Detergent-solubilized *Escherichia coli* cytochrome bo_3 ubiquinol oxidase: a monomeric, not a dimeric complex

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Abstract The protein molecular weight, M_r, and hydrodynamic radius, R_s, of Triton X-100-solubilized *Escherichia coli* cytochrome bo_3 were evaluated by computer fitting of sedimentation velocity data with finite element solutions to the Lamm equation. Detergent-solubilized cytochrome bo_3 sediments as a homogeneous species with an $s_{20,w}$ of 6.75 s and a $D_{20,w}$ of 3.71×10^{-7} cm^2/s, corresponding to a R_s of 5.8 nm and a M_r of 144 000 ± 3500. The protein molecular weight agrees very well with the value of 143 929 calculated from the four known subunit sequences and the value of 143 025 measured by MALDI mass spectrometry for the histidine-tagged enzyme. We conclude that detergent-solubilized *E. coli* ubiquinol oxidase is a monomeric complex of the four known subunits.

Key words: Cytochrome *bo* ubiquinol oxidase; Sedimentation velocity; Sedimentation equilibrium; Computer analysis; *Escherichia coli*

1. Introduction

Cytochrome bo_3 is one of two quinol oxidases of the *Escherichia coli* aerobic respiratory chain. It is a member of the heme-copper oxidase family [1] and like all other members of this conserved family, catalyzes the four-electron reduction of O_2 to water and generates a proton motive force across the membrane [2–4]. It consists of four non-identical protein subunits with gene product molecular weights of 74 367, 34 911, 22 622 and 12 029 [5], corresponding to the molecular weight of 143 929 for the monomeric enzyme. The masses of the individual subunits have been confirmed by MALDI mass spectrometry, which yields values of 141 990 [6] and 143 025 [7] for the post-translationally processed and His-tagged complexes, respectively.

Cytochrome bo_3 may or may not dimerize, as do most of the mitochondrial electron transport complexes. Mitochondrial cytochrome bc_1 and cytochrome *c* oxidase, for example, crystallize as dimers [8–10] and each is thought to be dimeric within the mitochondrial inner membrane [11,12]. The functional consequences of dimerization are not well understood. The cytochrome bc_1 dimer is thought to be the functional unit [13,14] and both electron and ubiquinol transfer between cytochrome bc_1 monomers is postulated to be mechanistically important [8,9]. The functional importance of the cytochrome *c* oxidase dimeric state is less clear, but proton translocation activity of both the prokaryotic and eukaryotic enzymes is believed to require a dimeric complex [15–17].

The inherent tendency of the mitochondrial complexes to dimerize is usually evident after detergent solubilization [18–21]. For cytochrome bc_1, this is especially true [18], but cytochrome *c* oxidase can also be dimeric, particularly at low detergent concentrations [19,21]. As the detergent concentration is raised, cytochrome *c* oxidase usually becomes a mixture of monomers and dimers and ultimately mono-disperse monomers [19–21].

The oligomeric state of *E. coli* cytochrome bo_3, either in situ or as a detergent-solubilized complex, is much less clear than its mitochondrial counterparts. Like mitochondrial cytochrome *c* oxidase, it is dimeric in two-dimensional crystalline arrays [22] and has been reported to be a mixture of monomers and dimers after solubilization with octyl β-D-glucopyranoside [23]. In the present study, we have rigorously analyzed the oligomeric state of detergent-solubilized *E. coli* cytochrome bo_3 by fitting sedimentation data to the Lamm equation. We find that detergent-solubilized cytochrome bo_3 can be homogeneous and monomeric or highly aggregated, but there was no evidence that it is ever dimeric.

2. Materials and methods

Cytochrome bo_3 ubiquinol oxidase containing a His-tag on subunit II was isolated from *E. coli* in one step using Ni^{2+} affinity chromatography and was of the same purity and activity as described previously [24]. The last step of the purification in which the enzyme is dialyzed into sarcosyl to remove Triton X-100 was omitted so that the Triton X-100-solubilized protein could be analyzed by analytical ultracentrifugation.

Sedimentation velocity and equilibrium experiments were performed using a Beckman XL-A ultracentrifuge equipped with absorbance optics. Sedimentation velocity studies were done at 27 000 rpm, 20°C and analyzed using the van Holde-Weischet procedure to establish the detergent concentration that is required for protein homogeneity, i.e. nearly identical values for the sedimentation coefficient across the entire boundary [25]. These analyses were done using the UltraScan computer software developed by Borries Demeler. Use of this program has been described previously [18] and is available together with tutorial examples on the WWW server at http://biochem.uthscsa.edu/UltraScan/. Once homogeneity was established, the entire sedimentation velocity data set was analyzed by the finite element analysis procedure of Demeler and Saber [26] to give best-fit values for both the sedimentation coefficient and diffusion coefficient. Applications of this method for global fitting of velocity data are available in Robinson et al. [27] and tutorial examples are given at the above web site.

Sedimentation equilibrium experiments were done at 9000 rpm and 10°C. Equilibrium was reached within 24 h and verified by examining scans after 48 h. The UltraScan software of Borries Demeler was again used to analyze the sedimentation equilibrium data. Best-fits were obtained assuming a single homogeneous species.

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Fitting sedimentation velocity data to the Lamm equation or sedimentation equilibrium each evaluates the effective hydrodynamic mass, $M_{pr}(1-\varphi'\rho_0)$, where:

$$M_{pr}(1-\varphi'\rho_0) = M_{pr}(1-\overline{\upsilon}_{pr}\rho_0) + \nu_{det}M_{det}(1-\overline{\upsilon}_{det}\rho_0) + \nu_{PL}M_{PL}(1-\overline{\upsilon}_{PL}\rho_0) + ...$$

In other words, the dry protein molecular weight, M_{pr}, is obtained directly from $M_{pr}(1-\varphi'\rho_0)$, provided that the partial specific volumes of protein, $_{pr}$, detergent, $_{det}$ and phospholipid, $_{PL}$, are known together with the mol detergent and mol phospholipid bound per mol protein, i.e. ν_{det} and ν_{PL}, respectively. Refer to [18,27,28] for a discussion of the assumptions involved in calculation of M_{pr} from $M_{pr}(1-\varphi'\rho_0)$. The values used in this study were: $\overline{\upsilon}_{pr} = 0.740$ cm^3/g, which was calculated from the amino acid sequences of the four subunits, assuming one copy of each; $\overline{\upsilon}_{det} = 0.908$ cm^3/g for the partial specific volume of Triton X-100 and $\overline{\upsilon}_{PL} = 1.00$ cm^3/g for the partial specific volume of phospholipid (the contribution of PL to the effective mass was ignored since the partial specific volume of PL is close to unity).

The only parameter that is not known, but significantly affects evaluation of M_{pr}, is the detergent binding. Because of this uncertainty, the sedimentation velocity or equilibrium data were fitted without correction for binding, i.e. the partial specific volume was set to zero when analyzing data with the UltraScan program. This sets $(1-\varphi'\rho_0) = 1$ and generates best-fit values for s_{obs} and D_{obs}. The two coefficients are then used to evaluate the effective hydrodynamic mass, $M_{pr}(1-\varphi'\rho_0)$, and the hydrodynamic or Stokes' radius, R_s. $M_{pr}(1-\varphi'\rho_0)$ is then used to evaluate M_{pr} using the above equation and assuming reasonable values for the amount of bound detergent. We assumed that bound Triton X-100 could be as low as 135 or as high as 165 mol bound per mol protein, which would introduce an uncertainty in M_r of 2.5%. The lower value is slightly larger than the binding of Triton X-100 to cytochrome b_5 (120 mol Triton X-100 per mol protein [29]), a protein that contains a single membrane spanning helix. The larger value corresponds to the binding of Triton X-100 to bovine cytochrome *c* oxidase (170–175 mol Triton X-100 bound per mol oxidase monomer [19]), an enzyme that contains 28 transmembrane helices per monomer [10]. The amount of Triton X-100 bound to *E. coli* cytochrome bo_3 oxidase should be between these extremes since its mass is 2/3 that of the bovine enzyme. The resulting small error introduced into the value of M_{pr} from this uncertainty in bound detergent is inconsequent in terms of establishing whether the complex is monomeric or dimeric.

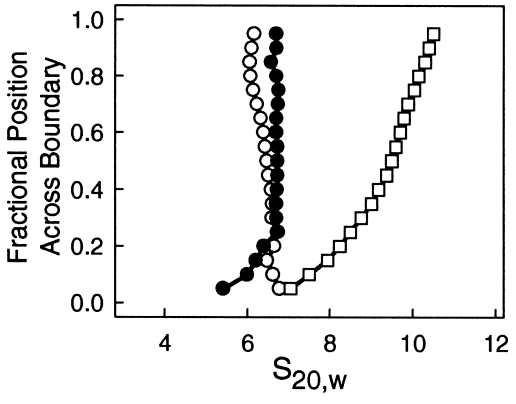

Fig. 1. Sedimentation velocity analysis of cytochrome bo_3 as analyzed by the van Holde-Weischet method to correct for diffusion. Each line represents the distribution of sedimentation coefficients at fractional positions across the sedimenting boundary. Cytochrome bo_3 (0.5 mg protein per ml) was solubilized in 50 mM potassium phosphate buffer, pH 8.1, containing: (a) 1.0 mg/ml Triton X-100 (open squares), (b) 5 mg/ml Triton X-100 (filled circles) or (c) 10 mg/ml Triton X-100 (open circles). Primary data scans of the absorbance at 408 nm versus the radial position were collected at 8 min intervals during centrifugation at 27 000 rpm at 20°C. Near identity of sedimentation coefficients across the boundary indicates sample homogeneity.

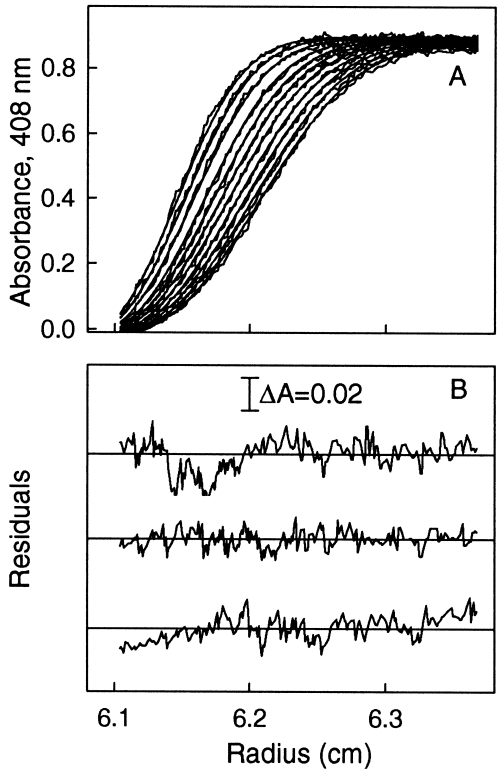

Fig. 2. Finite-element analysis fit of sedimentation and diffusion coefficients to cytochrome bo_3 sedimentation velocity data. Conditions were the same as described in the legend to Fig. 1 for cytochrome bo_3 solubilized in 5 mg/ml Triton X-100. The data at fractional positions between 0.20 and 0.85 were analyzed. A: Velocity data (jagged line) overlaid with finite-element solution for a single, ideal component system (smooth line). Scans were taken at 8 min intervals after reaching speed. Scans 5–16 are shown (the fourth scan was not scanned correctly and is not shown). B: Representative residual plots for the fitting by finite-element analysis to the data. Residuals are shown for scans 5, 11 and 16. Residuals for the other data scans were similar.

3. Results

Homogeneous, mono-disperse cytochrome bo_3 oxidase is essential before sedimentation velocity data can be fitted by finite element analysis to the Lamm equation. Not only must the preparation be pure, but also the concentration of detergent that is required to obtain and maintain mono-disperse enzyme must be determined. Van Holde-Weischet analysis of sedimentation velocity data is ideally suited to this purpose. Normally, sarcosyl (0.05 mg/mg protein) is exchanged for the Triton X-100 as the final step in purification of cytochrome bo_3 oxidase. However, enzyme preparations are a heterogeneous polydisperse mixture with sarcosyl as the solubilizing detergent (data not shown). This heterogeneity remains even if an excess of Triton X-100 is added to the sarcosyl-treated enzyme. Mono-disperse enzyme can be obtained if the sarcosyl exchange step is omitted. In pH 8.1 buffer containing either 5 or 10 mg Triton X-100 per mg cytochrome bo_3, homogeneity was achieved as is evident from the nearly constant values obtained for $s_{20,w}$ across the boundary (Fig. 1, filled and open circles). Decreasing the amount of Triton X-100 to 2 mg per mg protein or less significantly increased heterogeneity as is evident from the increase in $s_{20,w}$ across the boundary (Fig. 1, open squares).

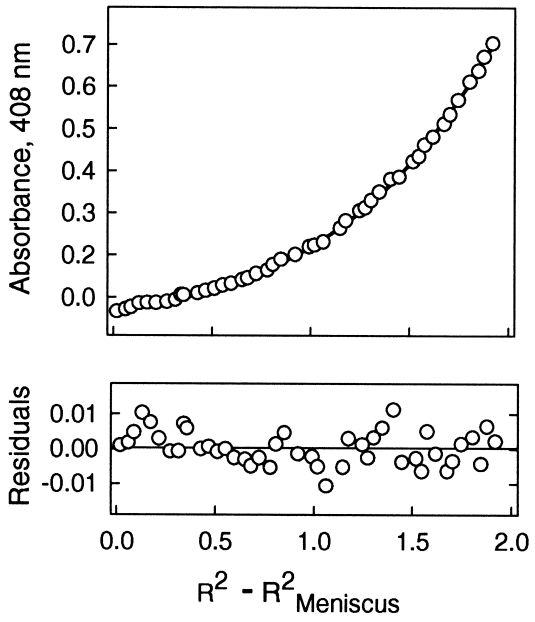

Fig. 3. Sedimentation equilibrium analysis of cytochrome bo_3. The sample (0.15 mg/ml protein) in 50 mM potassium phosphate buffer at pH 8.1 contained 0.75 mg/ml Triton X-100. The ratio of mg detergent to mg protein is the same as for the data fitted in Fig. 2, i.e. ratio of mg Triton X-100 to mg protein of five. Data were collected at 408 nm after centrifugation for 24 h at 9000 rpm and 10°C. Top panel: experimental data (open circles) and non-linear least-squares fit to an ideal, single component model (solid line). Bottom panel: distribution of residuals.

Once homogeneity is obtained, the same data can be fitted to the Lamm equation using finite element analysis to obtain best-fit values for s_{obs} and D_{obs} (refer to Section 2). High quality fits were obtained by this analysis procedure with nearly random residuals (Fig. 2). The fitted lines in Fig. 2 were generated from the best-fit values of 6.75 s and 3.71×10^{-7} cm^2/s for the observed sedimentation and diffusion coefficients, respectively. These two coefficients result in an effective hydrodynamic mass, $M_{pr}(1-\varphi'\rho_0)$ of 44 100, which can be used to calculate the protein molecular weight, provided that the stoichiometry of detergent binding to protein is known. We believe that a stoichiometry between 135 and 165 mol Triton X-100 bound per mol *E. coli* oxidase is reasonable (refer to Section 2 for rationale), which results in a protein molecular weight of 144 000 ± 3500.

The effective hydrodynamic mass and protein molecular weight of Triton X-100-solubilized cytochrome bo_3 oxidase were also determined by sedimentation equilibrium to verify that the fitting of the sedimentation velocity data to the Lamm equation yields a reliable value for the protein molecular weight. The equilibrium studies were conducted under the same detergent to protein ratio that was employed in the velocity experiments, i.e. 5–10 mg Triton X-100 per mg protein. Sedimentation equilibrium data were collected and fitted to a single non-interacting model (Fig. 3). From these data, $M_{pr}(1-\varphi'\rho_0)$ was determined to be 43 000 ± 2000, which is within the experimental error of the value evaluated from the sedimentation velocity data. Assuming the same uncertainty in detergent binding as above, the protein molecular weight would be 140 000 ± 8000.

4. Discussion

The protein molecular weight, M_r, and hydrodynamic radius, R_s, of detergent-solubilized *E. coli* cytochrome bo_3 ubiquinol oxidase were successfully evaluated by a new approach: computer fitting of sedimentation velocity data with finite element analysis solutions to the Lamm equation. Iterative fitting generates best-fit values for both the sedimentation and diffusion coefficients were then used to calculate the two molecular parameters. The validity of the technique was confirmed by comparing molecular weights obtained from sedimentation velocity and sedimentation equilibrium data. The two values are in excellent agreement (144 000 and 140 000, respectively) and both are within the experimental error of the value calculated from the known sequence data (143 929) and that experimentally determined by MALDI mass spectrometry (143 025) for the purified subunits, including His-tagged subunit II [5,7].

We conclude that detergent-solubilized *E. coli* cytochrome bo_3 ubiquinol oxidase exists either as a stable monomeric complex with a protein molecular weight of 144 000 or that it non-specifically associates to form non-defined aggregates. Because the value for M_r is nearly identical to that calculated from the known sequence data or from the MALDI mass spectrometry data, we also conclude that the Triton X-100-solubilized complex contains a single copy of each subunit. Unlike mitochondrial terminal oxidases, no evidence of a dimeric form is detected. Cytochrome *c* oxidase purified from the bacterium *Paracoccus denitrificans* is also monomeric, not dimeric, when solubilized in octyl-pentaoxyethylene ether [30]. Mitochondrial inner membrane electron transport complexes, however, are often dimeric even after solubilization by detergent [16,18,19,21]. Mitochondrial oxidases are much more complicated than bacterial oxidases since they contain an additional 10 nuclearly encoded subunits. However, structures of their three core subunits are surprisingly similar to bacterial oxidases [10,22,31]. The functional and structural roles of the additional subunits are not known, but at least with bovine cytochrome *c* oxidase, two of these, subunits VIa and VIb, directly participate in the self-association of monomers within crystallized oxidase [10]. These contacts obviously are not present in the crystallized bacterial terminal oxidases [22,31]. The absence of these two subunits as well as the other small nuclearly encoded subunits may explain the absence of dimeric forms with these enzymes.

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