ISOLATION OF A HEME BINDING SUBUNIT FROM BOVINE HEART CYTOCHROME c OXIDASE

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SUMMARY--A subunit which retains heme has been isolated and purified up to a homogenous form on polyacrylamide gel electrophoretic column in the presence of sodium dodecyl sulfate and β-mercaptoethanol from cytochrome oxidase. The separation of the subunit does not rely on any detergent except cholate used in the preparation of cytochrome oxidase. The purification involves a reaction with pyridine, pH precipitation, and DEAE-cellulose column chromatography. The purified subunit has a molecular weight of 11,600 daltons and contains more than 40 nmol Fe per mg protein; the lower iron content than the calculated value is apparently due to the loss of heme a in the course of the purification. The subunit is freely soluble in aqueous solution at neutral pH to give a dark green color. Spectral properties and amino acid composition of this subunit have been studied.

Many reports (for example, Refs. 1-4) on the subunit of cytochrome oxidase have appeared in the literature. A very critical point to be revealed is which subunit(s) binds the heme. It is generally agreed that the active cytochrome oxidase is composed of cytochromes a and a_3, as originally defined by the discoverers (5), although their prosthetic groups are most probably identical, i.e. heme a. Our recent evidence suggests that there are two heme binding subunits in the oxidase; they are subunits of molecular weights of 40,000 and 11,600 daltons. This communication reports the isolation, purification and some properties of the latter.

MATERIALS AND METHODS

Lipid deficient cytochrome oxidase, which contained less than 0.1% phospholipid, was prepared according to the method developed in this laboratory (6). The final purified enzyme, with heme a content of 12 nmol per mg protein, was taken up in 50 mM phosphate buffer containing 1% cholate to a final protein concentration of 65 mg/ml. The preparation was kept at -20° until use.

Heme concentration in the native enzyme was determined by spectrophotometric method (6) and in the isolated subunit by analysis of iron (7). Protein was estimated as usual by biuret method in the presence of hydrogen peroxide (8). All spectra were obtained in a Cary spectrophotometer, model 14, at room temperature.

RESULTS AND DISCUSSION

Isolation of the subunit containing heme a--The key steps involved in the isolation are reaction with pyridine, precipitation with acid, fractionation by ammonium sulfate and DEAE-cellulose column chromatography. All operations were conducted at 0-4°, unless otherwise indicated.

Twenty ml of lipid-deficient cytochrome oxidase were diluted to a protein concentration of 15 mg per ml by 65 ml of 50 mM phosphate buffer, pH 7.4. To the solution was added slowly an equal volume of pyridine with constant stirring. Stirring was continued for 20 minutes. The mixture was then centrifuged for one hour at 25,000 x g. All of the subunits of molecular weight of 40,000 and most of the subunit of molecular weight 21,000, together with the undissociated oxidase, were removed in the precipitate. The supernatant solution, which contained the heme a-binding subunit, other smaller subunits and free heme a, was diluted with 340 ml of water. The diluted solution was allowed to stand for 20 minutes. The precipitate was removed by centrifugation at 25,000 x g for 20 minutes. The precipitate thus obtained contained most of the free heme a and the unwanted proteins.

The supernatant solution was acidified with 10 N HCl to pH 4.5 and solid ammonium sulfate was added to 25% saturation. After 20 minutes the precipitate thus formed was collected by centrifugation. The supernatant solution was either discarded or saved for the further isolation of other subunits. The precipitate was dissolved in 25 ml of 50 mM phosphate buffer, pH 7.4, containing 4 M urea. The pH was adjusted to 7.4 by the addition of N NaOH. The solution was then brought to pH 8.5 by the further addition of NaOH before the pH fractionation. The precipitate collected between pH 5.8 and 4.5 by the addition of N HCl was dissolved in about 20 ml of 5 mM phosphate buffer containing 4 M urea, pH 7.4. A DEAE-cellulose column (0.9 x 15 cm) was equilibrated with 5 mM phosphate buffer containing 4 M urea. The acid fraction containing the heme subunit was placed onto the column. The column was then washed with two bed volumes of

TABLE I. Amino Acid Composition* of the Isolated Heme Binding Subunit of Cytochrome Oxidase

Amino acids	μmole/mg subunit	Mole/ subunit**	Amino acids	μmole/mg subunit	Mole/ subunit**
Lysine	0.45	5.2	Glycine	0.52	6.0
Histidine	0.22	2.6	Alanine	0.60	7.0
Arginine	0.46	5.3	Valine	0.58	6.7
Aspartic acid	0.86	10.0	Methionine	0.09	1.0
Threonine	0.39	4.5	Isoleucine	0.43	5.0
Serine	0.25	2.9	Leucine	0.74	8.6
Glutamic acid	0.86	10.0	Tyrosine	0.26	3.0
Proline	0.48	5.6	Phenylalanine	0.25	2.9

Cystine and tryptophan were not determined.

*These values are identical for the 23,000 and 11,600 dalton subunits as described in the text. The protein was hydrolyzed with 6 N HCl under argon in the presence of 0.05% thioglycolic acid at 110° for 24, 48, and 72 hours and the amino acids were determined in an automatic amino acid analyzer, Beckman model 120-C. The values for serine, threonine and valine are calculated by zero time extrapolation of hydrolysis (cf. Table III of Ref. 1).

**These values are calculated based on the molecular weight of 11,600 daltons determined by SDS-gel electrophoresis as described in the text. It may be cautioned that the molecular weight by such kind of determination must be considered provisional.

5 mM phosphate-4 M urea and eluted by a linear gradient of KCl formed from 100 ml of 5 mM buffer containing 4 M urea and 100 ml of 0.3 M KCl in the same buffer. The heme subunit was collected between 0.2 and 0.3 M KCl. The subunit precipitated after dialysis against 5 mM phosphate buffer, pH 5.2, for 4 hours. The precipitate was redissolved in 5 ml of 50 mM phosphate buffer, pH 7.4. The yield was found to be about 20% of the subunit of the oxidase used.

Properties of the isolated heme subunit--The purified heme subunit contained about 40 nmol heme per mg protein. The molecular weight was found to be 11,600 daltons by the conventional polyacrylamide gel electrophoresis

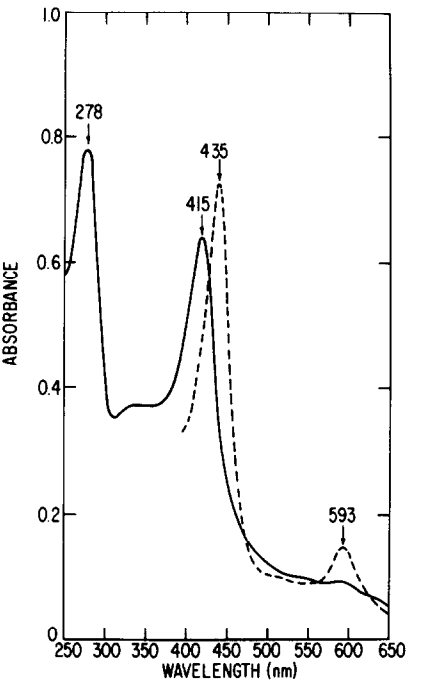

Fig. 1. Absorption spectra of an isolated heme subunit from cytochrome oxidase. The sample contained 0.38 mg of protein per ml of 50 mM phosphate buffer, pH 7.4, optical path 1 cm. Solid line, the oxidized form; dotted line, the dithionite-reduced form.

in the presence of sodium dodecyl sulfate[1]. The subunit was highly soluble in aqueous solution even in the absence of urea or detergent. However, the preparation was highly polymerized. It was eluted in the void volume of Sephadex G-150 column in the absence of dispersing detergent. When the subunit was treated with SDS in the absence of β-mercaptoethanol[1] at 37° and followed by SDS-polyacrylamide electrophoresis, two protein components with molecular weights of 23,000 and 11,6000 daltons were found. The latter was found to be associated with heme. However, when β-ME was present during the SDS treatment, only one band with molecular weight of 11,600 daltons was observed. These observations indicate that when heme a was released in the course of preparation of the subunit the latter becomes dimerized. This fact

[1]Abbreviations: SDS, sodium dodecyl sulfate; β-ME, β-mercaptoethanol.

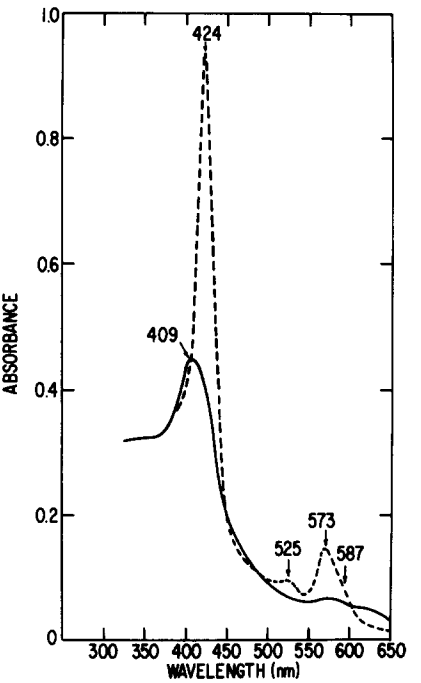

Fig. 2. Alkaline pyridine-hemochromogen spectra of an isolated heme subunit. The reaction mixture in a final volume of 1 ml contained 0.28 mg protein, 0.15 ml pyridine, and 0.1 ml of N NaOH, optical path 1 cm. Solid line, the oxidized form; dotted line the dithionite reduced form.

explains the lower heme content of the final product, compared with the predicted or calculated value of 86 nmol per mg protein. Dimerization may have been the result of the formation of disulfide linkages as the dimerized form could be cleaved by treatment with β-ME. The argument that the higher molecular weight species (dimer) may not belong to the same heme binding subunit can be excluded by the fact that both fractions show identical amino acid composition. The amino acid composition is summarized in Table I. Although it is plausible that the low heme content of the final product makes the idea of the two subunits binding one heme prosthetic group, the results[2]

[2]Unpublished results from this laboratory, see also the results of 1:1 ratio of all subunits in cytochrome oxidase in yeast (e.g. Ref. 9)

of one to one ratio of all subunits of oxidase does not seem to support the possibility.

Figure 1 shows the absorption spectra of the isolated heme subunit. Soret absorption of the oxidized form was located at 415 nm with a broad α-band at 590 nm. Upon reduction by dithionite, the Soret peak shifted to 435 nm and the α-band to 593 nm. These spectral characteristics indicated that heme environment had been drastically altered from that in native cytochrome oxidase. Furthermore, the alkaline pyridine hemochromgen spectrum (Fig. 2) shows α-peak at 573 nm with a shoulder at 587 nm, which was different from that of native oxidase with an α-band at 587 nm. The nature of the binding between heme a and protein is not clear at present and is currently under investigation. It is possible that one of the vinyl group might have been modified or even linked to the protein moiety through the formation of a thio-ether linkage, alternatively the vinyl group might be oxidized to the acetyl form as in diacetyldeutero-heme IX whose pyridine hemochromgen spectrum shows α-peak of 573 (10). This oxidation may be effected from the reaction of the Schiff base formed between the formyl group of the heme a and an ε-amino group of the protein moiety (11). Both processes may render the heme group to acid-acetone non-extractable.

Taking the evidence obtained from the topological studies (12) into consideration, this subunit might reside on the cytoplasmic side of the membrane. It was not unlikely that the isolated heme subunit may have originated from cytochrome a rather than a_3. These considerations are based on the deduction that the heme prosthetic groups and copper are bound to specific protein subunits rather than "caged" by the protein moieties.

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