht, J. (1982) J. Biol. Chem. 257, 2-10
Kuboymas, M., Yong, F. C., and King, T. E. (1972) J. Biol. Chem. 247, 6375-6383
Laemmli, U. K., and Hubbard, W. D. (1984) J. Phys. Chem. 88, 1163-1167
Lamm, O., and Poison, A. (1986) Biochem. J. 20, 529-541
Le Maitre, M., Rivas, E., and Moller, J. V. (1980) Anal. Biochem. 106, 12-21
Le Maitre, M., Kwee, S. J., Anderson, P. M., and Moller, J. V. (1983) Eur. J. Biochem. 129, 525-535
Love, B., Chant, S. H. P., and Stotz, E. (1970) J. Biol. Chem. 245, 6664-6674
Ludwig, B. (1980) Biochim. Biophys. Acta 594, 177-189
Ludwig, B., Downer, N. W., and Capaldi, R. A. (1979) Biochemistry 18, 1401-1407
Margoliash, E., and Schechter, A. (1986) Adv. Protein Chem. 21, 113-115
Merle, P., and Kadenbach, B. (1980) Eur. J. Biochem. 106, 499-507
Millet, F., Darley-Usmar, V., and Capaldi, R. A. (1985) Biochemistry 24, 3857-3862
Millet, F., Dejong, K., Paulson, L., and Capaldi, R. A. (1980) Biochemistry 19, 254-255
Mitchell, P., and Moyle, J. (1983) FEBS Lett. 157, 167-178
Mitchell, P., Kollai, R., and Azai, A. (1983) Biochem. Biophys. Res. Commun. 114, 822-828
Nakay, Y., Scheckter, R. N., Reynolds, J. A., and Tanford, C. (1976) Biochemistry 15, 3884-3890
Reynolds, J. A., and Tanford, C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 4467-4474
Reynolds, J. A., and Capaldi, R. A. (1977) Biochemistry 16, 376-381
Rosevear, P., Van Asten, T., Baxter, J., and Ferguson-Miller, S. (1980) Biochemistry 19, 4105-4110
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Eur. J. Biochem. 110, 251-268
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56
Saraste, M., Penttila, T., and Wikstrom, M. (1981) Biochim. Biophys. Acta 668, 45-56