Detergent-Solubilized Monomeric and Dimeric Cytochrome bc_1 Isolated from Bovine Heart†

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ABSTRACT: Mitochondrial cytochrome bc_1 complex, isolated from frozen bovine heart, was solubilized with five different "non-denaturing" detergents: dodecyl maltoside, octaethylene glycol monododecyl ether ($C_{12}E_8$), Triton X-100, Tween 20, and sodium cholate. The hydrodynamic properties of the solubilized complex III's were then investigated by sedimentation analysis. Complex III exists as a stable and monodisperse dimer when it is solubilized in low ionic strength buffer with a low concentration of any of the five detergents. At pH 7.8, 20 °C, the protein sediments as a homogeneous species with an s_{obs} of about 14 S. The protein molecular weight of the 14S particle, after correction for bound detergent, is 465 000 ± 30 000 as measured by sedimentation equilibrium analysis. The aggregation state and/or homogeneity of cytochrome bc_1 is strongly dependent upon the concentration of the solubilizing detergent and ionic strength. The enzyme becomes a homogeneous, monomeric complex with a protein molecular weight of 235 000 ± 20 000 and an s_{obs} of 10–10.5 S after it is solubilized in high concentrations of Tween 20 (more than 5 mg/mg of protein) and sodium chloride (more than 0.5 M). However, a heterogeneous mixture of subcomplexes is produced upon solubilization of the complex with high concentrations of the other detergents and 0.5 M NaCl. Monomerization of cytochrome bc_1 by Tween 20 and 0.5 M NaCl has no effect on either the spectral properties, the subunit composition, or the enzymatic activity and is reversible since the dimeric 14S particle is regenerated upon removal of the high concentration of salt. Pretreatment of complex III with antimycin A also stabilizes the enzyme and permits the generation of the homogeneous monomeric complex by the other detergents with high ionic strength; e.g., a homogeneous 10S particle with a protein molecular weight of 230 000 ± 20 000 is also generated with 10 mg of dodecyl maltoside/mg of protein and 1 M sodium chloride if the complex is first inhibited by antimycin A.

Ubiquinol–cytochrome *c* oxidoreductase (EC 1.10.2.2, also named bc_1 complex or complex III) is one of the multisubunit, electron transport complexes of the inner mitochondrial membrane. The general function of cytochrome bc_1 is to catalyze the transfer of electrons from ubiquinol to cytochrome *c* and to couple this electron transfer to the translocation of protons across the membrane (Rieske, 1976; Trumpower, 1990). Ubiquinol–cytochrome *c* reductase of bovine heart mitochondria consists of 11 nonidentical subunits as determined by gel electrophoresis in sodium dodecyl sulfate (Schägger et al., 1986). Three subunits, subunits III, IV, and V, contain all of the functional prosthetic groups: cytochrome *b*, cytochrome c_1, and the high-potential iron–sulfur center, respectively.

The amino acid sequence and, therefore, the protein molecular weight of each subunit of beef heart cytochrome bc_1 complex have been obtained either directly by protein sequencing (Wakabayashi et al., 1982a,b, 1985; Schägger et al., 1983, 1987; Borchart et al., 1985, 1986) or indirectly from DNA gene sequences (Anderson et al., 1982; Gencic et al., 1991). The molecular mass of subunit XI has been measured by fast atom bombardment mass spectrometry (Terzi et al., 1991), and molecular masses of all of the subunits except cytochrome *b* (subunit III) have recently been determined by electrospray ionization mass spectrometry (ESI/MS) (Musatov & Robinson, 1994). The mass spectrometry masses of five subunits (II, VI, VIII, IX, and XI) were in excellent agreement with the masses predicted from the sequences, i.e., within ±2 mass units per 10 kDa; however, none of the other five subunits (I, IV, V, VII, and X) had molecular masses that agreed with the published sequence data. The molecular mass of subunit I was the most different, with a value of 49 236 Da, rather than 35 833 Da as is calculated from the reported DNA sequence (Gencic et al., 1991). Using these corrected molecular masses, the minimum molecular weight of the mitochondrial complex, containing 1 copy of each of the 11 subunits, would be 243 006–243 154, depending upon which of the two isoforms of subunits V and X are present (Musatov & Robinson, 1994).

However, when the mitochondrial complex is dissolved in the nonionic detergent Triton X-100, at neutral pH, a protein molecular weight that is about twice this value is obtained by sedimentation or gel filtration methods, i.e., the native complex is dimeric (von Jagow et al., 1977; Weiss & Kolb, 1979). Electron microscopy of two-dimensional membrane crystals and neutron scattering data also suggest a dimeric form for complex III in membrane environments (Perkins & Weiss, 1983; Leonard et al., 1981). On the basis of this structural evidence, recent functional models for the Q-cycle and for the "half of the sites reactivity" have evoked the dimeric form of cytochrome bc_1 (Mitchell & Moyle, 1985; de Vries, 1986; Schmitt & Trumpower, 1990).

Here we report isolation of moderate to large amounts of active and pure dimeric complex III from frozen bovine heart that is structurally and functionally indistinguishable from preparations of the complex made from freshly prepared

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mitochondria. A new procedure is also described for the reversible generation of an active, monomeric complex from the dimeric form of cytochrome bc_1. Analytical ultracentrifuge experiments in a Beckman XL-A ultracentrifuge indicate that the aggregation state of cytochrome bc_1 is complex and depends not only upon the type of nondenaturing detergent, but also on the ionic strength and the presence or absence of electron transport inhibitors.

MATERIALS AND METHODS

Materials. Sodium deoxycholate and sodium cholate were purchased from Sigma Chemical Co. and were recrystallized before use. Dodecyl maltoside was obtained from Anatrace, Inc. Triton X-100 (specially purified) was obtained from Boehringer Mannheim GmbH; protein grade Tween 20 was from Calbiochem. Decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) and sodium borohydride were from Sigma Chemical Co. Electrophoresis grade acrylamide and bis(acrylamide) were purchased from Serva and Kodak Chemical Co., respectively, and were used without further purification. All other chemicals were ACS reagent grade.

Purification of the Cytochrome bc_1 Complex. Extraction of cytochrome bc_1 complex from Keilin–Hartree heart muscle particles [prepared from frozen hearts as described by Yonetani (1960)] was adapted from the method of Schägger et al. (1986) with the following minor modifications. Heart muscle particles were suspended at 26 mg of protein/mL in 20 mM Na-MOPS[1] buffer, pH 7.4, containing 1.75% (w/v) Triton X-100 and 0.60 M NaCl with the pH maintained at pH 7.4 by the addition of 1 M NaOH. The suspension was centrifuged for 75 min at 41 500 rpm in a 50.2 Ti rotor (156000g at r_{av}) in a Beckman L5-50B centrifuge, and the supernatant was carefully decanted and discarded. The pellet was solubilized by the addition of buffer containing 2% Triton X-100, 0.60 M NaCl, and 0.30 M sucrose. After incubation at 4 °C for 10 min, the suspension was centrifuged for 60 min at 34 000 rpm (105000g at r_{av}), and the dark red supernatant was removed, diluted with 0.25 volume of 10 mM Tris-HCl buffer at pH 8.0, and dialyzed overnight against 10 volumes of the same buffer. The resulting turbid suspension was centrifuged for 75 min at 41 500 rpm and the dark red pellet resuspended by homogenization in 20 mM Tris-Cl buffer, pH 8.0, containing 0.66 M sucrose and 0.001 M histidine (TSH buffer) with a glass/Teflon homogenizer. This resuspended product is equivalent to the precipitated S-1 fraction of Rieske (1967) and was further purified by ammonium acetate and ammonium sulfate precipitation from deoxycholate and cholate as described by Rieske (1967). After the final ammonium acetate precipitation, the floating layer of red protein was carefully removed, dissolved in the TSH buffer, dialyzed 2–3 h against TSH buffer, and frozen at −70 °C until it was used.

Quantitation of the Cytochrome bc_1 Complex Composition. Cytochrome c_1 concentrations were calculated from the difference spectrum of ascorbate-reduced minus oxidized forms, using an extinction coefficient of 17.5 mM^{-1} cm^{-1} for $A_{552.5}$–A_{540} (Yu et al., 1972). Cytochrome b concentrations were calculated from the resulting difference spectrum of the dithionite-reduced minus the ascorbate-reduced enzyme, using an extinction coefficient of 28.5 mM^{-1} cm^{-1} for A_{562}–A_{577} (Berden & Slater, 1970). Protein concentrations were measured by the Biuret method (Gornall et al., 1949). Ubiquinone was extracted and quantified as described by Kröger (1978). Total phospholipid was determined by the procedure of Chen et al. (1956) after digestion of either the entire protein–lipid complex or the chloroform/methanol-extracted phospholipids in perchloric acid according to Marinetti (1962).

Enzymatic Activity. The rate at which cytochrome c was enzymatically reduced by cytochrome bc_1 was measured spectrophotometrically at 550 nm (ϵ_{550} = 18.5 mM^{-1} cm^{-1}) at 25 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM EDTA/2 mM NaN_3 as described by Nellson and Gellerfors (1978) except that 60 μM decylubiquinol rather than duroquinol was used as the substrate.

Subunit Composition. C_{18} reverse phase HPLC (Musatov & Robinson, 1994) and two different sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) systems (Schägger & von Jagow, 1987; Robinson et al., 1980) were used for evaluation of the subunit composition of cytochrome bc_1. Densitometric scans of the Coomassie Blue stained SDS–PAGE gels were obtained by (1) capturing a digitized picture of the stained gel using a Philips monochrome CCD camera that was connected to an Apple Macintosh IIsi computer via a QuickCapture frame grabber interface computer board purchased from Data Translation, Inc., and (2) analysis of the captured video image to yield the appropriate densitometric scans using version 1.49 of the National Institutes of Health image software run on a Macintosh IIsi computer. To confirm that none of the 11 subunits of the cytochrome bc_1 complex had dissociated during monomerization, the subunit composition of monomeric enzyme was determined by both SDS–PAGE and C_{18} reverse phase HPLC after reisolation of the monomeric complex as a red pellet by centrifugation for 2 h at 199000g in a Beckman Airfuge.

Analytical Ultracentrifugation. The sedimentation coefficient and protein molecular weight of the detergent-solubilized complexes were determined by sedimentation velocity and sedimentation equilibrium in a Beckman Optima XL-A analytical ultracentrifuge. Sedimentation coefficients at various positions across the sedimenting boundary were determined after correction for diffusion as described by van Holde and Weischet (1978). This data analysis was done using programs written by Dr. Borries Demeler at this institution for use with a PC microcomputer running Origin 2.94 under Microsoft Windows. Sedimentation equilibrium data were analyzed by multiple least-squares fitting of the absorbance vs R^2 data to a monomer–dimer model to yield the two values for $M_{pr}(1 - \varphi'\rho)$ and their absorbances at the meniscus (A_{men}). The protein molecular weight, M_{pr}, of the detergent-solubilized complex was then evaluated from M_{pr}-$(1 - \varphi'\rho)$ after correction for bound detergent and phospholipid as described by Tanford et al. (1974), i.e., $M_{pr}(1 - \phi'\rho) = M_{pr}[(1 - \bar{v}_{pr}\rho) + \delta_{det}(1 - \bar{v}_{det}\rho) + \delta_{PL}(1 - \bar{v}_{PL}\rho)]$, where $\bar{v}_{pr}$, $\bar{v}_{det}$, and $\bar{v}_{PL}$ are the partial specific volumes of protein, detergent, and phospholipid, respectively, δ_{det} and δ_{PL} are the milligrams of bound detergent and bound phospholipid per milligram of protein, and ρ is the density of solvent. The advantage of this type of analysis is that the molecular weight of the unhydrated protein complex is directly measured without a contribution from either the bound detergent or the bound phospholipid. The percentage of monomer and dimer was determined from the total integrated absorbance of each species within the cell which was evaluated from each $M_{pr}(1$

[1] Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; Na-MOPS buffer, 3-(*N*-morpholino)propanesulfonic acid titrated to the appropriate pH with NaOH; Tris-HCl buffer, tris(hydroxymethyl)-aminomethane base tritrated to the appropriate pH with hydrochloric acid; EDTA, ethylenediaminetetraacetic acid; TSH buffer, 20 mM Tris-HCl buffer, pH 8.0, containing 0.66 M sucrose and 0.001 M histidine.

$-\varphi'\rho$) and A_{men} pair by integration of the sedimentation equilibrium equation between the meniscus and the bottom of the cell. In this analysis, a protein partial specific volume of 0.737 cm^3/g (von Jagow et al., 1977) was used for the calculation of protein molecular weight and for correction of s_{obs} to $s_{20,w}$. The contribution of the bound phospholipid to the hydrodynamic mass, $M_{pr}(1-\varphi'\rho)$, is negligible since the partial specific volumes of phospholipids are between 0.965 and 1.015 cm^3/g (Steele et al., 1978), making $\delta_{PL}(1-\bar{v}_{PL}\rho)$ very close to zero. The magnitude of the correction for the contribution of bound detergent varied depending upon the detergent that was used. In $C_{12}E_8$, the correction for bound detergent is also nearly zero. $C_{12}E_8$ has a partial specific volume of 0.973 cm^3/g (Steele et al., 1978); therefore, $\delta_{det}(1-\bar{v}_{det}\rho)$ is small relative to $1-\bar{v}_{pr}\rho$ because δ_{det} is almost certainly less than 0.5 g/g. This makes $M_{pr}(1-\varphi'\rho)$ approximately equal to $M_{pr}(1-\bar{v}_{pr}\rho)$ for the $C_{12}E_8$-solubilized protein–phospholipid complex. Completely neglecting the contribution of bound $C_{12}E_8$ would result in approximately a 5% error in M_{pr}. With dodecyl maltoside, Tween 20, and Triton X-100, the analysis is slightly more involved. The partial specific volumes are 0.83, 0.869, and 0.908 cm^3/g, respectively; therefore, bound detergent does contribute to $M_{pr}(1-\varphi'\rho)$. For these detergents, δ_{det} was assumed to be 0.3–0.5 g/g for dodecyl maltoside and 0.5–0.7 g/g for Tween 20 or Triton X-100,[2] i.e., $\delta_{det}(1-\bar{v}_{det}\rho)$ is 0.05–0.06, 0.07–0.09, and 0.05–0.08, respectively. On the basis of these assumptions, $1-\varphi'\rho$ would be 0.33 ± 0.03 in each of the three detergents, a value that should introduce no more than a 10% error in the evaluation of the protein molecular weight. An error of this magnitude certainly is not enough to hinder the identification of a monomeric or dimeric complex.

All analytical ultracentrifugation experiments were done in the same buffer, i.e., 20 mM Tris-HCl/1 mM EDTA, pH 7.8. Before each analytical ultracentrifuge analysis, the cytochrome bc_1 complex was dialyzed several hours against this standard buffer which contained a low concentration of the appropriate detergent, e.g., 1 mg of detergent/mg of protein. Salt and any additional amount of detergent were added 1 h before centrifugation. Detergents were employed throughout the analysis at concentrations above their critical micellar concentrations in order to maintain the protein in a soluble state.

RESULTS

Cytochrome bc_1 complex isolated from Keilin–Hartree particles was as active and pure as complex that has been isolated from freshly prepared mitochondria. In a typical preparation, 500 g of frozen beef heart yields 70–100 mg of pure complex. The properties of this enzyme are very similar to those previously reported for enzyme that is isolated from freshly isolated mitochondria. The contents of cytochrome *b* and cytochrome c_1 in our preparations are 6.6 ± 0.4 nmol/mg of protein and 3.5 ± 0.3 nmol/mg of protein, respectively, and the complex contains the typical 11 subunits known to comprise the bovine heart enzyme (Figure 1 and Figure 7A). The complex also contains 200–300 nmol of phospholipid, contains less than 1 nmol of quinone/mg of protein, and has an enzymatic activity of 260–280 μmol of cyt *c* oxidized s^{-1} (μmol of cyt bc_1)$^{-1}$.

[2] This value is based upon the known binding of two of these detergents to cytochrome *c* oxidase, an analogous multisubunit, transmembrane, inner membrane, electron transport complex. For cytochrome *c* oxidase, δ_{det} = 0.35 g of detergent/g of protein for dodecyl maltoside and δ_{det} = 0.50–0.67 g of detergent/g of protein for Triton X-100.

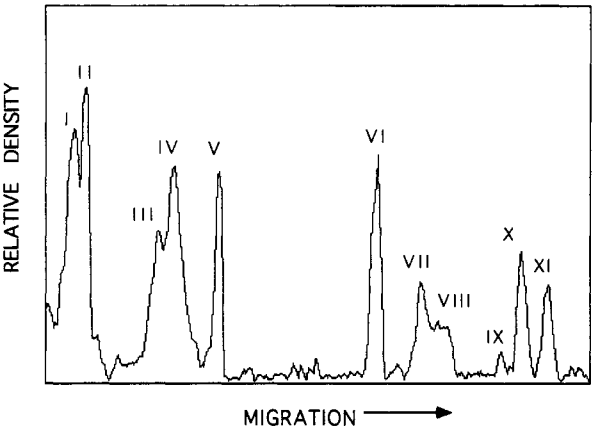

FIGURE 1: Separation of the subunits of purified cytochrome bc_1 complex by SDS–PAGE. Approximately 25 μg of protein was loaded and separated on a 14.5 cm long slab gel using the Tricine buffer system of Schägger and von Jagow (1987).

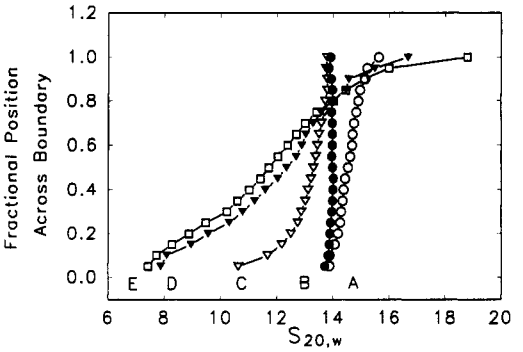

FIGURE 2: Dependence of the sedimentation coefficients on the concentration of $C_{12}E_8$. Each line represents the distribution of sedimentation coefficients at fractional positions across the sedimenting boundary; i.e., sedimentation coefficients at 0.25, 0.5, and 0.75 would be obtained by analyzing the data with an absorbance that is one-fourth, half, and three fourths of the absorbance observed in the plateau region of the sedimenting material. Cytochrome bc_1 complex (0.5 mg/mL) was solubilized in 20 mM Tris-HCl buffer at pH 7.8, containing 1 mM EDTA, 0.1 M NaCl, and (A) (○) 1.0 mg of $C_{12}E_8$/mg of protein, (B) (●) 2.5 mg/mg of protein, (C) (△) 5.0 mg/mg of protein, (D) (▲) 7.5 mg/mg of protein, and (E) (□) 10.0 mg/mg of protein. Primary data scans of the 416 nm absorbance vs radial position were collected at 8 min intervals during centrifugation of the samples at 22 000 rpm and 20 °C. Data were analyzed by the van Holde–Weischet analysis procedure as described under Materials and Methods.

Dimeric Cytochrome bc_1. Preparations of cytochrome bc_1, in standard low ionic strength buffer (I = 0.02–0.10 M) containing relatively low concentrations of $C_{12}E_8$, sodium cholate, dodecyl maltoside, or Triton X-100, were judged to be homogeneous as analyzed by sedimentation velocity experiments. For example, enzyme that is solubilized in pH 7.8 buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 1.0–2.5 mg of $C_{12}E_8$/mg protein is homogeneous and has an $s_{20,w}$ of 14–14.5 S (Figure 2, curves A and B). Similar sedimentation coefficients are obtained when the enzyme is dissolved in the same buffer, but containing sodium cholate (2 mg/mg of protein), dodecyl maltoside (1 mg/mg of protein), or Triton X-100 (2.5 mg/mg of protein); $s_{20,w}$ values were 14.1, 14.6, and 14.8 S, respectively. These small variations in the sedimentation coefficients are probably due to small differences in the amount of bound detergent, the differential partial specific volumes of the four detergents, and the influence of bound detergent upon the size of the solubilized complex. The dependence of the hydrodynamic properties of complex III upon the detergent concentration was examined between

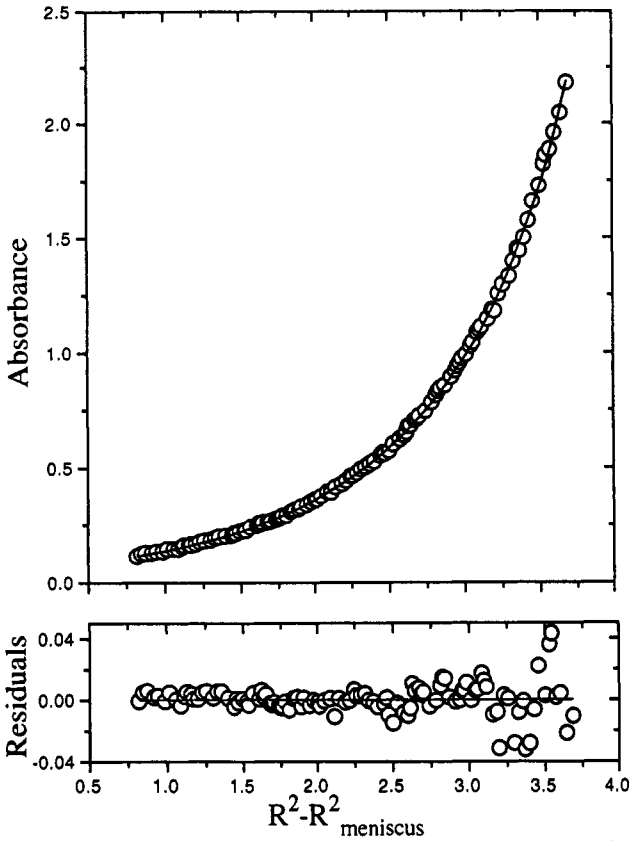

FIGURE 3: Sedimentation equilibrium analysis of cytochrome bc_1 complex solubilized in 20 mM Tris-HCl buffer at pH 7.8, containing 1 mM EDTA and 1.0 mg of $C_{12}E_8$/mg of protein. The sample (100 μL) contained 0.16 mg of protein/mL. The open circles are the experimental data, and the solid line is the least-squares fit to the data using an ideal, noninteracting monomer–dimer model. The lower graph is the distribution of residuals. The 416 nm absorbance data were taken after centrifugation for 24 h at 6000 rpm and 10 °C.

1 and 10 mg of detergent/mg of protein in buffer containing 0.1 M NaCl. Once again, the complex is homogeneous in $C_{12}E_8$ with the sedimentation coefficient essentially independent of the ratio of detergent to protein from 1.0 to 2.5 mg/mg (Figure 2, curves A and B). However, the complex becomes very heterogeneous at higher detergent to protein ratios (Figure 2, curves C, D, and E). Similar behavior is observed in the other detergents. Because each of the detergent-solubilized complexes is homogeneous when the protein to detergent ratio is less than 2.5, it is possible to determine the protein molecular weight and, therefore, the aggregation state of the complex in each detergent solution by equilibrium centrifugation. This analysis is simplest to analyze in $C_{12}E_8$ because the partial specific volumes of the bound $C_{12}E_8$ ($\bar{v}$ = 0.973 cm^3/g) and the bound phospholipids ($\bar{v}$ = 0.965–1.015 cm^3/g) are both close to the density of water and do not contribute significantly to the hydrodynamic mass (refer to Materials and Methods). Sedimentation equilibrium data for the complex that was solubilized in $C_{12}E_8$ were collected and fitted to a single or to a double monomer–dimer model (Figure 3). At least 90% of the enzyme is dimeric with a protein molecular weight of 465 000 ± 30 000, a value that is similar to that previously reported by von Jagow et al. (1977) and Weiss and Kolb (1979). It should be emphasized that this molecular weight is that of the assembled unhydrated heme containing protein subunits and does not include either the bound detergent or the phospholipids. This molecular weight is about twice that obtained from summation of the ESI/MS subunit masses, i.e., 243 006–243 154 (Musatov & Robinson, 1994), and

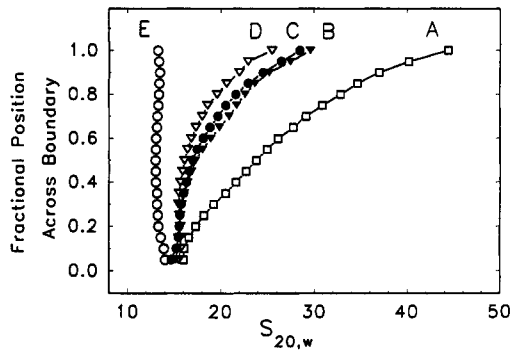

FIGURE 4: Distribution of sedimentation coefficients as a function of the concentration of Tween 20. Data analysis was the same as described in the legend to Figure 2. Cytochrome bc_1 complex (0.5 mg/mL) was solubilized in 20 mM Tris-Cl buffer at pH 7.8, containing 1 mM EDTA, 0.1 M NaCl, and (A) (□) 1.0 mg Tween 20/mg of protein, (B) (▲) 2.5 mg/mg of protein, (C) (●) 5.0 mg/mg of protein, (D) (△) 7.5 mg/mg of protein, and (E) (○) 10.0 mg/mg of protein. The rotor speed was 22 000 rpm at 20 °C. Data were collected at 416 nm.

indicates the $C_{12}E_8$-solubilized complex is dimeric. On the basis of the protein molecular weight and observed sedimentation coefficients in each detergent, our preparations of the beef heart cytochrome bc_1 complex are dimeric when solubilized in low ionic strength buffer, $I < 0.1$ M, containing low concentrations of any of these nondenaturing detergents. The Stokes radius of these detergent-solubilized dimers is 7.5–8.0 nm.

The sedimentation equilibrium experiments agree with the above sedimentation velocity experiments in that large increases in the concentration of detergent do not generate monomeric enzyme, but cause a general disruption of the structure of the protein complex. For example, complex that is analyzed by sedimentation equilibrium after solubilization in 10 mg of dodecyl maltoside/mg of protein and 0.1 M NaCl, i.e., corresponding to the sedimentation velocity experiment in Figure 2, curve E, is heterogeneous and consists of at least two components with molecular weights of 66 000 and 360 000.

Very different results are obtained for the complex when it is solubilized in the standard buffer containing 0.1 M NaCl and 1 mg of Tween 20/mg of protein (Figure 4, curve A). In Tween 20, also a nondenaturing detergent, the enzyme is extremely heterogeneous. The sedimentation coefficient, s_{obs}, varies between 15 and 45 S across the boundary, which indicates that a large amount of aggregation occurs in Tween 20 at low ionic strength. Increasing the ratio of Tween 20 to protein decreases the heterogeneity of cytochrome bc_1 (Figure 4, curves B, C, and D), and the protein becomes homogeneous with an s_{obs} of about 13.6 S in 10 mg of Tween 20/mg of protein (Figure 4, curve E), a value similar to that obtained for the dimeric enzyme in the other detergents.

Increasing the ionic strength has very little effect upon the aggregation state of the cytochrome bc_1 complex if the concentration of any of these detergents is kept below 2.5 mg/mg of protein. For example, the sedimentation coefficient is nearly independent of the concentration of NaCl between 0.1 and 1.0 M with 1 mg of $C_{12}E_8$/mg of protein; s_{obs} only decreased from 14 to 13 S as the concentration of NaCl was raised from 0.1 to 1.0 M, mostly due to an increased solvent density.

Formation of Monomeric Cytochrome bc_1. Although the cytochrome bc_1 complex is the most heterogeneous in Tween 20 solutions at relatively low ionic strength, a combination of a high ionic strength (1 M NaCl) and moderately large concentrations of Tween 20 (5–10 mg/mg of protein) leads

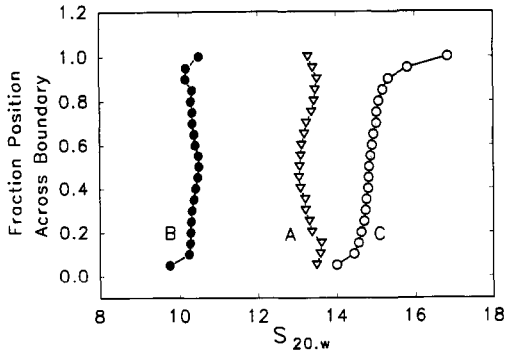

FIGURE 5: Sedimentation velocity analysis of monomeric (B) and dimeric (A, C) forms of cytochrome bc_1. The buffer in each experiment was 20 mM Tris-Cl buffer at pH 7.8 containing 1 mM EDTA. (A) (▲) Cytochrome bc_1 complex (0.16 mg/mL) with 0.1 M NaCl and 1.6 mg/mL Tween 20. (B) (●) Cytochrome bc_1 complex (0.16 mg/mL) with 1.0 M NaCl and 1.6 mg/mL Tween 20. (C) (○) The same sample as (B), but the concentration of NaCl was decreased by dialysis to 0.1 M NaCl prior to analysis by sedimentation velocity. The rotor speed was 22 000 rpm at 20 °C. Data were collected at 416 nm.

to a homogeneous complex with s_{obs} = 10 ± 0.5 S (Figure 5, curve B). Such a sedimentation coefficient is consistent with monomerization of the complex. The concentrations of NaCl, protein, and detergent, as well as the incubation time, all affect the monomerization by Tween 20. Incubation times longer than 4 h, or large detergent to protein ratios, do not produce monomeric cytochrome bc_1, but cause a general disruption of the complex; e.g., after incubation of 0.16 mg/mL cytochrome bc_1 with 10 mg/mL Tween 20 for 24 h at 4 °C, s_{obs} = 3 S.

The dissociation of dimeric cytochrome bc_1 into monomers by high salt and Tween 20 is fully reversible. When the [NaCl] is decreased by dialysis to 0.1 M, the sedimentation coefficient of the monomeric form of the enzyme returns to 14.6 S (Figure 5, curve C), a value similar to that originally obtained for the dimeric enzyme (Figure 5, curve A). The generation of homogeneous dimeric complex from the monomeric, Tween 20 solubilized enzyme is especially interesting since cytochrome bc_1 originally formed large aggregates in Tween 20 at low ionic strength.

To accurately determine whether the Tween 20, 10S particle is indeed the monomeric form of the enzyme, the protein molecular weight was measured by sedimentation equilibrium under these conditions. Once again, the equilibrium data only fitted well to a monomer–dimer model (Figure 6). Fitting to such a model suggests that the monomer and dimer are not interacting and are not in rapid equilibrium. Comparison of the integrated concentrations of monomer and dimer throughout the entire cell indicates that 81% of the complex is monomeric with a protein molecular weight of 235 000 ± 20 000 and 19% is dimeric with a molecular weight of 465 000 ± 30 000. At first, the appearance of any dimer form seems surprising since the complex was judged to be homogeneous by sedimentation velocity under these solution conditions. However, the detergent to protein ratio is not constant throughout the equilibrium cell. The value of $M_{pr}(1 - \bar{v}_{pr}\rho)$ is much larger than $M_{mic}(1 - \bar{v}_{mic}\rho)$, where M_{mic} and $\bar{v}_{mic}$ are the molecular weight and partial specific volume of the detergent micelle, respectively. Therefore, centrifugation redistributes the protein more than the detergent micelles. Although the sample originally had a detergent to protein ratio of 6, after 24 h at 6000 rpm the detergent to protein ratio

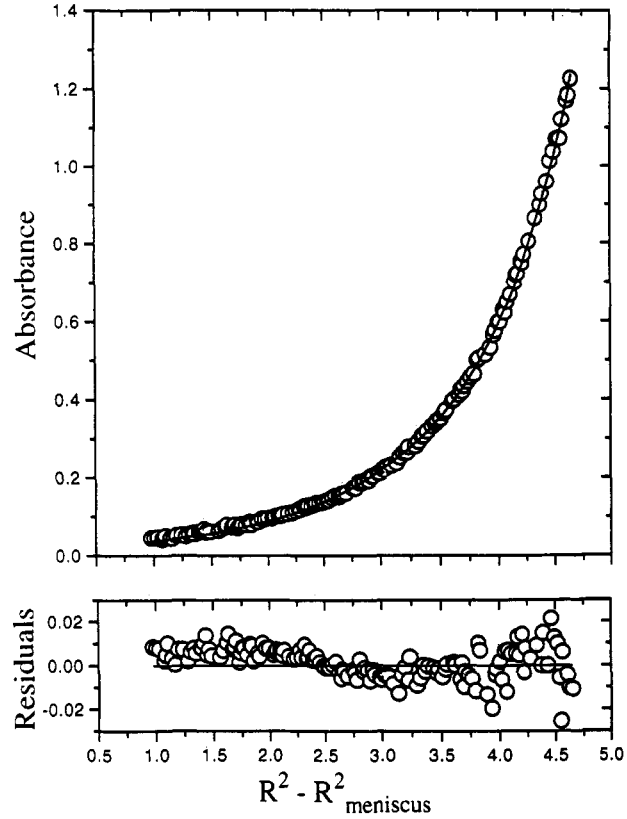

FIGURE 6: Sedimentation equilibrium analysis of monomeric cytochrome bc_1. The sample, 100 μL, was similar to that in Figure 5A; i.e., the enzyme, 0.16 mg/mL, was in 20 mM Tris-HCl buffer at pH 7.8, containing 1 mM EDTA, 1.0 M NaCl, and 0.96 mg/mL Tween 20. Data were collected after centrifugation for 24 h at 6000 rpm and 10 °C. In the upper graph, the open circles are the experimental 416 nm data; the solid line is the least-squares fit to the data using an ideal, noninteracting monomer–dimer model. In the lower graph is the distribution of residuals, i.e., the difference between the experimental data and the theoretical fit to the data.

at the meniscus would be 75 while at the cell bottom it would only be 0.60. Presumably, the dimeric complex is regenerated at the bottom of the cell because of the much lower relative concentration of detergent. Attempts to decrease the amount of dimeric enzyme by increasing the detergent concentration were unsuccessful because low molecular weight species were generated, probably due to the very high detergent to protein ratios in the top portion of the cell. In every sedimentation equilibrium experiment in which this unwanted degradation of monomeric cytochrome bc_1 did not occur, at least 15% of the complex was dimeric.

Formation of monomeric cytochrome bc_1 does not occur in high ionic strength buffer in any of the other nondenaturing detergents unless the complex is stabilized with antimycin A. As was the case with low ionic strength buffer, high concentrations of dodecyl maltoside, Triton X-100, or $C_{12}E_8$ in high ionic strength buffer cause unwanted degradation of the complex with the generation of low molecular weight species, probably due to dissociation of some subunits. These conditions generally result in an s_{obs} of about 6 S, when monitored at 416 nm, and are very heterogeneous when monitored at 280 nm. However, with the antimycin A inhibited complex, a homogeneous population with an s_{obs} of about 10 S is obtained in solutions containing 5 mg of dodecyl maltoside/mg of protein together with 0.5–1.0 M NaCl. Antimycin A must stabilize cytochrome bc_1 and prevent the dissociation of subunits that occur with the noninhibited enzyme. Sedimentation equilibrium analysis of the antimycin

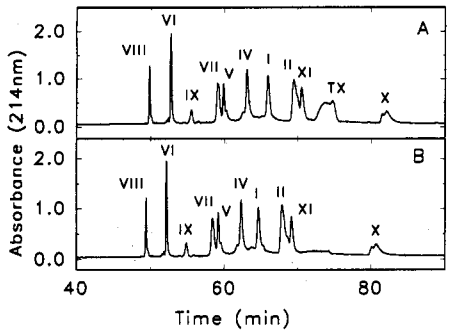

FIGURE 7: Reverse-phase HPLC determination of the subunit content of dimeric and monomeric cytochrome bc_1. All the HPLC experimental procedures, including elution gradients and the identification of the subunit eluting in each peak, were as described previously (Musatov & Robinson, 1994). Panel A: Analysis of dimeric cytochrome bc_1. Cytochrome bc_1 as isolated (0.7 mg of protein) was denatured in 0.2% TFA/water and injected onto a 5 μm, 300 Å pore size, Vydac C_{18} column (0.46 × 25 cm). The elution peak at about 70 min was due to a small amount of residual Triton X-100 that remained in the preparation from the isolation procedure. Panel B: Analysis of monomeric cytochrome bc_1. Enzyme (0.7 mg) was solubilized in 20 mM Tris-HCl buffer containing 1 mM EDTA, 7 mg of Tween 20, and 1 M NaCl. After reisolation of the monomeric complex as a red pellet by centrifugation as described under Materials and Methods, the complex was denatured in 0.2% TFA/water and analyzed identically to the dimeric enzyme in Panel A.

A inhibited complex in dodecyl maltoside gives results that are similar to what is obtained with the noninhibited complex in Tween 20; i.e., at least 60% of the complex is monomeric with a protein molecular weight of 235 000 ± 20 000, the remainder being dimeric with a molecular weight of 465 000 ± 30 000.

Spectral Analysis, Subunit Composition, and Enzymatic Activity of Monomeric Cytochrome bc_1. The spectral properties of enzyme that has been isolated from frozen heart tissue are nearly identical to those previously reported for the enzyme isolated from freshly prepared mitochondria. The completely oxidized form of the cytochrome bc_1 complex exhibits maxima at 415 nm and a broad band at 525 nm. The visible absorption spectrum of dimeric cytochrome bc_1 is also unaltered upon monomerization by Tween 20 (10 mg/mg with 1 M NaCl). Clearly, the heme prosthetic groups are not perturbed by the dissociation of the dimeric enzyme into monomers. The monomeric enzyme has exactly the same electron transport activity when it is directly diluted into the decylubiquinol assay mixture as the dimeric enzyme, 260–280 μmol of cyt *c* oxidized s^{-1} (μmol of cyt $bc_1)^{-1}$. The monomeric enzyme also exhibits the expected reaction of ferrocytochrome *b* with antimycin A, producing the typical inhibitor-induced red shift in the cytochrome *b* spectrum. The subunit composition of the Tween 20/1 M NaCl-treated enzyme is not affected by the monomerization process and is identical to the dimeric enzyme; i.e., it still contains the 11 subunits that are characteristic of the cytochrome bc_1 complex as determined by either C_{18} reverse phase HPLC (Figure 7) or SDS–PAGE (data not shown).

DISCUSSION

The mitochondrial cytochrome bc_1 complex is generally accepted to be a dimer of 400 000–500 000 daltons that contains 2 copies of each of the 11 subunits. This dimeric structural model is based upon (1) velocity and equilibrium sedimentation studies of a Triton X-100 solubilized bovine complex missing the iron–sulfur protein (von Jagow et al., 1977), (2) sedimentation velocity and gel filtration studies of Triton X-100 solubilized *Neurospora* cytochrome bc_1 (Weiss & Kolb, 1979), (3) the 3-D structure of the *Neurospora* complex by electron microscopy of 2-D crystalline sheets (Leonard et al., 1981), and (4) neutron scattering data of the *Neurospora* complex (Perkins & Weiss, 1983). In general, the present work agrees with the dimeric model, but the aggregation state and self-association of the bovine cytochrome bc_1 complex are more complicated than was previously believed. We find that cytochrome bc_1 is not always dimeric, but can be highly aggregated, dimeric, monomeric; or a heterogeneous mixture of subcomplexes with molecular weights smaller than monomers. The type of complex that is generated depends upon the type and concentration of the solubilizing detergent, the ionic strength, the presence or absence of electron transport inhibitors, and most probably the pH.

In low or high ionic strength buffer, containing low concentrations of most nondenaturing detergents, e.g., $C_{12}E_8$, sodium cholate, dodecyl maltoside, or Triton X-100, bovine cytochrome bc_1 is homogeneous and dimeric with a protein molecular weight of 465 000 ± 30 000 and a sedimentation coefficient of 14–15 S. These values are similar to those obtained by von Jagow et al. (1977) and de Vries (1986) for the Triton X-100 and dodecyl maltoside solubilized complexes, respectively. However, as pointed out by de Vries, the stability of dimeric cytochrome bc_1 in low ionic strength buffer containing dodecyl maltoside is inconsistent with the gel filtration results of Nalecz et al. (1985), which indicated that very low ionic strength favors monomerization of dodecyl maltoside solubilized cytochrome bc_1. Clearly, analytical ultracentrifugation experiments are much more reliable than either gel filtration or sucrose gradient methods for determining the aggregation state of detergent-solubilized membrane proteins.

High concentrations of these detergents, e.g., >5 mg/mg of protein, however, do disrupt the dimeric complex and produce a heterogeneous mixture of particles. This mixture may be a mixture of monomers and dimers, but is most likely a disrupted complex from which subunits have been dissociated. With each of these detergents, we were never successful in generating homogeneous monomeric cytochrome bc_1 unless the complex was first stabilized by inhibition with antimycin A, an inhibitor known to stabilize the cytochrome bc_1 complex (Rieske et al., 1967). After inhibition by antimycin A, high concentrations of these detergents no longer cause disruption of the complex and instead produce a significant amount of monomeric complex having a protein molecular weight of approximately 230 000.

In contrast to the detergents discussed above, Tween 20 acts quite differently. In low ionic strength buffer, low concentrations of Tween 20 (<2.5 mg/mg of protein) are ineffective at solubilizing cytochrome bc_1 and produce a highly heterogeneous mixture of complexes with sedimentation coefficients from 15 to 45 S. Only with high concentrations of detergent, e.g., >10 mg/mg of protein, does Tween 20 produce a homogeneous, dimeric complex of 14 S, i.e., the same species formed in low concentrations of the other detergents. As we suggested years ago (Robinson & Capaldi, 1977), Tween 20 is a very mild nonionic detergent that is quite ineffective at disrupting protein–protein interactions. In the case of cytochrome bc_1, almost no dissociation of subunits occurs when the dimers are solubilized by very high concentrations of Tween 20 in low ionic strength buffer. The unusual stability of cytochrome bc_1 in Tween 20 may explain the observation of Kubota et al. (1992) that Tween 20 is the

detergent of choice to improve the accuracy and reproducibility of the cytochrome bc_1 assay. Perhaps the dilution of cytochrome bc_1 into other detergents during preincubations and enzymatic assays may cause unwanted dissociation of subunits, something that does not occur in Tween 20.

The stability of cytochrome bc_1 complex in Tween 20 permitted us to probe the effects of high ionic strength upon the dissociation of the cytochrome bc_1 dimers. In contrast to the other detergents, very high ionic strength, e.g., 1 M NaCl, in the presence of high concentrations of Tween 20 reversibly dissociates dimeric cytochrome bc_1 into homogeneous and active monomers. Although active monomeric cytochrome bc_1 was recently isolated from the alga *Polytomella* spp. (Gutiérrez-Cirlos et al., 1994), active, monomeric, mitochondrial cytochrome bc_1 has not been isolated except by the low ionic strength prodedure of Nalecz et al. (1985), a result that appears to be inconsistent with both the present study and that of de Vries (1986). The active 10S monomeric cytochrome bc_1 that we prepared has a protein molecular weight of 235 000 ± 20 000, almost exactly half the molecular weight of the dimers and within experimental error of the value of 243 006–243 154 calculated from the data of Musatov and Robinson (1994). Because Tween 20 is much milder that the other detergents, monomeric cytochrome bc_1 could be easily produced without first stabilizing the complex by inhibition with antimycin A. As mentioned above, monomerization of cytochrome bc_1 is not caused by high ionic strength alone since the dimers that were generated in low concentrations of all of the other detergents were stable at these high ionic strengths. The reversibility of the high salt, Tween 20 monomerization process is unusual in that dimers are generally quite difficult to form from detergent-solubilized monomers. We had previously thought, based upon our studies on bovine cytochrome *c* oxidase, that the lack of reversibility of monomerization of membrane complexes was due to steric hindrance caused by the masking of the hydrophobic surfaces by bound detergent. Tween 20 may be unique in that it does not block these sites, and the finding suggests that we should test the reversibility of cytochrome *c* oxidase monomerization in Tween 20.

One of the more intriguing observations is the full electron transport activity of the monomeric, Tween 20 solubilized enzyme when it is diluted into the decylubiquinol assay. This result implies that the monomer is fully active, but it is, of course, impossible to rule out the possibility that the monomeric enzyme dimerizes upon dilution into the assay buffer. The decreased ionic strength when the enzyme is diluted in the assay mixture would certainly favor the reassociation of the monomers into dimers, but the large dilution and resultant decrease in enzyme concentration should have the opposite effect. If the monomeric complex is fully active, it poses interesting possibilities for testing the recent proposal that the dimeric form of cytochrome bc_1 is essential for coupled electron transport (de Vries, 1986; Schmitt & Trumpower, 1990).

The last major finding of the present study is the isolation of a fully active cytochrome bc_1 complex from frozen bovine heart that contains all 11 subunits. In contrast to cytochrome *c* oxidase and succinate:cytochrome *c* oxidoreductase (complex II–III), both of which are routinely isolated as highly pure and active enzymes from frozen bovine heart [e.g., see Mahapatro and Robinson (1990) and Yu and Yu (1980)], complex III is usually isolated only from freshly prepared mitochondria. In fact, Kubota et al. (1992) have stated that "the freshness of the beef heart seems critical for consistency of the preparation". Indeed, the isolation of the cytochrome bc_1 comples from frozen bovine heart is quite difficult and the use of published purification procedures, e.g., Rieske (1964), or Engel et al. (1980), was unsuccessful and resulted in final products with a low cytochrome *b* content and a low cytochrome *b* to cytochrome c_1 ratio. However, the quality of the extracted enzyme is significantly improved when Triton X-100 is used in the initial extraction of the enzyme from Keilin–Hartree particles, i.e., a procedure similar to that used by Schägger et al. (1986) for the extraction of the enzyme from freshly prepared mitochondria. Subsequent purification of complex III from deoxycholate and cholate by the method of Rieske (1967) then becomes routine and reproducible.

The composition and enzymatic activity of the enzyme isolated by this procedure are virtually identical to values that have been reported for complex III isolated from freshly prepared mitochondria. Interestingly, the contents of uniquinone and phospholipids in our preparations are intermediate between the values reported for cytochrome bc_1 isolated by the Schägger and Rieske methods. The unusually high yields of cytochrome bc_1 that are obtained from frozen heart, e.g., 70–100 mg/500 g of frozen heart, together with the newly available Beckman XL-A analytical ultracentrifuge have made the present structural analysis studies possible.

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