RECONSTITUTION OF CYTOCHROME c OXIDASE FROM AN APO-ENZYME AND CU(I)

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It has not yet been possible to prove that the copper which occurs in cytochrome c oxidase is essential to its activity (1). We wish to report now that an apo-enzyme of low copper content can be prepared from purified cytochrome c oxidase in the presence of reduced cytochrome c and bathocuproine sulfonate under strictly anaerobic conditions. This apo-enzyme is relatively inactive. Upon restoring the copper with Cu(I) acetonitrile perchlorate, the enzyme regains more than its original activity. In this preliminary communication, the preparation of apo-enzyme and its reconstitution are described, and a summary of the properties of oxidase, apo-oxidase, and reconstituted oxidase is given.

Methods. Cytochrome c oxidase was prepared from beef heart particles by the method of Fowler et al. (2) modified by Vanneste (3). Cytochrome c oxidase activity was assayed by the method of Smith and Camerino (4). Reduced cytochrome c (Sigma type V) was prepared by the technique of Horie and Morrison (5). Heme a was estimated by the method of Van Gelder and Slater (6). Cytochrome a_3 was estimated from the CO difference spectrum (428.5mμ-445mμ, $A = 148\ mM^{-1}cm^{-1}$) (3). Pyridine hemochromogen a was determined by the method of Morrison and Horie (7). Protein was determined by the biuret procedure with correction for heme absorption. Copper was determined with bathocuproine sulfonate after complete digestion with H_2SO_4 and H_2O_2. Cu(II) was also determined by electron spin resonance spectroscopy using Cu(II) EDTA as a standard; when necessary, the Beinert denaturation procedure was employed

(8). $Cu(I)(CH_3CN)_4 \cdot ClO_4$ was prepared according to the method of Hemmerich and Sigwart (9). We are indebted to Dr. Andreas Zuberbuhler for this preparation.

Apo-Enzyme. A typical reaction system consisted of 8 ml. 0.05 M phosphate buffer, pH 7.0, containing 1% Tween 80, 0.2 ml. 10% sodium deoxycholate, 2 ml. 0.003 M bathocuproine sulfonate in H_2O, 80 - 100 mumoles of cytochrome c oxidase in Tris-sucrose-histidine buffer, pH 8.0 (2), and 500 mumoles of cytochrome c in a final volume of 12 ml. The system was very carefully deoxygenated at 0°, but was allowed to come to room temperature (15 minutes) before adding 3 ml. deoxygenated 0.5 M ascorbate containing 0.001 M EDTA and adjusted to pH 7.0. The reaction was allowed to proceed for 5 minutes. Saturated ammonium sulfate (7.5 ml. pH 7.8) was then added. After 5 minutes, the precipitate was centrifuged and dissolved in 4 ml. 0.1 M phosphate buffer, pH 7.4, containing 1% Tween 80. The solution was dialyzed against 2 liters of 0.02 M phosphate buffer containing 1% Tween 80 for 3 hours (0°). For ESR study, two or more such preparations were combined, reprecipitated with ammonium sulfate, and dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4, containing 1% Tween 80.

Reconstitution. $Cu(CH_3CN)_4 \cdot ClO_4$, 0.001 M in acetonitrile, was added to apo-enzyme in Tween-phosphate buffer, pH 7.4, to a molar ratio to heme a of one-to-one. The reaction mixture was incubated aerobically at 4°; the original activity of the enzyme was recovered in 30 minutes. Continued increase of activity was observed for three hours. This reconstitution has been repeated ten times without failure. No activity was observed in the following controls: (1) reconstituted enzyme plus ascorbate only, (2) cytochrome c plus ascorbate only, (3) complete assay system plus apo-enzyme, (4) complete assay system with reconstituted boiled enzyme, (5) complete assay system minus enzyme plus bovine serum albumin plus $Cu(I)(CH_3CN)_4$, (6) complete assay system minus enzyme plus Cu(I) bathocuproine sulfonate, and others. In all experiments with reconstituted cytochrome c oxidase except study of reconsti-

tution rates, enzyme was reprecipitated with ammonium sulfate and redissolved in phosphate-Tween buffer pH 7.4.

Properties of enzyme, apo-enzyme, and reconstituted enzyme. The compositions and activities of cytochrome c oxidase and the apo-enzyme and reconstituted oxidase prepared from it are summarized in Table I. The following notable characteristics of the apo- and reconstituted oxidase were observed:

(1) The activities of the fully reconstituted enzymes were three- to four-fold greater than the activity of the original enzymes. We conclude that the original enzyme, a representative purified cytochrome c oxidase, was unsaturated with respect to copper.

(2) Apo-enzyme contained 45% of the original total copper per heme a, but less than 15% of the activity. We conclude that detergent-dispersed oxidase is an aggregate containing internal and exposed active sites. Apo-enzyme appears to have lost essential exposed copper. The activity of apo-enzyme could only be observed at high protein concentrations, upon which it showed non-linear dependence. We suggest that at high protein concentrations sub-unit interchange occurs with the exposure of internal active sites.

(3) No outstanding qualitative differences existed among the optical spectra of the oxidized, reduced, and CO-complexed states of original, apo- and reconstituted enzymes. However, cytochrome a_3 in apo-enzyme was reduced more slowly than cytochrome a_3 in the original or reconstituted enzymes. It is probable that the presence of exposed or external copper facilitates reduction of cytochrome a_3. The 830 mμ band is now under study.

(4) No outstanding difference existed among the ESR spectra of original, apo-, or reconstituted enzymes. Reconstitution appeared to result in replacement of copper at the ligand site which gives rise to the typical signal of ESR-detectable copper in cytochrome c oxidase, and no other.

(5) The ratio of ESR-detectable to ESR-indetectable copper was the same in original and apo-enzymes. This supports the conclusion in paragraph 2. ESR-detectable and ESR-indetectable copper appear to have been removed in proportion to their ratio of occurrence.

Table I.

Characteristics of Cytochrome c Oxidase, Apo-Enzyme, and Reconstituted Oxidase

Preparation	Heme a/Cytochrome a_3	ESR-Detectable Cu(II) umoles/heme a	Cu/Heme a	Activity, sec^{-1}
I	2.5	0.66	1.06	440
II	2.2	0.47	1.03	457
Apo-I	2.3	0.32	0.45	44[a]
Apo-II	2.1	0.22	0.49	68[a]
Apo-II (another preparation)	2.08	0.29	0.48	80[a]
Reconstituted-I	3.1	0.40	1.42[b]	1,500
Reconstituted-II	3.75			1,500
Reconstituted-II (another preparation)	3.0	0.55	1.91[b]	1,350

a. No activity of apo-enzyme could be observed at enzyme (heme a) or protein concentrations at the same levels as used in the assays of original enzyme or reconstituted enzyme. In order to observe activity, 25-30 times that enzyme concentration was used.

b. From ESR-detectable Cu(II) after denaturation by procedure of Beinert and Palmer (8). May contain some Cu(II) arising from Cu(I) BCS.

(6) The ratio of cytochrome a3 to total heme a decreased in passing from original to reconstituted enzyme. The significance of this change and its relationship to copper content of the enzyme is being examined.

(7) The preparation of apo-enzyme failed in the presence of oxygen. The preparation of apo-enzyme was much less efficient in the presence of anaerobic ascorbate only compared to anaerobic ascorbate plus cytochrome c.

(8) The apo-enzyme and reconstituted enzyme contained bound BCS and BCS-Cu(I), respectively, and it is possible that $Cu(CH_3CN)_4$ may have reactivated apo-enzyme by removing inhibitory BCS rather than by replacing essential copper. However, this is improbable for the following reasons: (1) BCS does not inhibit cytochrome c oxidase activity at concentrations ten-fold greater than used here for copper removal (10, 11). (2) BCS has no effect upon the activity of dithionite-reduced cytochrome c oxidase (12). (3) BCS-containing oxidase prepared by the aerobic apo-enzyme procedure (see (7) above) is fully active.

Summary

Under anaerobic conditions, copper can be removed from reduced cytochrome c oxidase with concomitant loss of enzymic activity. Upon replacement of copper with Cu(I) acetonitrile, more than the original activity is regained. It is therefore probable that the enzymic activity of cytochrome c oxidase is copper-dependent.

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