ISOLATION AND PROPERTIES OF MAMMALIAN CYTOCHROME c_1*

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Since the discovery of cytochrome c_1 in 1940 by Yakushiji and Okunuki (1940), numerous attempts to purify the hemoprotein have been reported (Yakushiji and Okunuki, 1941; Glaze and Morrison, 1960; and Sekuzu et al, 1960). The meager data supplied for most of these preparations render difficult any assessment of their purity, yield, and ability to undergo enzymic oxidation or reduction. Up to the present time the cytochrome c_1 prepared in this laboratory by Green et al (1959) was the purest reported; it contained 14.7 μmoles of heme per g of protein. Sekuzu et al (1960) have recently reported on a preparation of cytochrome c_1 which contained 10.1 μmoles of iron per g of protein (no statement was made concerning the contribution of non-heme iron to this figure). Their preparation was also oxidizable and enzymically reducible, although no kinetic studies were presented. By the procedure which follows, cytochrome c_1 was prepared from beef heart mitochondria in a form in which it could readily be reduced by a variety of enzyme preparations. The preparation of highest purity contained 26.2 μmoles of heme per g of protein (average purity 18.5); the highest yield was 23% (average yield 14%).

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All operations in the isolation procedure were performed at 0-5° unless otherwise specified, and all centrifugations were carried out in a Spinco Model L Ultracentrifuge at 30,000 r.p.m. Mitochondria were prepared by the method of Crane et al (1956) for "KCl mitochondria", and the final protein concentration adjusted to 65 mg/ml. A solution of sodium deoxycholate was added to beef heart mitochondria to give 0.4 mg of bile salt per mg of protein. After centrifugation of this suspension, the residue was discarded, and to the supernatant were added a solution of sodium cholate (to give 1.0 mg per mg protein), Duponol C* (to give a concentration of 2%) and solid ammonium sulfate (to give 17 mg/ml). The suspension was centrifuged, and the residue was again discarded. Solid ammonium sulfate was added to the supernatant to give 45% saturation, and the suspension was centrifuged. The sediment was dissolved in 0.1 M phosphate buffer, pH 7.4. Saturated ammonium sulfate solution was added to a final concentration of 5%, and the solution was heated to 40° for 5 minutes. The precipitate was removed by centrifugation and discarded. To the supernatant were added solutions of sodium cholate (to give 3 mg/mg protein) and saturated ammonium sulfate (to give 21% saturation). The turbid suspension was heated to 30° and the precipitate removed by centrifugation and discarded. This procedure of heating to 30° was repeated three more times with successively higher concentrations of ammonium sulfate (26%, 30% and 36%). To the final supernatant (which contained 2.5 μmoles of cytochrome c_1 heme per g of protein) was added a solution of sodium cholate to give 0.02 g/ml. The pH was then adjusted to 7.1 and the solution was frozen. After thawing, the suspension was centrifuged, and the pink precipitate which had formed during the freezing process (a cytochrome c_1-cholate complex) was collected and washed several times with 2.0 M KCl. The precipitate was then dissolved in 0.01 M phosphate buffer, pH 7.4. The hemoprotein was adsorbed onto calcium phosphate gel which had been prepared by the method of Singer (1950). After washing the protein gel suspension eight times with 0.01 M phosphate buffer, pH 7.4, the hemoprotein was eluted from the gel with 1.0 M phosphate buffer, pH 7.4.

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The composition and some properties of cytochrome c_1 are summarized in Table I. Solutions of the purified hemoprotein were frozen and thawed repeatedly in the absence of any detergent without loss of solubility and without change in spectral or enzymatic characteristics.

The reduction of cytochrome c_1 was followed at 38° in a Beckman DU spectrophotometer, in the presence of a variety of enzymes: BHM* and ETP** (Crane et al, 1956), and SDC*** (Green et al, 1955). Succinate, DPNH, and reduced Coenzyme Q were employed as substrates. Reduction of cytochrome c_1 was obtained in every case (except that SDC was not tested with DPNH or with reduced Coenzyme Q), and the reduction was consistently sensitive to antimycin A. Since no attempt was made to purify an enzyme system with respect to cytochrome c_1 reductase activity, the rates were not very high (the highest rate observed, ca. 0.70 μmole/min/mg protein, was attained with ETP as the enzyme and reduced Coenzyme Q as the substrate).

When catalytic amounts of cytochrome c were added to a solution of reduced cytochrome c_1 and purified cytochrome oxidase, the rate of oxidation of the cytochrome c_1 determined spectrophotometrically at 38° was 5 μmoles/min/mg of oxidase (500 μmoles/min/μmole of oxidase). However, further experiments showed that a non-enzymic oxidation of reduced cytochrome c_1 by cytochrome c may fully account for the above results. Equimolar quantities of reduced cytochrome c_1 and oxidized cytochrome c were mixed at 0° and separated within two minutes. Separation of the two hemoproteins was accomplished by adsorption of the mixture onto a column of carboxymethylcellulose, and elution of cytochrome c_1 with distilled water, followed by elution of cytochrome c with 1.0 M phosphate buffer, pH 7.4. More than 90% of each hemoprotein was recovered. When a solution of each hemoprotein alone was adsorbed on and eluted from carboxymethylcellulose,

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* Beef heart mitochondria.

** Electron transport particle.

*** Succinic dehydrogenase complex.

TABLE I

Composition and Properties of Cytochrome $\underline{c}_1$

	$\underline{c}_1$*	$\underline{c}_1$**
Iron	23.5 μmoles/g***	26.2 μmoles/g
Heme****	23.5 μmoles/g	26.2 μmoles/g
Total flavin*****	None	None
Lipid******	3.6%	----
Sodium Cholate*******	< 5 μg/mg	< 5 μg/mg
Minimum Mol. Wt. (calculated on the basis of iron content)	42,000	38,000
Absorption Bands (Reduced form)		
Alpha	554	554
Beta	524	524
Soret	418	418
Spectral Shift with CO	None	None

* The preparation of cytochrome $\underline{c}_1$ which could be obtained routinely.

** The preparation of cytochrome $\underline{c}_1$ with the highest purity obtained.

*** Protein was determined gravimetrically.

**** The extinction coefficient used for cytochrome $\underline{c}_1$ (554 mμ-540 mμ) was 17.5×10^3 cm^{-2} (Green et al, 1959).

***** Total flavin was determined by the method previously reported by Green et al (1955).

****** Lipid was determined by total phosphorus analysis, assuming the lipid to contain 4% phosphorus.

******* Cholate was determined by the method of Mosbach et al (1954).

the recovery was also more than 90%, and no change in the state of oxidation or reduction accompanied passage through the column. The results of these experiments showed that within two minutes of contact between the two hemoproteins, 47% of the cytochrome $\underline{c}_1$ became oxidized while 48% of the cytochrome $\underline{c}$ became reduced. No change in this proportion was noted when the two hemoproteins were left in contact for as long as 8 minutes. Electron transfer between the two hemoproteins also occurred when oxidized cytochrome $\underline{c}_1$ was mixed with reduced cytochrome $\underline{c}$. In view of these observations it is possible that studies on the oxidation of reduced cytochrome $\underline{c}_1$ by cytochrome oxidase in the presence of catalytic quantities of cytochrome $\underline{c}$ have no bearing on the sequence of events within the intact mitochondrion.

More details of the isolation procedure, of the physicochemical properties, and of the oxidation and enzymic reduction of cytochrome $\underline{c}_1$ will be reported in a later communication.

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