THE ISOLATION OF CYTOCHROME b FROM BEEF HEART MITOCHONDRIA*

Ralph Bomstein, Robert Goldberger** and Howard Tisdale

Institute for Enzyme Research
University of Wisconsin
Madison, Wisconsin

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Since the identification of cytochrome b by Keilin (1925) there have been numerous reports of the isolation of this hemoprotein in various stages of purity from mammalian heart muscle (Hübscher et al, 1954; Widmer et al, 1954; Sekuzu and Okunuki, 1956; and Feldman and Wainio (1959). The preparations reported by Sekuzu and Okunuki (1956) and by Feldman and Wainio (1959), obtained through the use of proteolytic and lipolytic enzymes, were shown to be free from associated enzymatic activities and from contamination by other cytochromes. However, the incomplete physical data given by both groups render rigid assessment of purity a difficult matter. In the present work an account is given of a procedure for the isolation and purification from beef heart mitochondria of a cytochrome b that appears by spectral, ultracentrifugal, and chemical standards to be the purest yet reported. In contrast to the methods used by other groups (Sekuzu and Okumuki, 1956; and Feldman and Wainio, 1959), this method avoids the damage to the protein which may attend exposure to proteolytic and impure lipolytic enzymes.

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** Post-doctoral Trainee of the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin.

Beef heart mitochondria, prepared as previously described (Crane et al, 1956), were diluted to a protein concentration of 30 mg/ml by homogenization with 0.9% KCl. A solution of sodium deoxycholate was added to give a final detergent concentration 1.35%. The suspension was then centrifuged in a Spinco Model L preparative ultracentrifuge in a #30 rotor (all centrifugations were made in this way unless otherwise specified) for 60 minutes. The supernatant was made 1.7% (W/V) with respect to sodium cholate, 2% (W/V) with respect to Duponol C* (sodium lauryl sulfate), and 30% saturated with respect to ammonium sulfate (solid). The turbid suspension was then centrifuged. Solid ammonium sulfate was added to the supernatant to 50% saturation, and the suspension centrifuged. The resulting orange sediment was dissolved in sufficient 0.1 M phosphate buffer, pH 7.4, to give a protein concentration of 15 mg/ml. The solution was heated to 40°C. for 5 minutes and the resulting precipitate removed by centrifugation. Solid ammonium sulfate (10.6 g/100 ml) was added to the supernatant; the suspension was heated at 35° for 5 minutes and then centrifuged. Sodium cholate (3 mg/mg protein) and ammonium sulfate (6.9 g/100 ml) were added to the supernatant and the solution was heated at 40° for 5 minutes. Centrifugation of this suspension gave a blood-red sediment which was essentially cytochrome b and represented 40% of the cytochrome b present in the starting material. It had a heme:protein ratio of 12.2 μM/g based on a molar extinction coefficient of 20.7 $mM^{-1} cm^{-1}$.** Examination of the direct spectrum of the material isolated at this point in the procedure failed to reveal the presence of any cytochrome other than cytochrome b. However, application of the differential heme method of Basford (1957) enabled detection of trace amounts of cytochrome c_1.

This material was purified further by the following procedure. The blood-red sediment was homogenized in a sufficient quantity of 0.25 M sucrose to give a protein concentration of 10 mg/ml. This suspension was clarified by adding

* E. I. Dupont, Wilmington, Delaware

** This figure was calculated from the value obtained by B. Chance (1952) for the pyridine hemochromogen of cytochrome b.

Duponol C to a final detergent concentration of 0.3% (W/V). Ammonium sulfate (2.6 g/100 ml) was added, the suspension was centrifuged, and supernatant discarded. The final preparation of cytochrome b was then obtained by repeating the above procedure with sucrose, Duponol C and ammonium sulfate, and homogenization of the residue in 0.10 M phosphate buffer, pH 7.4. All operations in the isolation procedure were performed at 0-5° unless otherwise specified.

The cytochrome b thus obtained had 36 μM of heme per gram of protein, and represented 30% of all the cytochrome b in the original suspension of beef heart mitochondria. The iron content of this preparation, determined by the method of Smith et al (1952), was 0.18%. The absorption maxima (see Fig. 1) in the dithionite-reduced form were at 562, 532 and 429 mμ. The preparation contained less than 0.04% phospholipid phosphorous, and less than 1 mole of flavin per 65 moles of heme. No cytochrome a, c or c_1 heme was detected upon application of the differential heme method. It was auto-oxidizable and had no succinic or DPNH dehydrogenase activity associated with it. It could be dispersed with 0.001 M Duponol C, but precipitated at 5% saturation with ammonium sulfate. Sedimentation studies* of such a dispersion in a Spinco Model E analytical ultracentrifuge indicated the presence of large aggregates with "molecular weights" of at least two million. After solubilization of the cytochrome b in 0.015 M cetyldimethylethylammonium bromide (CDEAB), however, precipitation with ammonium sulfate was accomplished only at 70% saturation. In analytical ultracentrifuge studies of the CDEAB-solubilized cytochrome b (CDEAB at 0.004 M) the hemoprotein sedimented with a single boundary with which all the color was associated. The sedimentation coefficient s_{-10}, was calculated to be 2.6 Svedberg units. From this value and from data obtained using the Archibald approach to equilibrium method, an average molecular weight of 20,000 was calculated. (The partial specific volume, .748, determined by Strittmatter and Velick (1956) was used in calculating this molecular weight.) Calculation of the molecular weight from iron

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analysis gave a value of 30,000; from spectral data, 28,000. The lack of agreement may well indicate that the cytochrome b obtained needs further purification.

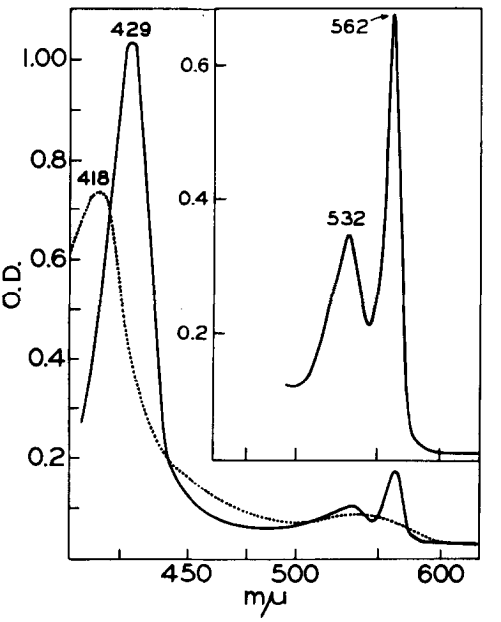

Fig. 1. Direct Absorption spectra of cytochrome b recorded with a Beckman DK-2 spectrophotometer. The cytochrome was dispersed with M/1000 Duponol C in M/100 phosphate buffer, pH 7.4, at final concentrations of 0.20 mg. protein per ml. (complete spectrum) and 0.88 mg. protein per ml. (insert).

The insolubility of the cytochrome b in the absence of CDEAB is apparently an intrinsic property of this hemoprotein, and is a consequence of the formation of large aggregates rather than a reflection of heat denaturation. This conclusion is reached from the following considerations. Cytochrome b was prepared by us in one step without the use of heat from an enzymatically functional cytochrome b-c_1 complex. This preparation was also insoluble in buffers, in sodium cholate and in sodium deoxycholate, but could be dispersed with Duponol C. Further studies on the isolation and purification of cytochrome b will be reported in a later communication.

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