THE EXISTENCE OF AN UBIQUINONE BINDING PROTEIN IN THE RECONSTITUTIVELY ACTIVE CYTOCHROME b-c_1 COMPLEX

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SUMMARY--A protein, tentatively named apo-Q-protein I, with molecular weight of 15,000[1] from the reconstitutively active cytochrome b-c_1 complex has been identified as being responsible for electron transfer between succinate dehydrogenase and ubiquinone. The identification was based on the chemical modification, proteolytic enzyme digestion, and isolation and purification of the protein to nearly pure form.

A very useful characteristic of the cytochrome b-c_1 complex (cf. Ref. 1) is its capability to reconstitute with soluble succinate dehydrogenase (SDH)[2] to form succinate-cytochrome c reductase. The reconstituted reductase (1) is antimycin-A sensitive and identical with the intact reductase in all the properties tested. On the other hand, Complex III (2), whose content of cytochromes b and c_1 is comparable to the b-c_1 complex, is completely inactive toward SDH. The difference between these two preparations is the lack or inactivation of one essential component in Complex III. Such a component is apparently required for the communication of electrons between SDH and ubiquinone (3). Recently we have investigated the b-c_1 complex with the aid of proteolytic enzyme digestion in addition to the direct isolation of the protein into a partially pure form. For simplicity, we refer to this protein as apo-Q-protein I, because we have some evidence to suggest the existence of another apo-Q-protein in the other regions

[1] All the molecular weights given should be considered only as tentative ones because they are determined by SDS gel eletrophoretic technique rather than by more accurate methods.

[2] Abbreviations used: SDH, succinate dehydrogenase; Q, ubiquinone (coenzyme Q), the subscript represents the isoprene unit on position 3 of the benzoquoid ring; PHMB, p-hydroxymercuric benzoate; SDS, sodium dodecyl sulfate; QH_2, fully reduced Q.

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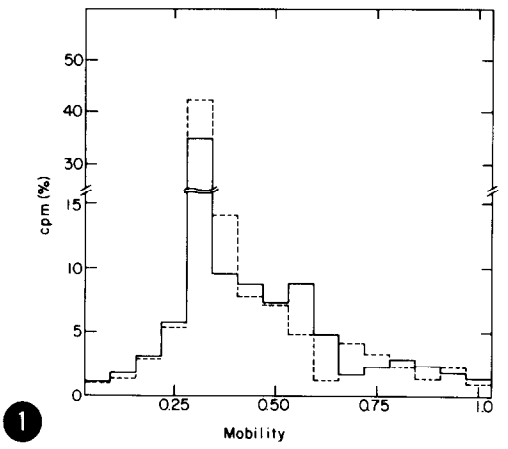
(functionally speaking) of the cytochrome $\underline{b}$-$\underline{c}_1$ complex. This communication reports the evidence for the identification of apo-Q-protein I.

MATERIALS AND METHODS

The soluble cytochrome $\underline{b}$-$\underline{c}_1$ complex (1), and succinate dehydrogenase (4) were prepared from the beef heart muscle preparation (5) according to the methods reported from this laboratory. Previously reported methods were used to prepare Complex II (6) and Complex III (2) and to estimate enzymic activities of succinate → cytochrome $\underline{c}$ (1), succinate → ubiquinone (6) and QH_2 → cytochrome $\underline{c}$ (2) at room temperature. Q_2 was synthesized from Q_0 and geraniol by the method of Shunk et al. (7) with modifications. Analytical polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (8). Radioactivity was measured by use of a Beckman LS-100 liquid scintillation counter. Horse cytochrome $\underline{c}$, type III, chymotrypsin and phenylmethyl sulfonylfluoride, a proteolytic enzyme inhibitor (all from Sigma); sodium dodecyl sulfate (SDS) (Matheson Coleman and Bell); ^{14}C-PHMB (Mann); reagents for polyacrylamide gel electrophoresis (Canalco); and other chemicals in the highest available purity were purchased commercially.

RESULTS AND DISCUSSION

We found that the inactivation of reconstitutive activity of the soluble cytochrome $\underline{b}$-$\underline{c}_1$ complex was directly proportional to the first half of the alkylable sulfhydryl groups of the complex. It became completely inactive toward SDH when half of the about 70 nmoles of PHMB reacting groups per mg protein were bound to the reagent. Under the same conditions no significant loss of QH_2-cytochrome $\underline{c}$ reductase activity was observed. Therefore, partial (50%) and complete (100%) alkylations of the $\underline{b}$-$\underline{c}_1$ complex with ^{14}C-PHMB were followed by analytical polyacrylamide gel electrophoresis in the presence of SDS and ^{14}C distribution on the gel column was then determined. We were able to identify the possible candidates for apo-Q-protein I. Figure 1 shows the ^{14}C distribution of the ^{14}C-PHMB-treated $\underline{b}$-$\underline{c}_1$ complex; the dotted line shows the results of complete alkylation and the solid line represents the first 50% alkylation. The radioactivity resulted from the initial reaction is present mainly in three regions with electrophoretic mobility of around 0.3, 0.6, and 0.9 which correspond to the subunits with molecular weights of 53,000-50,000, 28,000, and 15,000, respectively. These results indicate that the subunits of the $\underline{b}$-$\underline{c}_1$ complex with these molecular weights are the possible candidates for the apo-Q-protein I.



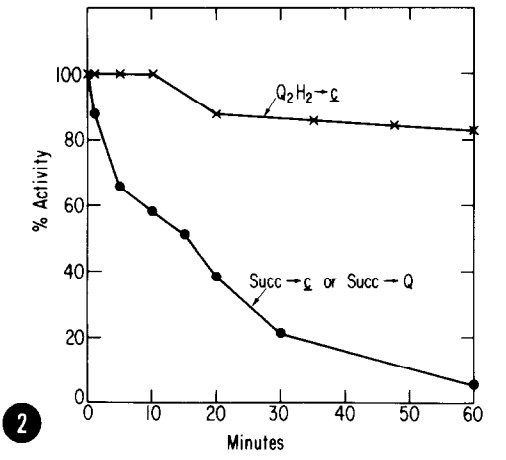


Fig. 1. Distribution of sulfhydryl groups among the subunits of the soluble cytochrome b-c complex. Solid line represents the distribution of ^{14}C isotope on the analytical polyacrylamide gel electrophoretic column of the sample which has been treated with a limited amount (35 nmoles per mg protein) of ^{14}C-PHMB. Dotted line represents the samples which have been completely alkylated with ^{14}C-PHMB. The electrophoresis was done according to Weber and Osborn, except no β-mercaptoethanol was used during the dissociation of enzyme with SDS.

Fig. 2. Chymotrypsin digestion of the cytochrome $b\text{-}c_1$ complex. The soluble cytochrome $b\text{-}c_1$ complex was made in 50 mM phosphate buffer containing 0.5% sodium cholate and 20 mM $(NH_4)_2\ SO_4$. Four ml of the enzyme solution (6 mg/ml) were preincubated at room temperature for 10 min before adding 0.12 ml of chymotrypsin solution (1 mg/ml). The ratio between chymotrypson and the $b\text{-}c_1$ complex was 1:200. The mixture was incubated at room temperature. 0.5 ml aliquots of the mixture were withdrawn at indicated times and mixed with 5 µl of the chymotrypsin inhibitor, and the mixture placed in ice immediately. The phenyl methylsulfonylfluoride solution was made in ethanol at 87 mg/ml. The cold, inhibitor-treated samples were used for assay of $QH_2 \rightarrow$ cytochrome c activity directly or reconstituted with excess of SDH for the assay of succinate $\rightarrow$ ubiquinone or succinate $\rightarrow$ cytochrome c activities. Proper controls were conducted at the same time. Q symbolized ubiquinone; Q_2H_2, reduced ubiquinone and the subscript 2 represents Q with 2 isopyrene units; c, cytochrome c; and succ, succinate.

Since the soluble $b\text{-}c_1$ complex contains relatively high concentration of the PHMB-reacting group the results of the alkylation study alone cannot be used as the sole evidence for identification. Thus experiments from other standpoints were designed.

Limited digestion of the soluble $b\text{-}c_1$ complex (6 mg/ml) with chymo-

TABLE I. Reconstitution of Succinate-Q Reductase from Apo-Q-Protein I and Soluble Succinate Dehydrogenase

Addition	Specific Activity
	μmoles/min/mg protein
(1) Apo-Q-protein I	0
(2) Succinate dehydrogenase	0.04
(3) (1) + (2)	30.5*

*Sixty micrograms of apo-Q-protein I were added to 2 mg (i.e. in excess) of SDH and then diluted to 0.5 ml. The final medium was 50 mM Na-K-phosphate buffer, pH 7.4. Aliquots were then withdrawn and assayed for succinate-Q reductase activity at room temperature (about 23°). The specific activity was expressed in μmoles of succinate oxidized per minute per mg of apo-Q-protein.

trypsin (chymotrypsin:b-c_1 complex = 1:200) at room temperature gave the pattern of the enzymic activities for QH_2 → cytochrome c and succinate → Q, as shown in Fig. 2. The succinate → Q activity was measured after the preparation was reconstituted with excess of SDH (cf. Table I). It is clear that these two activities were not affected in parallel. QH_2-cytochrome c reductase is much more resistant to chymotryptic digestion than the reconstituted succinate-cytochrome c or succinate-Q reductase. Figure 3 (upper and middle tracings) compares the densitometric tracing of analytical SDS-polyacrylamide gel electrophoresis of the intact and the chymotrypsin-digested soluble cytochrome b-c_1 complex. A significant decrease in the amount of subunits with molecular weights of 53,000-50,000, and 28,000 were observed in the digested sample. Two new peaks with molecular weights of around 20,000 and 16,000 appeared in the products. These peaks apparently resulted from the proteolytic degradation of higher molecular weight proteins.

Although the subunit with molecular weight of 28,000 has been considered as a candidate based on the ^{14}C alkylation and the digestion of chymotrypsin

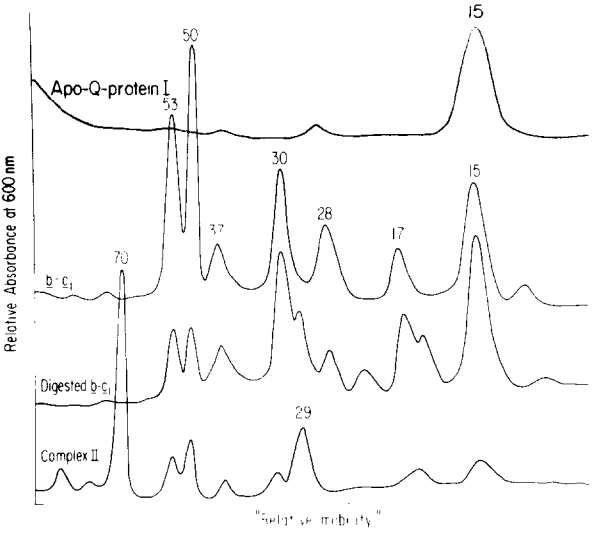


Fig. 3. Densitometric tracing of analytical SDS-gel electrophoresis of the purified apo-Q-protein I, the intact and chymotrypsin digested cytochrome $\underline{b}$-$\underline{c}_1$ complex and "intact" Complex II. The third tracing is the chymotrypsin digested one. Digest conditions are identical to those in Fig. 2. Digestion time, 1 hour. 0.1 mg protein was used in electrophoresis in all cases.

experiments, its participation in succinate-Q reductase activity was, however, easily ruled out. Since Complex II is active as succinate-ubiquinone reductase, it must contain apo-Q-protein I. As indicated in the lower tracing of Fig. 3, Complex II lacks the subunit with molecular weight of 28,000. The low concentration of 53,000 and 50,000 in Complex II also raised questions about the role of these subunits as apo-Q-protein I.

The possible role of the high molecular weight subunits as apo-Q-protein I was eventually eliminated by direct isolation of the protein to a partially purified form. When the soluble $\underline{b}$-$\underline{c}_1$ complex was treated with non-ionic detergents, such as Triton X-100 (1.5%), in the presence of urea (2 M) followed by a calcium phosphate column filtration, we obtained a subfraction of the soluble $\underline{b}$-$\underline{c}_1$ complex, which contained no subunits with molecular weight of 53,000, yet showed full activity toward the reconstitution with SDH to form succinate-ubiquinone reductase, and SDS gel electrophoresis of

this fraction showed three subunits with molecular weights of 50,000, 37,000 and 15,000.

Although all of these three subunits are present in Complex II, the concentrations of subunits with molecular weight of 50,000 and 37,000 are well below the one to one molar ratio as compared with flavoprotein and iron-protein of SDH in such a complex. In terms of molar ratio, the 15,000 subunit is a better candidate than the 50,000 subunit for apo-Q-protein I because the former has approximately one to one stoichiometry to the subunits of SDH. Indeed, when the crude apo-Q-protein I preparation was further fractionated with ammonium sulfate, a fraction between 25 and 35 saturation, which retained all the activity, was found to contain mainly 15,000 subunits. The densitometric tracing of SDS-polyacrylamide gel electrophoresis of purified apo-Q-protein is depicted in the top curve of Fig. 3. On the other hand, a fraction, precipitated up to 25% ammonium sulfate saturation, enriched with 50,000 and 37,000 subunits was completely inactive toward the reconstitution with SDH to form succinate-Q reductase. The reconstitutive activity of isolated apo-Q-protein I with SDH is given in Table I.

Since the isolated apo-Q-protein contains only protein with molecular weight of 15,000, we conclude that apo-Q-protein I is the one with molecular weight of 15,000 in the soluble $\underline{b}$-$\underline{c}_1$ complex. It has been shown that there are many moles (with respect to the minimum number of the $\underline{c}_1$ subunit of 30,000 (cf. Ref. 3)) of 15,000 dalton subunits present in the $\underline{b}$-$\underline{c}_1$ complex. It is obvious that only one of these subunits present in the complex is apo-Q-protein I.

In addition, since there is only one protein needed to convert the SDH into succinate-Q reductase, it is predicted that the subunit structure of succinate-ubiquinone reductase is very simple, $\underline{i.e.}$ no more than three subunits are involved, because active SDH contains only two subunits (9).

Since apo-Q-protein I contains no heme nor does purified SDH, it may now be conclusively stated that the electron transfer reaction between

succinate and Q does not involve cytochromes at all. The inactive cytochromes in Complex II are present due, most probably, to its method of preparation. In this connection it should be mentioned that all the complexes named by Green and co-workers (Ref. 2 and references cited therein) are only artificially defined by their methods of preparation. There is no evidence whatsoever to show the actual occurrence of these 4 Complexes, as 4 separate entities, required in electron transport. Thus, it is not surprising to see that Complex II, although having been named succinate-Q reductase, contains 1 mole of cytochrome b per mole of flavin and varying amounts of c_1, yet neither of these cytochromes undergoes oxidation-reduction. The cytochromes b and c_1 present in Complex II should be considered only as the denatured contaminants.

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