Triton X-100 Induced Dissociation of Beef Heart Cytochrome *c* Oxidase into Monomers†

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Received October 30, 1985

ABSTRACT: Purified beef heart cytochrome *c* oxidase, when solubilized with at least 5 mg of Triton X-100/mg of protein, was found to be a monodisperse complex containing 180 molecules of bound Triton X-100 with a protein molecular weight of 200 000, a Stokes radius of 66–72 Å, and an $s^0_{20,w}$ = 8.70 S. These values were determined by (1) measurement of the protein molecular weight by sedimentation equilibrium in the presence of D_2O, (2) evaluation of the sedimentation coefficient, $s^0_{20,w}$, by sedimentation velocity with correction for its dependence upon the concentration of protein and detergent, and (3) measurement of the effective radius by calibrated Sephacryl S-300 gel chromatography. The monomeric complex was judged to be homogeneous and monodisperse since (1) the effective mass of the complex was independent of the protein concentration throughout the sedimentation equilibrium cell and (2) a single protein schlieren peak was observed during sedimentation velocity. These results are interpreted in terms of a fully active monomeric complex that exhibits typical biphasic cytochrome *c* kinetics and contains 2 heme *a* groups and stoichiometric amounts of the 12 subunits normally associated with cytochrome *c* oxidase. With lower concentrations of Triton X-100, cytochrome *c* oxidase dimers and higher aggregates can be present together with the monomeric complex. Monomers and dimers can be separated by sedimentation velocity but cannot be separated by Sephacryl S-300 gel filtration, probably because the size of the Triton X-100 solubilized dimer is not more than 20% larger than the Triton X-100 solubilized monomer. These results suggest that homogeneous, dimeric, Triton X-100 solubilized cytochrome *c* oxidase is very difficult to isolate as a stable species.

Beef heart cytochrome *c* oxidase is an intrinsic, inner mitochondrial membrane complex that contains four prosthetic groups per functional unit: two hemes and two coppers (Wikström et al., 1981). Since the purified complex contains 9–12 nmol of heme *a* and 11–14 nmol of copper per milligram of protein (Hartzell et al., 1978), the minimum protein molecular weight of the functional complex is 150 000–220 000. It is generally agreed that cytochrome *c* oxidase contains a single copy of at least eight nonidentical protein subunits but there is increasing evidence that four additional polypeptides that stain poorly after polyacrylamide gel electrophoresis are also true subunits of the complex (Kadenbach et al., 1983a,b). The amino acid sequence of each of these 12 polypeptides has been determined by either protein or DNA sequencing techniques [Anderson et al., 1982; Buse et al., 1985; also see the review by Azzi (1980)]. If cytochrome *c* oxidase is assumed to contain a single copy of each of the 8 nonidentical subunits, the minimum protein molecular weight of the complex would be 160 000; alternatively, if the complex is assumed to contain a single copy of each of the 12 proposed subunits, the minimum molecular weight would be 200 000.

Both the true molecular weight and the aggregation state of purified, detergent-solubilized cytochrome *c* oxidase have recently received a great deal of attention. Several lines of evidence support the idea that cytochrome *c* oxidase may exist as a dimer in the inner mitochondrial membrane, e.g., functional studies on the association of cytochrome *c* with the purified enzyme (Bisson et al., 1980) and three-dimensional studies of negatively stained vesicle crystals of the complex (Henderson et al., 1977; Deatherage et al., 1982). A wide variety of hydrodynamic approaches have also been used to study this problem including sedimentation equilibrium corrected for detergent binding, sucrose gradient centrifugation that is sometimes corrected for detergent binding, but not corrected for preferential water binding, and gel filtration calibrated with water-soluble molecular weight standards. By these methods, protein molecular weights between 130 000 and 400 000 have been obtained and used to support models ranging from a monomeric 6-subunit enzyme to a dimeric 12-subunit enzyme [e.g., see Robinson & Capaldi (1977), Saraste et al. (1981), Georgevich et al. (1983), Suarez et al. (1984), and Bolli et al. (1985)].

In an attempt to clarify both the minimal molecular weight and the aggregation state of a functional cytochrome *c* oxidase complex, we have reinvestigated the aggregation state of the Triton X-100 (TX)[1] solubilized cytochrome *c* oxidase complex containing 10 nmol of heme a/mg of protein and all of the normal subunits including subunit III. Using the techniques of (1) sedimentation velocity, (2) gel filtration, and (3) sedimentation equilibrium in H_2O/D_2O mixtures to correct for bound detergent, we have found that monodisperse preparations of TX-solubilized cytochrome *c* oxidase complex contain only cytochrome aa_3 "monomers" with a protein molecular weight of 200 000 while preparations that contain cytochrome aa_3 "dimers" are polydisperse mixtures containing significant amounts of either "monomers" or higher aggregation states of the complex.

†This investigation was supported by research grants from the National Institutes of Health (GM 24795) and The Robert A. Welch Foundation (AQ 907).

[1] Abbreviations: TX, Triton X-100; $M_p(1 - \phi'\rho)$, effective mass of protein which is equal to $M_p(1 - \bar{v}_p\rho) + \nu M_d(1 - \bar{v}_p\rho)$; M_p, protein molecular weight; M_d, detergent molecular weight; $\bar{v}_p$, partial specific volume of protein; $\bar{v}_d$, partial specific volume of detergent; ν, moles of detergent bound per mole of protein; R_s, Stokes radius; Tris-HCl, tris(hydroxymethyl)aminomethane base titrated to the appropriate pH with HCl; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-2328$01.50/0

Experimental Procedures

Materials

Cytochrome *c* oxidase was isolated by the method of Fowler et al. (1962) as modified by Capaldi and Hayashi (1972) from Keilin-Hartree beef heart muscle particles that had been prepared according to Yonetani (1960). After the final ammonium sulfate precipitation, the cytochrome *c* oxidase precipitate was dissolved in 0.02 M Tris-HCl buffer at pH 8 containing 0.09 M NaCl, 1 mM EDTA, and 15 mM TX, dialyzed against 50 volumes of this buffer, and stored in liquid N_2 until used. The isolated complex contained 9.5–10.3 nmol of heme *a*/mg of protein on the basis of $\Delta\epsilon_{605-630nm(reduced)} = 16.5\ mM^{-1}\ cm^{-1}$ for heme *a* (Griffiths & Wharton, 1961) and protein determination according to Gornall et al. (1949). Typical preparations also had a molecular activity of 320–350 s^{-1} when assayed in 0.025 M phosphate buffer at pH 7.0 containing 10 mM lauryl maltoside.

Triton X-100 was purchased from Research Products International, treated with sodium hydrosulfide to destroy any contaminating peroxides (Strittmatter et al., 1978), and phase-separated into chloroform to remove excess dithionite and its oxidation products. After removal of all traces of chloroform by a 20-μm vacuum, it was stored at −40 °C until it was used. Lauryl maltoside was obtained from Calbiochem; Sephacryl S-300, cytochrome *c* (type III), and 99.8% deuterium oxide were purchased from Sigma Chemical Co.

Methods

All measurements were done at pH 8.1 in 0.02 M Tris-HCl buffer containing 1 mM EDTA and 0.09 M NaCl with TX added to the appropriate concentration. Cytochrome *c* oxidase and TX concentrations were measured spectrophotometrically on the basis of $\epsilon_{422} = 1.54 \times 10^5\ M^{-1}\ cm^{-1}$ (van Gelder, 1978) and $\epsilon_{277} = 1.50 \times 10^3\ M^{-1}\ cm^{-1}$ (Robinson & Tanford, 1975), respectively. Glycerol gradient delipidation of cytochrome *c* oxidase by TX was performed as previously described (Robinson et al., 1980) with the exception that 0.2 M Tris-HCl buffer at pH 8.0 containing 0.1 mM EDTA rather than the 0.02 M Tris-HCl buffer was used during the 5% TX incubation and centrifugation. With this altered buffer system, a single glycerol gradient centrifugation was sufficient to remove all of the phospholipids except for the three tightly bound cardiolipins. Quantitation of the amount of bound phospholipid, analysis of the protein subunit composition by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis in 2 M urea, and determination of the cytochrome *c* oxidase activity were each done as described previously (Robinson et al., 1980) with one exception: the data collection during spectrophotometric activity measurements and calculation of molecular activities were done automatically by an Apple II Plus microcomputer that was connected via an "Adalab" A/D converter (Interactive Microware) to the analog output of the spectrophotometer. Steady-state kinetic assays were done polarographically with a Clark oxygen electrode at 25 °C in 25 mM Tris–acetate buffer at pH 7.9 containing 0.7 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 7 mM ascorbate, 0.05–30 μM cytochrome *c*, and 5 mg/mL Tween 80 as described by Brautigan et al. (1978).

Gel Filtration. Sephacryl S-300 gel filtration was done at 5 °C with a 0.9 × 94 cm column flowing at ca. 8 mL/h. The flow rate was controlled with a peristaltic pump, and the elution volume in grams for each eluting protein peak was accurately determined by weighing the effluent contained in every 10 fractions that were collected. Standardization of the gel filtration column was done according to Ackers (1975) with myoglobin, chymotrypsin, hemoglobin, catalase, and thyroglobulin as standards having known Stokes radii. K_d was calculated relative to the void volume and total volume markers blue dextran and 2-mercaptoethanol. The inverse error function of $1 - K_d$ was evaluated as described by Ackers. The cholate-solubilized preparation of cytochrome *c* oxidase that was used in the Sephacryl S-300 gel filtration experiments was prepared as follows. The TX in the original "stock" enzyme was exchanged for cholate with an Ultragel AcA 44 gel filtration column equilibrated with 10 mM cholate (prior to gel filtration, 10 mg of cholate was added per milligram of TX).

Sedimentation Methods. These measurements were done with a Beckman Model E analytical ultracentrifuge equipped with both schlieren optics and a photoelectric scanner. Schlieren photographs were used to calculate the apparent sedimentation coefficients as described by Chervenka (1973). $s_{20,w}$ values were calculated from the measured *s* value by correcting for the solvent viscosity at 20 °C, which was measured with a Cannon-Ubbelohde semimicro viscometer, and the solvent density, which was measured with a Mettler-Anton Paar Model DMA 60 density meter. The sedimentation equilibrium measurements that were done in D_2O to increase the solvent density were analyzed as described by Reynolds and Tanford (1976). After the addition of D_2O to a sample, the solvent density was measured with a Mettler-Anton Paar Model DMA 60 density meter with the temperature set to an accuracy of 0.05 °C and controlled to a precision of 0.5 mdeg centigrade with a Tronac Model 405 and Model PTC-40 constant-temperature water and precision temperature controller. Although this approach measures the solution rather than the solvent density, the error in the density is less than 0.3 parts per 1000 which does not affect the final result. The following partial specific volumes were used to evaluate the protein molecular weight: cytochrome *c* oxidase, $\bar{v}_p = 0.743$ (Robinson & Capaldi, 1977); TX, $\bar{v}_p = 0.903$, measured at 25 °C with the Mettler-Anton Paar density meter as described by Tanford et al. (1974). High-speed sedimentation equilibrium data were collected with the photoelectric scanner set at 420 nm, and the data were analyzed by a linear regression fit to the ln *A* vs. R^2 plot with an Apple II Plus microcomputer. Elaborate methods of calculating base-line corrections were not necessary since the data were collected by using the photoelectric scanner at 420 nm rather than interference optics. Point by point calculation of the protein molecular weight as a function of the protein concentration throughout the sedimentation equilibrium centrifuge cell was performed as suggested by Teller et al. (1973) to determine protein homogeneity. Calculations were done by using a nonlinear regression program, "CURFIT", and a point by point differentiation–integration program, "VIDICHART", that were purchased for the Apple II Plus microcomputer from Interactive Microware, Inc. Point by point calculations of the effective mass, $M_p(1 - \phi'\rho)$, were done by (1) reading about 50–55 equally spaced data points from the scanner tracing, (2) using all points with $A_{420} > 0.04$ to calculate ln *A* and R^2 (ca. 30 points), (3) smoothing the ln *A* vs. R^2 data by fitting a fifth-degree polynomial to the plot of ln *A* vs. R^2 with nonlinear regression analysis, (4) recalculating smoothed values of ln *A* from the fitted line at each of the original R^2 values, and (5) calculating $M_p(1 - \phi'\rho)$ from the slope at each value of R^2 by using the differentiation program.

Results

Effect of Triton X-100 Concentration on the Size of Cytochrome c Oxidase. Incubation of purified cytochrome *c* oxidase with 2 mg of TX/mg of protein followed by Sepharose

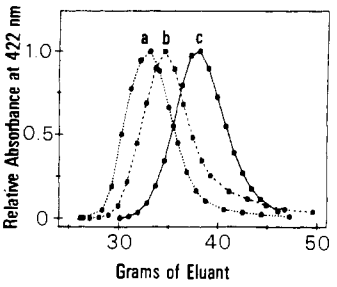

FIGURE 1: Effect of TX concentration on the elution of cytochrome *c* oxidase from a Sephacryl S-300 gel filtration column (0.9 × 94 cm) equilibrated with 1 mg/mL TX at pH 8.0. The 1-mL samples that were applied to the column contained 5 mg/mL protein, 5–10% glycerol, and the following TX concentrations: (curve a) 5 mg/mL TX; (curve b) 10 mg/mL TX; (curve c) 50 mg/mL TX. In each case, the flow rate was 7–8 mL/h, and 0.9-g fractions were collected. The absorbance at 422 nm has been normalized to facilitate comparison of the elution peaks; the maximum absorbance at 422 nm for each sample was (curve a) A_{max} = 0.445, (curve b) A_{max} = 0.541, and (curve c) A_{max} = 0.502.

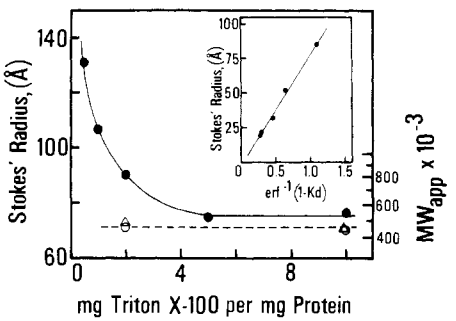

FIGURE 2: Effect of TX concentration on the Stokes radius of cytochrome *c* oxidase as determined by Sephacryl S-300 gel filtration. The abscissa values indicate the milligrams of TX per milligram of protein that were present in the sample applied to the column; in each case, the sample (1 mL) contained 5 mg/mL protein and 5–10% glycerol. Three experimental conditions are shown: (1) the data represented by the closed circles (●) were collected by using the original dialyzed "stock" enzyme and a column that was equilibrated with pH 8.0 buffer containing 1 mg/mL TX; (2) the data shown by the open triangles (△) were obtained by using the cholate-solubilized sample of cytochrome *c* oxidase (see Methods) with the S-300 column equilibrated with 1 mg/mL TX; (3) the data illustrated by the open circles (○) were obtained by using the original dialyzed stock enzyme, but the column had been equilibrated with pH 8.0 buffer containing 10 mg/mL TX. All of the experimental conditions and methods were identical with those described in Figure 1 with the exception of the amount of TX that was used. The amount of TX did not affect the calibration obtained with proteins of known Stokes radii; the calibration curve is shown in the inset. The apparent molecular weight scale shown on the right side of the figure gives the calibration of the column based upon the molecular weights of the spherical, globular, water-soluble protein standards; the values were calculated from the values of R_s from the relationship log M_p = 2.473 log R_s + 1.0811 which is the equation based upon a linear regression fit obtained with 10 globular proteins.

4B gel filtration in the presence of 1 mM TX has been used to prepare "dimeric" TX-solubilized cytochrome *c* oxidase (Robinson & Capaldi, 1977). However, as shown in Figure 1, increasing or decreasing the concentration of TX in the sample applied to such a column greatly affects the size of the complex as judged from its elution behavior from a Sephacryl S-300 column equilibrated with 1 mg of TX/mL (1.5 mM TX). With less than 1 mg of TX/mg of protein, the sample was chromatographically heterogeneous since a shoulder occurred on the trailing edge of the peak (data not shown); however, with TX concentrations greater than 1 mg of TX/mg of protein, symmetrical elution peaks were always obtained (Figure 1). As the TX concentration was increased from 1 to 10 mg of Tx/mg of protein, the elution position of the complex was retarded, indicating that the size of the complex had decreased, i.e., dissociation of the dimeric complex had occurred. The higher concentrations of TX did not cause a decrease in one peak and the generation of a second later eluting peak as would be expected for the conversion of stable dimers into stable monomers, but rather the symmetrical elution peak monotonically shifted to larger elution volumes. Apparently, the different sized species are in rapid enough equilibrium compared with their rate of separation by gel filtration to prevent their separation, or the monomer and dimer are not sufficiently different in size to be separated by gel filtration.

The effect of increasing the TX concentration upon the average size of the TX–protein complex, i.e., Stokes radius (R_s), is shown in Figure 2. Incubation of cytochrome *c* oxidase with low concentrations of TX prior to gel filtration in 1 mg of TX/mL resulted in a large soluble complex with R_s >130 Å. As the amount of TX in the incubation mixture was increased, the size of the complex decreased until a complex of 72–74 Å was obtained after incubation of the enzyme with more than 5 mg of TX/mg of protein. Alternatively, a TX–protein complex of nearly the same size, i.e., 70 Å, was obtained after incubation of the complex with as little as 2 mg of TX/mg of protein provided that (a) the complex was exposed to high concentrations of sodium cholate prior to its transfer into TX or (b) the TX concentration in the elution buffer was increased to 10 mg of TX/mL.

The decreased size of the TX–cytochrome *c* oxidase complex after exposure to these high detergent concentrations was not the result of TX extraction of either phospholipid or specific subunits from the complex, e.g., subunit III, since neither the subunit composition nor the phospholipid content of the complex was significantly altered by any of the procedures (Table I and Figure 3). The molecular activity of cytochrome *c* oxidase was also not significantly altered by any of the treatments provided the enzyme was assayed in a detergent capable of supporting full activity, e.g., lauryl maltoside (Table I). Samples treated with the highest concentrations of TX, e.g., 10 mg of TX/mg of protein, also exhibited the typical biphasic cytochrome *c* kinetics when assayed polarographically in detergents that support full activity. Analysis of the biphasic Eadie–Hofstee plot resulted in values of 90 nM and 2 μM for the high- and low-affinity K_m, respectively, and 60 and 200 s^{-1} for the high- and low-affinity turnover number, respectively. Assuming that reaggregation of the complex did not occur during its dilution to 1.5 nM during the spectrophotometric enzymatic assays, or to 15 nM in the polarographic kinetic assays, the completely dissociated cytochrome *c* oxidase complex was fully active and kinetically identical with the values

Table I: Phospholipid Content and Molecular Activity after Triton X-100 Incubation and Sephacryl S-300 Gel Filtration

mg of TX/mg of protein in sample	phospholipid content (P/heme aa_3)	molecular act. in lauryl maltoside (s^{-1})
0.5	19	410
1.0	19	390
2.0	19	400
5.0	16	400
10.0	14	310
2.0[a]	16	390

[a] Prior to gel filtration on Sephacryl S-300 in 1.5 mM Triton X-100, the cytochrome *c* oxidase was (1) dissolved in 5 mg of cholate/mg of protein and (2) eluted from an Ultragel AcA 44 gel filtration column in 10 mM cholate, and (3) 2.0 mg of TX was added per milligram of protein to the pooled fractions (5 mg of protein/mL) and applied to the Sephacryl S-300 column.

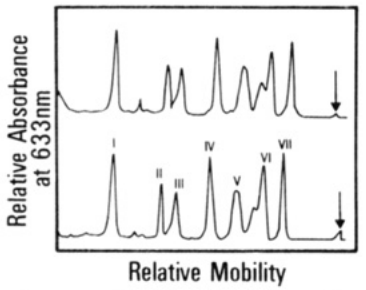

FIGURE 3: Subunit composition of cytochrome *c* oxidase samples eluted from Sephacryl S-300. Each panel illustrates the densiometric scan that was obtained for a cytochrome *c* oxidase sample after discontinuous polyacrylamide slab gel electrophoresis in dodecyl sulfate and 2 M urea after being stained with Coomassie blue. The top scan was obtained with complex to which 0.5 mg of TX/mg of protein was added prior to its elution from a column equilibrated with 1 mg/mL TX; i.e., this sample had a Stokes radius of 130 Å. The bottom scan was obtained with complex to which 10 mg of TX/mg of protein was added prior to its elution from the same column; i.e., this sample had a Stokes radius of 75 Å. In each case, the arrow indicates the migration of the tracking dye which had migrated 9.5 cm from the top of the running gel. In both samples, two stained bands were clearly visible in the area labeled "V"; three stained bands were also evident in the area labeled "VI". The subunits are numbered according to Downer et al. (1976).

reported for the dimeric and more aggregated forms of the enzyme.

Homogeneity and Sedimentation Coefficient of the Triton X-100–Cytochrome c Oxidase Complex. From the data in Figure 2, it is apparent that 5–10 mg of TX/mg of protein is sufficient to completely dissociate cytochrome *c* oxidase as judged by gel filtration. At these high concentrations of TX, the detergent-solubilized complex was found to be a homogeneous, monodisperse preparation by sedimentation velocity as is evident from the single protein schlieren peak that was obtained with more than 5 mg of TX/mg of protein (Figure 4A). In agreement with the gel filtration results, concentrations of TX that were insufficient to generate a monodisperse complex, i.e., 2 mg of TX/mg of protein, resulted in the appearance of a second faster sedimenting protein species (Figure 4B).

To determine the dependence of the dissociation of cytochrome *c* oxidase upon the TX concentration, as monitored by sedimentation velocity, two studies were done as a function of TX concentration. At high concentrations of TX, increasing the concentration from 5 to 23 mg of TX/mg of protein greatly decreased the s_{obsd}. The decreased s_{obsd} was almost entirely due to increased solvent viscosity and was not due to further dissociation of the complex since $s_{20,w}$ for the complex decreased only 4% as the TX concentration was increased to 50 mg/mL (Table II). The dependence of the sedimentation coefficient upon the protein concentration was also measured by extrapolating the $s_{20,w}$ to zero protein concentration while keeping the TX concentration constant at 10 mg/mL. $s_{20,w}$ decreased approximately 5% as the protein concentration was increased from 0.9 to 5 mg/mL. From extrapolation of both TX and protein to zero concentration, $s^0_{20,w}$ for the monodisperse TX complex was determined to be 8.70 S. Using this value together with the R_s = 70 Å that was obtained by gel filtration, we calculated the effective mass of the TX–cytochrome *c* oxidase complex, $M_p(1 - \phi'\rho)$, to be 69 000 using the equation for the sedimentation coefficient of a detergent-solubilized protein (Tanford et al., 1974). Interpretation of $M_p(1 - \phi'\rho)$ in terms of the protein molecular weight and, therefore, the aggregation state of the complex is difficult unless the amount of bound detergent and the partial specific volumes of protein and detergent are known. Direct measurement of TX binding to the complex at these very high TX concentrations is impossible, but as will be shown by the sedimentation equilibrium experiments in D_2O, this value represents the monomeric complex with M_p 200 000 rather than dimeric cytochrome *c* oxidase; i.e., it is the single cytochrome aa_3–TX-solubilized complex. The value of 69 000 that is calculated for $M_p(1 - \phi'\rho)$ by combining the sedimentation

Table II: Effect of Triton X-100 on the Solvent Density, Viscosity, and Sedimentation Coefficient of Cytochrome *c* Oxidase[a]

Triton X-100 concn (mg/mL)	solvent density (g/mL)	rel viscosity of solvent	s_{obsd} (S)	$s_{20,w}$ (S)
5	1.003	1.048	8.10	8.68
10	1.004	1.075	7.84	8.63
20	1.005	1.136	7.14	8.29
46	1.007	1.384	5.71	8.39

[a] Measurements were made at 20.0 °C with 2 mg/mL protein in 0.02 M Tris-HCl buffer at pH 8.10 containing 0.09 M NaCl and 0.1 mM EDTA.

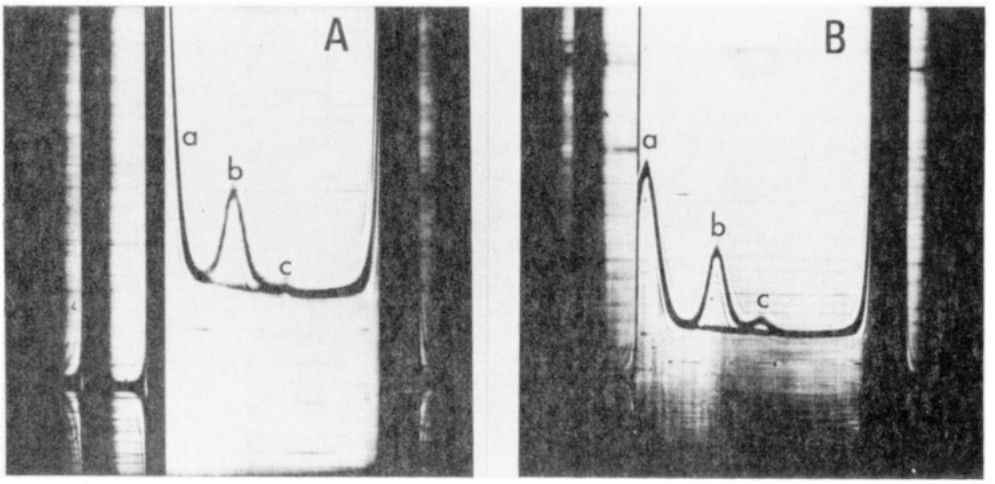

FIGURE 4: Sedimentation velocity patterns of cytochrome *c* oxidase obtained at 20 °C in pH 8.0 buffer: (A) 20 mg/mL TX and 2.3 mg/mL protein (8.7 mg of TX/mg of protein); (B) 10 mg/mL TX and 5 mg/mL protein (2 mg of TX/mg of protein). The schlieren pictures were taken 48 min (panel A) and 40 min (panel B) after the rotor reached the maximum speed of 51 800 rpm. In each panel, "a" indicates the schlieren peak corresponding to TX micelles which had an $s_{20,w}$ = 1.21 S in the experiment shown in panel B, "b" indicates the slower protein peak which had $s_{20,w}$ = 8.29 and 8.39 S in the experiments shown in panels A and B, respectively, and "c" represents the faster sedimenting protein peak which had an $s_{20,w}$ = 12.88 S in the experiment shown in panel B.

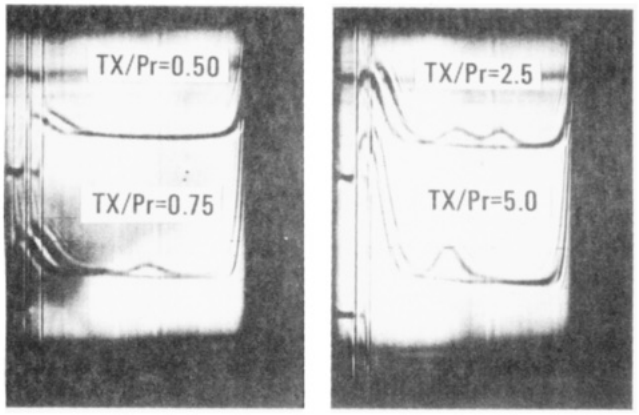

FIGURE 5: Dissociation of cytochrome *c* oxidase by Triton X-100 as measured by sedimentation velocity. Experimental conditions were 20 °C in pH 8.1 buffer and $I = 0.10$ with 1.9 mg/mL protein and the appropriate amount of TX. In each experimental run, two double-sector cells were used, one with a regular window and the other with a wedge window with a 1-deg positive offset. In each panel, the top schlieren picture is of the cell with the wedge window and the bottom one of the cell with the regular window. The reference in each case contained approximately the same concentration of TX as the sample but a slightly larger volume resulting in the double meniscus line at the left side of each picture. The peak at the left side of each figure, which gets larger with increased amounts of TX, is caused by the TX micelles. Both pictures were taken 75 min after the rotor reached a full speed of 38 750 rpm.

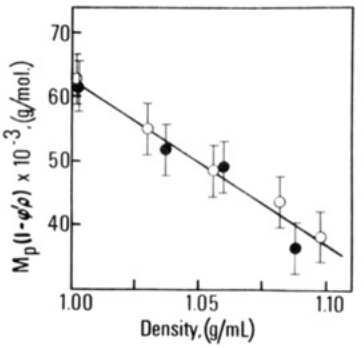

FIGURE 6: Effect of solvent density on the effective mass of TX-solubilized cytochrome *c* oxidase, $M_p(1 - \phi'\rho)$, as measured by sedimentation equilibrium. The data shown with the closed circles (●) were measured by using the purified, dialyzed stock cytochrome *c* oxidase that had been dissolved in pH 8.0 buffer containing 10 mg/mL TX and enough D_2O to obtain the desired solution density; the values represented by the open circles (○) were obtained with complex that had been (a) delipidated of all but the three tightly bound cardiolipins by using glycerol gradient centrifugation in 10 mg/mL TX, (b) equilibrated with 1 mg/mL TX by using DEAE-cellulose ion-exchange chromatography, and (c) dialyzed against 1 mg/mL TX, pH 8.0, buffer containing the appropriate amount of D_2O. In each case, the solution density was measured with a precision density meter. The data shown for the stock cytochrome *c* oxidase (●) are the average of the values obtained in two to three separate experiments that were centrifuged for 20 h at 10000 rpm; the data shown for the delipidated complex are the average of values from two to three separate experiments that were analyzed at both 10000 and 15000 rpm. In each experiment, 0.1 mL of sample was used, resulting in a 3-mm column height in the centrifugation cell. The error bars at each point indicate the experimental reproducibility. The line drawn through the data represents the linear regression fit to all the data.

velocity and gel filtration results also agrees very well with the value obtained by sedimentation equilibrium at a solvent density of 1.00 (refer to the next section on sedimentation and Figure 6). Alternatively, R_s can be calculated from $M_p(1 - \phi'\rho)$ that is obtained by sedimentation equilibrium and the sedimentation coefficient by using the equation for s given by Tanford et al. (1974) for a detergent-solubilized protein. By this approach, $R_s = 66$ Å.

The second sedimentation velocity experiment was performed at low TX concentrations, i.e., between 0.5 and 5.0 mg of TX/mg of protein, to permit a comparison of the dissociation of aggregated oxidase as monitored by sedimentation velocity with the data previously obtained by gel filtration. With [TX]/[protein] = 0.5, cytochrome *c* oxidase was highly aggregated and quickly pelleted on the bottom of the cell (Figure 5). Dimeric oxidase, with $s_{20,w} = 12.9$ S if measured in 10 mg/mL TX, began to appear when [TX]/[protein] = 0.75, but before a homogeneous dimeric species appeared, the monomeric species also was present; i.e., both monomeric and dimeric complexes are observed at [TX]/[protein] = 2.5 in Figure 5. Once again, a homogeneous complex was only observed at [TX]/[protein] $\geq$ 5.

Determination of Protein Molecular Weight by Sedimentation Equilibrium in H_2O/D_2O Mixtures. Once homogeneity of a protein has been established, sedimentation equilibrium is the most reliable method for determination of protein molecular weight since it directly measures the effective mass of a detergent-solubilized complex, $M_p(1 - \phi'\rho)$, where $M_p(1 - \phi'\rho) = M_p(1 - \bar{v}_p\rho) + \nu M_d(1 - \bar{v}_d\rho)$ (Tanford et al., 1974). To evaluate the protein molecular weight (M_p) from $M_p(1 - \phi'\rho)$, either the amount of detergent that is bound to the protein, ν, must be measured, or the influence of the bound detergent upon the measurement must be negated by increasing the solution density with D_2O (Reynolds & Tanford, 1976). The latter approach was used to evaluate the protein molecular weight of the homogeneous 66–70-Å TX-solubilized cytochrome *c* oxidase since this complex could only be prepared by methods that prevented determination of detergent binding, i.e., (1) addition of 5–10 mg of TX/mg of protein, in which case it was impossible to measure TX binding due to the very high free concentration of TX, or (2) treatment of 5 mg of protein/mL with 25–50 mg of TX/mL followed by Sephacryl S-300 gel filtration in 1 mg of TX/mL (Figure 2), in which case complete separation of excess TX micelles from the protein was impossible due to the large excess of TX and the incomplete separation of the protein and micelle peaks.

The effective mass, $M_p(1 - \phi'\rho)$, of two different preparations of cytochrome *c* oxidase was analyzed by sedimentation equilibrium as a function of the solvent density. One preparation of the enzyme contained ca. 30 mol of P/mol of cytochrome aa_3 complex before it was diluted into 10 mg of TX/mL (50 mg of TX/mg of protein) and analyzed by sedimentation equilibrium at four solvent densities; the other preparation was a delipidated complex containing only 5.5 mol of P/mol of cytochrome aa_3 (i.e., ca. three cardiolipins per complex) that had been equilibrated with 1 mg of TX/mL. With each preparation, nearly identical results were obtained (Figure 6). In such a plot, extrapolation of $M_p(1 - \phi'\rho)$ to a density of 1.1074 g/mL negates the contribution of TX to the effective mass and permits the direct determination of the protein molecular weight by using the equation given above for the definition of $M_p(1 - \phi'\rho)$ and a solvent density of 1.1074. The amount of detergent bound to the complex can be calculated from the slope and the intercept (Reynolds & Tanford, 1976). By this type of data analysis, the complex in 10 mg of TX/mL containing 30 mol of P per complex was found to have a protein molecular weight of 190000 ± 15000 while the delipidated complex in 1 mg/mL TX that contained only 3 mol of cardiolipin per complex had a protein molecular weight of 220000 ± 15000. Estimation of the bound TX is more subject to error since it is based upon both the intercept and the slope of the data in Figure 6, but the best fit to all of the data corresponds to about 180 ± 30 mol of TX bound/mol of cytochrome aa_3 complex.

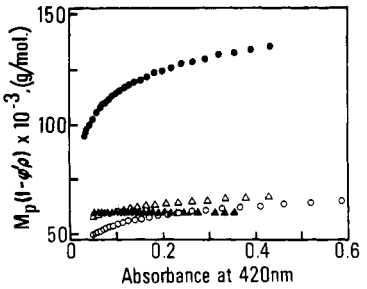

FIGURE 7: Dependence of the effective mass, $M_p(1 - \phi'\rho)$, of TX-solubilized cytochrome *c* oxidase on the protein concentration within the sedimentation equilibrium cell. In each experiment, 0.1 mL of the solution having a density of 1.002 was used to fill the cell; i.e., no D_2O had been added. Each plotted value represents a point by point determination of the slope of a fifth-order polynomial that had been fitted to the data plotted as ln A_{420} vs. R^2. The open triangles (△) represent the data obtained at 10 000 rpm with delipidated cytochrome *c* oxidase in 1 mg/mL TX (the preparation of this sample is described in the legend to Figure 6); the closed triangles (▲) represent the data obtained with the delipidated sample at 15 000 rpm. The open circles (○) represent data obtained with the stock cytochrome *c* oxidase in 10 mg/mL TX at 10 000 rpm. The closed circles (●) were calculated from the data shown in Figure 3 of Robinson and Capaldi (1977) that had been obtained for the dimeric TX-solubilized cytochrome *c* oxidase complex.

The above analysis is, of course, dependent upon a monodisperse TX-solubilized complex. At each density, the homogeneity of each of the two preparations was established by a point by point calculation of $M_p(1 - \phi'\rho)$ throughout the sedimentation equilibrium cell. The resulting values of $M_p(1 - \phi'\rho)$ were nearly independent of the speed of centrifugation, the sample preparation, the initial protein concentration, or the protein concentration within the cell, i.e., the radial position (Figure 7). Independence of $M_p(1 - \phi'\rho)$ from these variables proves that the preparations are truly homogeneous at each of the densities used in Figure 6; it also confirms the validity of using the whole cell average values for $M_p(1 - \phi'\rho)$ in this figure. A similar point by point evaluation of $M_p(1 - \phi'\rho)$ at each of the data points that had been previously used to determine the dimer molecular weight of the TX-solubilized cytochrome *c* oxidase complex (Robinson & Capaldi, 1977) was performed, and the values are included in Figure 7 for comparative purposes. Clearly, the previous preparation of cytochrome *c* oxidase was not entirely homogeneous and certainly contained monomeric and probably more highly aggregated oxidase. The usefulness of the point by point evaluation of $M_p(1 - \phi'\rho)$ rather than its evaluation from a linear regression fit to the entire data set is evident from this example since the sample heterogeneity was difficult to assess from the original ln A_{420} vs. R^2 plot.

DISCUSSION

Functional studies on cytochrome *c* oxidase are often interpreted in terms of a dimeric model for the complex, i.e., two multisubunit complexes each containing two *a* hemes and two coppers. For example, inhibition of the electron-transport activity with arylazidocytochrome *c* derivatives indicates "half of site" reactivity, suggesting that the complex exists as a dimer during the enzymatic assay (Bisson et al., 1980); the high-affinity cytochrome *c* binding site has been postulated to be at the interface between the monomers within the dimeric complex (Darley-Usmar et al., 1984).

In light of the present data, the intrinsic dimeric nature of cytochrome *c* oxidase, at least when it is solubilized with TX, should be questioned. The present data clearly document that the amount of TX that is used to solubilize the complex greatly affects its aggregation state. From both the gel filtration data in Figures 1 and 2 and the sedimentation velocity data in Figure 5, it can be concluded that solubilizing the aggregated complex with TX first forms some dimers, but monomers begin to form before all of the oxidase is converted to dimers. We have also found that individual preparations of the enzyme behave somewhat differently; however, it is very difficult to predict the concentration of TX that is necessary to form primarily dimers or 50% monomers and dimers. In the present study, the transition between dimers and monomers occurred at 1–2 mg of TX/mg of protein. The method previously used to prepare dimeric cytochrome *c* oxidase, i.e., 2 mg of TX/mg of protein, does not appear to produce a monodisperse, homogeneous detergent-solubilized complex (refer to Figures 2 and 6). To achieve monodisperse preparations, it is necessary to treat the complex with higher concentrations of TX, i.e., 5 mg of TX/mg of protein (refer to Figures 2, 4, and 5). The data in Figures 4, 6, and 7 clearly indicate that the homogeneous, TX complex is the monomeric species which contains two heme *a* groups; it has a protein molecular weight of 200 000, a R_s = 66–72 Å, and an $s^0_{20,w}$ = 8.70 S and includes approximately 180 molecules of bound TX.

When mixtures of monomers and dimers are present, e.g., with 2.5 mg of TX/mg of protein (refer to Figure 5), they could not be separated by gel filtration (refer to Figure 1, curve b). Two explanations for the inability of gel filtration to separate TX-solubilized monomers and dimers seem possible. First, the monomer and dimer may be in rapid enough equilibrium relative to the time required for the gel filtration experiments (12 h) to prevent their separation, but in slow equilibrium relative to the sedimentation velocity experiments (1 h). A second and probably more satisfactory explanation is that self-association of the cytochrome *c* oxidase complex does not greatly increase the R_s of the complex. The R_s of two identical touching spherical balls is only 30% larger than a single sphere [calculated from the Perrin equation in Tanford (1961) as described in Robinson & Tanford (1975)]. In the case of cytochrome *c* oxidase dimerization, R_s should increase less than 30% since (1) cytochrome *c* oxidase is known to be asymmetric and to self-associate side by side [this type of dimerization is more similar to the self-association of two prolate ellipsoids than it is to the self-association of two spheres; therefore, R_s would increase by only 25% [calculated from the Perrin equation in Tanford (1961)]] and (2) dimerization of a detergent-solubilized protein almost certainly displaces some bound detergent from the hydrophobic protein surfaces that are in contact, resulting in R_s increasing less than 25%. A simple geometric model based upon the known molecular dimensions of cytochrome *c* oxidase (approximately 115 × 50 × 50 Å) and assuming a 35–40-Å layer of bound TX (TX micelles have a R_s = 45 Å) indicates that slightly more than 25% of the detergent would be displaced by dimerization. Comparison of the TX binding values that were previously obtained with mainly dimeric oxidase, 180 molecules of TX bound per dimer (Robinson & Capaldi, 1977), and the identical value now obtained with the monomeric enzyme indicates very significant displacement of detergent upon dimerization. The sedimentation velocity experiments confirm the relatively small increase in R_s upon dimerization. The ratio of the sedimentation coefficient of the dimer and monomer is 1.54; thus, R_s increases by 30% if no detergent is displaced or increases by 18% if both monomer and dimer bind identical amounts of TX [calculated from the definition of the sedimentation coefficient and $M_p(1 - \phi'\rho)$ for detergent-solubilized proteins (Tanford et al., 1974)]. From these values, dimeric

cytochrome *c* oxidase would have an R_s = 78–85 Å compared with R_s = 66–70 Å for the monomeric complex; thus, it is not surprising that they are not separated by gel filtration.

It should also be noted that, based only upon the gel filtration data presented in Figure 2, the homogeneous TX-solubilized complex could easily have been misinterpreted in terms of a dimeric structure since it eluted from the Sephacryl S-300 column at the position expected for a globular, water-soluble protein with M_p = 500000. A similar discrepancy was observed for the lauryl maltoside solubilized monomeric complex because it eluted from a Sephacryl S-300 column with an apparent molecular weight of ~300000 (Ferguson-Miller et al., 1982; Suarez et al., 1984).

The value M_p = 200000 obtained for the protein molecular weight of the monomeric, TX-solubilized complex agrees very well with the value of 190000 reported for the lauryl maltoside solubilized complex by Suarez et al. (1984) and more recently by Bolli et al. (1985) although in the latter case dimeric oxidase was reported to be the predominant species with ionic strengths greater than 0.05 M. Each of these studies is also in excellent agreement with the value of 202787 that has been calculated by Buse from the amino acid sequences of the 12 subunits of cytochrome *c* oxidase (Buse et al., 1985) and lends support for the 12–13-subunit model for cytochrome *c* oxidase that has been proposed by Kadenbach et al. (1983a,b) and Buse et al. (1980, 1985).

The M_p value that we obtained for the TX-solubilized enzyme does not agree with the 129000–160000 value reported by Georgevich et al. (1983) for a TX-solubilized monomeric oxidase; however, they had purposely treated the complex with TX at slightly alkaline conditions to remove subunit III and some low molecular weight subunits. They also reported an M_p value of 326000 for the dimeric enzyme as measured by sedimentation equilibrium, which is significantly less than the expected value of 400000 based upon the current studies. Their value was based upon a whole cell average molecular weight calculated from a curving plot of ln A vs. R^2 which indicates contamination by lower molecular weight species, probably monomers. Such contamination would lower the calculated value for the molecular weight of the dimeric complex.

The only other recent sedimentation equilibrium data that appear to conflict with the present data are the studies of Saraste et al. (1981, 1982) which were done on the TX-solubilized, subunit III deficient complex (this complex may have been deficient in subunits a, b, and c). Interestingly, they reported a value for the protein molecular weight of 210000, for the sedimentation coefficient of 8.2 S, and for the Stokes radius of 65 Å. These values are nearly identical with those that we report, but due to a very high heme content (17–20 nmol/mg of protein), they concluded that they were studying the dimeric enzyme.

Although the present study documents the monomeric state of the TX-solubilized complex, the data tell us nothing about the aggregation state of cytochrome *c* oxidase within the mitochondrial inner membrane except that monomers cannot be excluded as a possibility. The approaches that were used in this study cannot be employed to investigate the aggregation state of cytochrome *c* oxidase within the inner mitochondrial membrane, or in fact within phospholipid vesicles. At present, we cannot assess whether TX solubilization of the aggregated complex, which was induced by ammonium sulfate precipitation of the enzyme during its purification, is reversing the self-association of monomeric enzyme or disrupting the native dimeric structure of the enzyme as it exists in situ. Nevertheless, the monomeric cytochrome *c* oxidase produced in the present study is functionally active when assayed in a detergent that can support electron-transport activity, and it does exhibit typical biphasic cytochrome *c* kinetics. We conclude, therefore, that the dimeric form of the complex is not a prerequisite for either of these processes.

Registry No. Triton X-100, 9002-93-1; cytochrome *c* oxidase, 9001-16-5.

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Light- and Nucleotide-Dependent Binding of Phosphodiesterase to Rod Disk Membranes: Correlation with Light-Scattering Changes and Vesicle Aggregation†

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Received October 11, 1985; Revised Manuscript Received December 17, 1985

ABSTRACT: Under conditions in which large guanosine cyclic 3′,5′-phosphate (cGMP)- and phosphodiesterase (PDE)-dependent changes in near-infrared transmission and vesicle aggregation and disaggregation occur, we have observed a striking change in the binding of PDE to rod disk membranes. The change in PDE binding is nucleotide and light dependent as are the light-scattering changes. The cGMP- and PDE-dependent light-scattering signal can be produced by a 500-nm light flash which bleaches $1/(1 \times 10^7)$ rhodopsin molecules. Mg ions are an essential cofactor for the nucleotide-dependent PDE binding and light-scattering changes. 3-Isobutyl-1-methylxanthine and other competitive inhibitors of PDE hydrolytic activity support increased PDE binding to the disk membrane, vesicle aggregation, and the light-scattering signal. However, treatments which block GTP-dependent activation of PDE hydrolytic activity (colchicine, GDP, or ethylenediaminetetraacetic acid) also block these phenomena. Thus, GTP-dependent activation of PDE rather than its hydrolytic activity appears to be correlated with the light-scattering signal.

Many near-infrared light-scattering signals have been reported to occur in rod outer segment membrane preparations in vitro (Hofmann et al., 1976; Uhl et al., 1978, 1979a,b; Bignetti et al., 1980; Kuhn et al., 1981; Vuong et al., 1984; Borys et al., 1983; Thacher, 1983; Lewis et al., 1983; Caretta & Stein, 1985; Kamps et al., 1985). Bignetti et al. reported a light-dependent decrease in turbidity of rod outer segment membrane suspensions in the presence of extrinsic membrane proteins and GTP. The binding and dissociation signals reported by Kuhn et al. (1981) and the P signal reported by Hofmann et al. (1976) were proposed to be related to the binding and guanine nucleotide dependent release of the G protein[1] from disk membranes. A larger infrared scattering signal (G+) also related to the presence of GTP and extrinsic membrane proteins has been reported by Lewis et al. (1983).

In a previous paper (Caretta & Stein, 1985), we described changes in near-infrared light scattering of rod outer segment (ROS) membranes dependent on bleached rhodopsin, G protein, PDE, GTP, and cGMP. Under the same conditions, disk vesicle–disk vesicle aggregation–disaggregation can be observed. We have continued our investigation of the cGMP- and PDE-dependent light-scattering change in order to identify the molecular events responsible for the light-scattering changes and the aggregation–disaggregation phenomenon. We have examined the partitioning of extrinsic membrane proteins between the membrane and supernatant fraction during light-scattering experiments. PDE binding to the membrane increases during the membrane aggregation (decreased infrared light transmission) phase, and PDE binding is reduced during the membrane disaggregation phase (increased infrared light transmission). Our results indicate that PDE undergoes a light- and nucleotide-dependent binding to the membranes which appears to correlate with both the aggregation dynamics of disk vesicles and the concurrent light-scattering changes.

Materials and Methods

Membranes were prepared as previously described (Caretta & Stein, 1985). Fresh calf eyes were dissected under dim red light (Kodak wratten filter 1), and the retinas were shaken

† This work was supported by National Institutes of Health Grant EY 05074-01A1 (P.J.S.), by AFOSR Grant 84-NL-024 (P.J.S.), by Research to Prevent Blindness, Inc., Grant EY00085, and by the Connecticut Lions.

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[1] Abbreviations: G protein, three polypeptide subunits of M_r 39 000, 37 000, and 6000 which comprise the GTPase; PDE, three polypeptide subunits of 94, 92, and 13 kilodaltons which comprise phosphodiesterase; cGMP, guanosine cyclic 3′,5′-phosphate; 8Br-cGMP, 8-bromoguanosine cyclic 3′,5′-phosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; ROS, rod outer segment(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0006-2960/86/0425-2335$01.50/0