PREPARATION AND PROPERTIES OF BOVINE HEART CYTOCHROME C OXIDASE WITH HIGH SOLUBILITY IN DETERGENT FREE MEDIA

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Summary. Cytochrome c oxidase is isolated from bovine heart by a procedure that involves differential precipitation, fractionation with ammonium sulfate in 0.5% cholate, and removal of residual cholate by molecular sieve chromatography. The oxidase is highly active and is unusually soluble in phosphate buffer without added detergent; solutions with several millimolar concentrations, yet low viscosities, are readily prepared. The preparation contains ca. 20% lipid with a Cu to Fe ratio of 1:1. Intensities of visible and Soret bands in oxidized and reduced states are ca. 25% lower than in the presence of detergent (0.75% Tween 20). Oxidized cytochrome c inhibits and binds more tightly than does the reduced species (K_I, 18 μM; K_m, 25 μM) as noted in mitochondria.

Introduction. The central role of cytochrome c oxidase in mitochondrial energy production has resulted in great interest in this complex lipoprotein (∿ 20% lipid) with multiple protein, heme, and copper components. Structure-function relationships have been probed in many studies but much remains unclear (1-5). Serious limitations for the application of several physical probes have been the low solubilities and the requirement of detergent to achieve even that level of solubility. We report here a convenient method for the isolation of the oxidase from bovine heart in a form which is very soluble in phosphate buffer without added detergent. The isolation of 1 to 1.5 g of oxidase in 48 hrs with exposure to cholate for less than 8 hrs is described. The high fluidity at millimolar concentrations makes it possible to carry out infrared, Mössbauer, magnetic resonance, magnetic susceptibility, and other studies to an extent not feasible with earlier preparations. Significant effects of detergent on the oxidase are also shown.

Methods and Materials. Keilin-Hartree particles from 1600 g of bovine heart mince were prepared essentially according to the procedure of Keilin and Hartree (6). All procedures were carried out at 0-4°. Ammonium sulfate

fractionations were done with solid $(NH_4)_2SO_4$ (Schwarz-Mann, enzyme grade) while maintaining the pH at 7.4 with 3N NaOH. Centrifugation was conducted at 30,000 x g for 10 min unless otherwise stated. Absorption spectra were recorded on a Cary 17 spectrophotometer at room temperature.

Protein was measured by the procedure of Lowry (7) using human serum albumin (Nutritional Biochemicals Corporation) as the standard. Copper was determined by the method of Wharton and Rader (8) and iron by the procedure of Doeg and Ziegler (9). Non-heme iron was estimated by the methods of Doeg and Ziegler (9) and Massey (10). Total phosphorus was determined by the procedure of Chen et al., (11). Oxidase activity was determined spectrophotometrically (12) as first order rates in terms of s^{-1}/mg protein/3 ml in 0.1 M Na phosphate buffer at pH 5.9 and 22° with 15 μM reduced cytochrome c; the reaction was initiated by addition of 10 μl of solution containing oxidase with 100 μg of protein per ml.

Results and Discussion. To prepare cytochrome c oxidase 460 ml Keilin-Hartree particles in 0.2 M phosphate buffer is treated with 40 ml of ∿ 40% cholate (in a specific case: total protein was 25.5 g; oxidase activity, 2.0)* and the mixture adjusted to 33% saturation in $(NH_4)_2SO_4$. After 30 min the precipitate is removed by centrifugation for 20 min. The supernatant containing the cytochromes is adjusted to 50% saturation in $(NH_4)_2SO_4$ and centrifuged for 20 min.

The precipitate is dissolved in 0.1 M phosphate buffer, 0.5% cholate, pH 7.4 to a concentration of 75 μM in heme a (total protein 8.55 g; activity 4.8; total heme a 11.5 mg) and dialyzed for 2 hrs against 0.04 M phosphate buffer pH 7.4. The turbid solution is centrifuged for 60 min at 45,000 x g and the supernatant discarded. The dialysis - centrifugation procedure results in removal of ∿ 70% of cytochromes b and c_1 with complete recovery of the oxidase and allows us to reduce the number of $(NH_4)_2SO_4$ fractionations for final puri-

*The progress of the isolation is reflected in values for total protein, oxidase activity, and total heme a enclosed within parentheses at several steps for a specific case.

fication. The precipitate is dissolved in 0.1 M phosphate buffer, 2% cholate, pH 7.4 (total protein 3.98 g; activity 6.7; total heme a 11.0 mg) and adjusted to 25% saturation in $(NH_4)_2SO_4$. After 30 min the precipitate is removed by centrifugation, the supernatant is brought to 45% saturation in $(NH_4)_2SO_4$ and centrifuged.

The precipitate is dissolved in 0.1 M phosphate buffer, 0.5% cholate, pH 7.4 at 60-70 μM in heme a (total protein 3.4 g; activity 7.4; total heme a 10.9 mg), adjusted to 25% saturation in $(NH_4)_2SO_4$ and left for 30 min. The precipitate (cytochromes b and c_1) is removed by centrifugation. The supernatant is adjusted to 40% saturation in $(NH_4)_2SO_4$ and centrifuged. This precipitate is dissolved in 0.1 M phosphate buffer, 0.5% cholate, pH 7.4 (total protein 1.9 g; activity 8.3; total heme a 10.9 mg) and fractionated as above except that the second fractionation is at 35% saturation in $(NH_4)_2SO_4$. Ammonium sulfate fractionation in the presence of cholate results in the precipitation of cholate leading to an increase in detergent concentration. Thus the use of a cholate concentration as low as 0.5% is necessary to maintain a low concentration of cholate.

The precipitate is dissolved in 0.1 M phosphate buffer, 0.75% Tween 20, pH 7.4 at a concentration of 50-60 μM in heme a (total protein 1.6 g; activity 9.3; total heme a 10.4 mg). The solution is adjusted to 25% saturation in $(NH_4)_2SO_4$, centrifuged, and the supernatant is brought to 35% saturation in $(NH_4)_2SO_4$. The precipitate (cytochrome c oxidase), collected by centrifugation, is dissolved in 0.1 M phosphate buffer, 0.75% Tween 20, pH 7.4 at a concentration of 20-25 mg/ml (total protein 0.91 g; activity 15.4; total heme a 10.0 mg).

The isolated oxidase is chromatographed on a Sephadex G-100-120 column (2.5 x 50 cm) equilibrated with 0.1 M phosphate buffer, 0.5% Tween 20, pH 7.4 in order to remove residual cholate. Helenius and Simons (13) have demonstrated that molecular sieve chromatography provides a convenient method for changing the detergent milieu of lipophilic proteins. The oxidase from the

column is precipitated at 33% $(NH_4)_2SO_4$. The precipitate is dissolved in 0.1 M phosphate buffer, 0.5% Tween 20, pH 7.4 and rechromatographed. The oxidase from the second chromatography is precipitated as above, dissolved in 0.01 M phosphate buffer pH 7.4 and dialyzed for 16 hrs against 0.01 M phosphate buffer pH 7.4

The spectrum of reduced versus oxidized oxidase in 0.01 M phosphate buffer pH 7.4 indicates the absence of other cytochromes (Figure 1). The pyri-

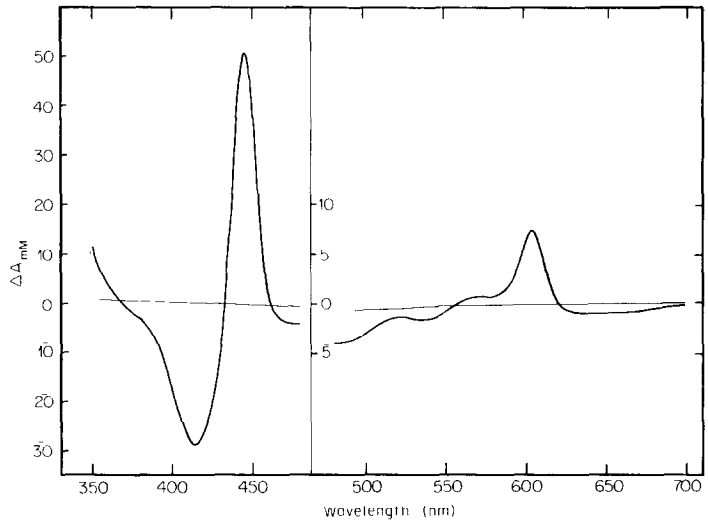

Figure 1: Absorption difference spectrum in visible and Soret regions of reduced cytochrome c oxidase vs oxidized oxidase in 0.01 M phosphate buffer showing the absence of other cytochromes. A_{mM} values are based on total iron.

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dine hemochromogen prepared directly from the oxidase revealed no hemes other than heme a. The chemical composition was similar to the best preparations from other methods. Our preparations uniformly exhibit copper:iron ratios near to 1.0 (in no instance greater than 1.1) and contain 9.7-11.7 nmoles copper/mg protein and 9.5-11.7 nmoles iron/mg protein. No non-heme iron was detected. The consistency of this method is shown by the fact our last 15 preparations have had 10.9 ± 0.1 nmoles iron/mg protein. The phospholipid content based on total phosphorus is 0.20 mg/mg protein. Uniformly activities of 18.6

TABLE I

Absorption Maxima And Extinction Coefficients of Cytochrome c Oxidase With And Without Detergent[a]

	Oxidized[b]					
Band maxima (nm)	418	515	545	598	660	830
A_{mM} with detergent	79	8.3	8.2	8.7	2.4	1.2
A_{mM} without detergent	59	6.3	6.3	6.6	2.0	1.1
	Reduced[c]					
Band maxima (nm)	443	517	560	603		
A_{mM} with detergent	100	7.2	7.7	19.3		
A_{mM} without detergent	78	5.3	6.3	14.5		

[a] The precipitated oxidase from the final chromatographic step was dissolved in either 0.1 M phosphate buffer, 0.75% Tween 20, pH 7.4 or 0.01 M phosphate buffer pH 7.4. A_{mM} values are based on total iron.
[b] As obtained.
[c] Reduction carried out with slight excess of sodium dithionite.

and 15.4 ± 0.3 were found in Na phosphate buffers of 0.075 M and 0.10 M concentrations respectively. These activities are high compared with values reported for roughly comparable experimental conditions (14): 4.5 (15), 5.64 (16), 14.2 (17) and 16 (14).

Yields of oxidase were typically about 1 g (900 to 1500 mg) which is greater than 2.5 times the yield obtained by the method of Kuboyma et al., (14).

The effect of detergent on visible-uv absorption maxima of oxidase is shown in Table I and Figure 2. The values of the detergent treated oxidase are similar to previously reported values (14,15); however without detergent there is ca. 25% decrease in intensity. Thus the use of Tween 20 for dissolution of the oxidase results in a hyperchromic effect without a wavelength shift. However, if the oxidase is originally dissolved in 0.01 M phosphate buffer pH 7.4, the subsequent addition of detergent (up to 0.75% Tween 20) is not observed to affect the spectrum. Myer (18) has indicated detergent may

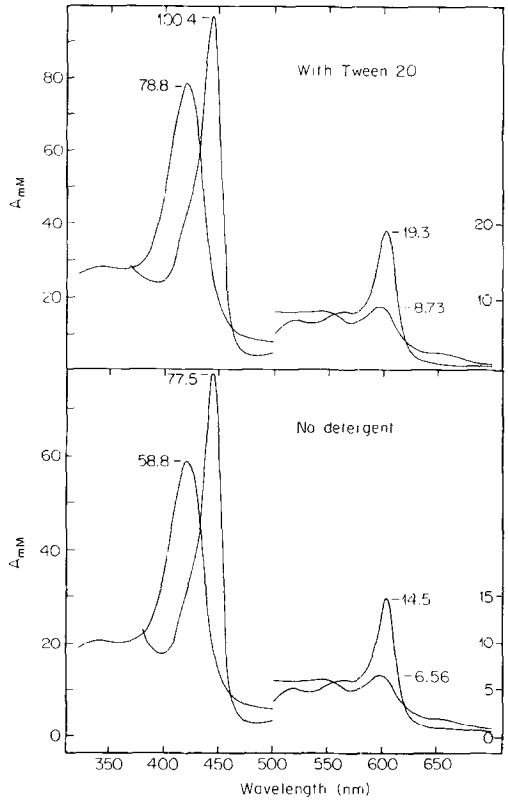

Figure 2: The absorption spectra in the visible and Soret regions of oxidized and reduced oxidase. The upper spectra are for oxidase dissolved in 0.1 M phosphate buffer, 0.75% Tween 20, pH 7.4. The lower spectra are for oxidase dissolved in 0.01 M phosphate buffer pH 7.4 (i.e., no detergent present). A_{mM} values are based on total iron.

induce alterations in the environment of the prosthetic group and has shown increased concentrations of detergent to increase ellipticity with no apparent alterations to the spectrum. The presence of detergents may well alter (reduce) π-π interactions between heme a molecules or heme a and protein. Since detergents can bind hydrophobically to lipoproteins (13), detergent treatment may remove lipid with exchange of bound lipid by bound detergent which may form micelle-like regions on the surface of the protein. Such exchanges can result in conformational differences between isolated and membrane bound oxidase. Our results suggest the conformation of the oxidase is affected by detergent.

The K_m and K_I values for ferro and ferri cytochrome c are 25 μM and 18 μM respectively. The K_m for ferrocytochrome c is similar to that obtained for Keilin-Hartree particles (19). In intact membranes Dutton et al., (20) indicate, based on a decrease in redox potential of bound cytochrome c, that the ferri form is bound more tightly. Nicholls and Chance (21) note this has not been the case for isolated oxidase which has been reported to bind both oxidation states equally well (22,23). However, our kinetic data are consistent with a tighter binding of the ferri form to the oxidase, as is the case for intact membranes, suggesting little modification of the enzyme has occurred during isolation. Thus our preparation in detergent-free media may represent a more native form than is the case in detergent solubilized preparations.

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