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# ISOLATION OF QP-C AND RECONSTITUTION OF THE $QH_2$-c REDUCTASE

By Tsing-ying Wang* and Tsoo E. King
Department of Chemistry and Laboratory of Bioenergetics
State University of New York at Albany, Albany, New York 12222

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Summary A ubiquinone protein, QP-C, which acts in the cytochrome b-$c_1$ region has been solubilized. The isolated QP-C shows one band of molecular weight 15,000 in polyacrylamide gel electrophoresis in sodium dodecyl sulfate and isoelectric focusing at the isoelectric point of pH 3.6. QP-C reconstitutes with the QP-C deficient b-$c_1$ complex to restore the $QH_2$-cytochrome c activity and recover the EPR signal of the complex.

## INTRODUCTION

It has been shown that the active forms of ubiquinone (Q)[1] are protein bound[1]. Evidence from many lines indicates that the mitochondrial respiratory chain contains at least three such QP acting at the different loci on the chain (1-3). QP-S has been isolated and can reconstitute with succinate dehydrogenase (SDH) to form succinate-Q reductase (4). The formation of the ubisemiquinone radical in these systems has been demonstrated (5). Recently, we have worked out a method to solubilize and purify another ubiquinone protein, or QP-C, acting in the cytochrome b-$c_1$ region. This paper reports the isolation of QP-C and subsequent reconstitution of $QH_2$-c reductase.

## MATERIALS AND METHODS

The cytochrome b-$c_1$-III complex or $QH_2$-cytochrome c reductase was prepared from the b-$c_1$-II complex (2), and its activity was determined by a previously published method (6). The QP-C deficient b-$c_1$-III complex or the b-$c_1$-IV complex was prepared from digestion of the b-$c_1$-III complex with immobilized chymotrypsin at 30° to the extent that approximately 25-30% of the

*Exchange scholar with The People's Republic of China, permanent address: Shanghai Institute of Biochemistry, Academia Sinica, Shanghai.

[1]Abbreviations used: DCIP, 2, 4, dichlorophenolindophenol; HPLC, high performance liquid chromatography; Q, ubiquinone; the subscript of Q denotes the number of isoprene units contained; $QH_2$, completely reduced Q; QP, ubiquinone protein; QP-C, the QP functions in the region of the cytochrome b-$c_1$ complex; QP-N, the QP in the NADH dehydrogenase respiratory segment; QP-S, the immediate electron acceptor of SDH; SDS, sodium dodecyl sulfate; and SDH, reconstitutively active succinate dehydrogenase.

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original activity remained. Usually 45-60 minutes were required, incubating mixture of immobilized chymotrypsin and the b-$c_1$-III complex in a ratio of 1 g wet weight of immobilized chymotrypsin which was obtained by filtration on a medium porosity sintered glass funnel per 4 ml of the b-$c_1$-III complex containing 6.6 mg. per ml. The mixture was filtered to remove the proteolytic enzyme. The immobilized chymotrypsin was prepared by reacting 20 mg of three times recrystallized enzyme per g CNBr-activated sepharose 4B (Pharmacia) in 0.1 M bicarbonate buffer pH 8.0 for 2 hrs at 22-24°. The excess of activating group was destroyed by addition of 0.2 M glycine buffer pH 8.0 according to the manufacturer's manual. $O_2$ was synthesized in this laboratory by a modification of the method of Shunk, et al. (7). $O_2H_2$ was prepared according to Engel, et al. (6).

QP-C activity was assayed by incubating the isolated QP-C rich fractions with the b-$c_1$-IV complex. The activity of $QH_2$-c reductase was determined with aliquots drawn from the incubation mixture by measuring the reduction of cytochrome c at room temperature. See the Results and Discussion section for further details. All other materials were procured from the highest purity commercially available. The water used was double distilled in an all glass apparatus and deionized.

The EPR measurements were made with Varian E4 as previously described (8). The machine setting was made at room temperature as described in the legend of Fig. 2 in ref. (8).

## RESULTS AND DISCUSSION

1. Solubilization and purification of QP-C -- Cytochrome b-$c_1$-III complex (about 120 mg) in 50 mM Tris buffer containing 0.32 M sucrose, pH 8.0 was treated with 1.6 M guanidine-HCl in the presence of 0.5% Lubrol-PX, 6.4% β-mercaptoethanol and 0.13 saturation of ammonium sulfate in a total volume of 5-6 ml at 0° for 30 minutes. The mixture was then fractionated with ammonium sulfate. The fraction collected between 0.23 and 0.48 saturation was dissolved in about 3 ml of Tris NaCl-Lubrol solution (25 mM-50 mM-0.5%) at pH 8.0. The solution showed three major protein peaks in an HPLC with a coated silica column. For actual separation, the solution was applied to a column (2.5 x 43 cm) of Ultrogel AcA-44 which had been equilibrated with the solution buffer. The column was eluted with the same buffer at a flow rate of 1.6 ml/10 min. and fractions, 1.6 ml each were collected. The elution profiles for protein, heme and QP-C activity are shown in Fig. 1. Based on results of SDS polyacrylamide gel electrophoresis, on the color of the fractions and the QP-C activity, the first peak was mainly cytochrome $c_1$; the second peak was QP-C; and the last peak was a low molecular weight protein. The SDS gel electrophoresis pattern of fraction no. 72 was shown in Fig. 2. The main band was observed at a position corresponding to a molecular weight of 15,000.

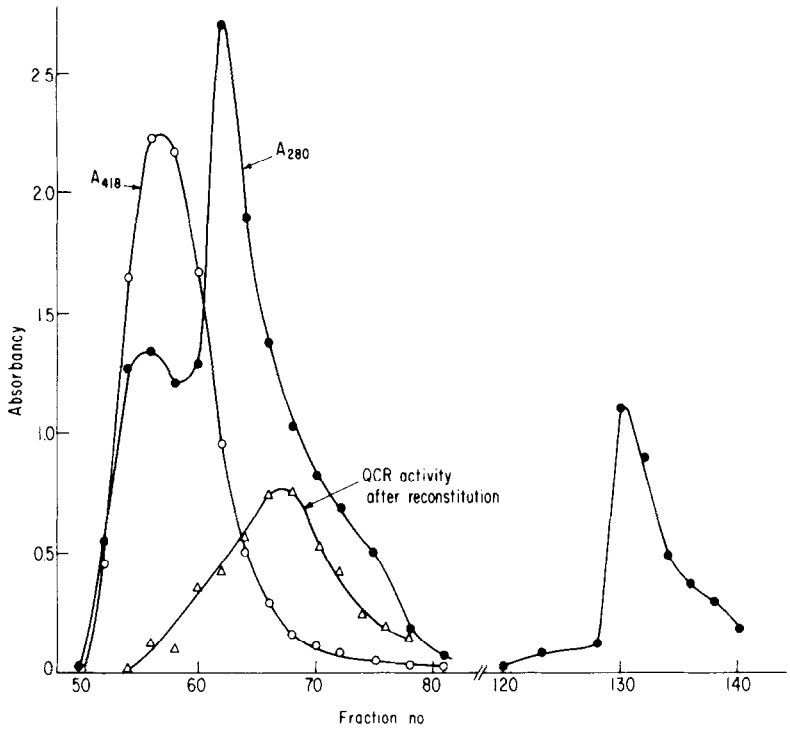


Fig. 1. Elution profile for the distribution of protein, heme and QP-C activity from Ultrogel AcA-44 column of a solubilized fraction of the $\underline{b}$-$\underline{c}_1$-III complex. Column size was 2.5 x 43 cm; flow rate of 1.6 ml/10 min/fraction. The QP-C activity was estimated by incubation 40 µl eluate with the $\underline{b}$-$\underline{c}_1$-IV complex at 35° for 1 hour. The mixture was chilled and diluted to 500 µl. 10 µl aliquots were assayed for $QH_2$-cytochrome $\underline{c}$ reductase activity at about 23°.

The molecular weight was confirmed with the sample containing lysozyme (internal marking). A trace amount of contaminated cytochrome $\underline{c}_1$ was also detected. This fraction was analyzed by isoelectric focusing and found to contain one band with an isoelectric point at approximately pH 3.6. (The arrow shows the position of QP-C; therefore, the approximate molecular weight of QP-C is 15,000; $\underline{cf}$. Fig. 2.) Although the yield was low and improvements of the method may be easily designed, the product was at least 95% pure.

2. Reconstitution of ubiquinone-cytochrome c reductase -- Aliquots of 30-60 µg of QP-C depleted $\underline{b}$-$\underline{c}_1$-III complex or the $\underline{b}$-$\underline{c}_1$-IV complex were mixed with varying amounts of QP-C in Tris-sucrose buffer pH 8.0 (0.05 M-0.25 M) in a total volume of 200 µl. These mixtures were incubated at 35° for one hour and then diluted to 500 µl with Tris-sucrose buffer after incubation. Ali-

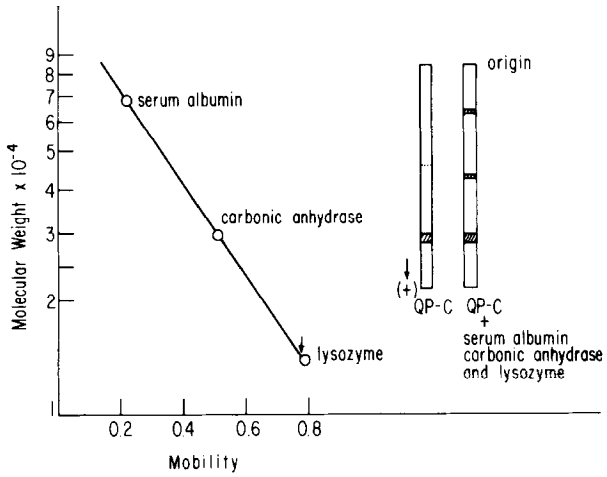


Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of QP-C. The sample after treatment in 2% SDS and 1% mercaptoethanol at 50° for 25 min was run on 10% acrylamide gel (5 mm inner diamter and 7 cm long) with 0.27% N, N' methylenebisacylamide cross-link. On the right of the figure are the drawings of gel patterns obtained after staining with Coomassie brilliant blue for the sample and the sample plus internal markers. The arrow on the left shows the position of QP-C.

quots were withdrawn for enzymic assay. Fig. 3 shows the restoration of $QH_2$-cytochrome c reductase of the b-$c_1$-IV complex by reconstitution with QP-C. QP-C failed to reconstitute succinate-Q reductase with SDH, as witnessed by the inactivity to catalyze the succinate-DCIP reaction, although both QP-S and QP-C possess the same monomeric molecular weight in SDS polyacrylamide gel electrophoresis. We have previously reported the multiplicity of the 15,000 molecular weight proteins (9).

Calculating from the results in Fig. 3, it can be seen that the molar ratio of QP-C to $c_1$ was more than one, based on the molecular weight of 15,000. Several explanations for a ratio of QP-C to $c_1$ greater than unity may be offered: (a) QP-C may not be a monomer in the active form; (b) some inactivation may have occurred during its preparation; (c) other 15,000 protein with a similar isoelectric point may be present; and (d) as suggested before (10), there may be two QP-C's that appear to be identical in the in vitro assay system. At present, we favor the last possibility. More explicitly, $Q_i$ and $Q_o$ which act on the protoplasmic and matrix sides, respectively (ref. 10 and

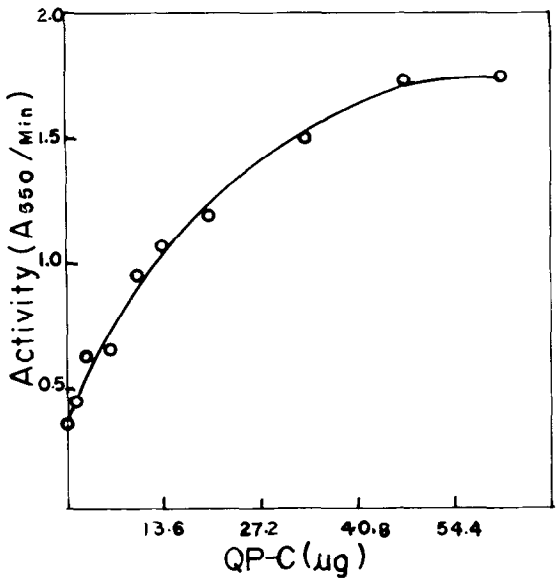


Fig. 3. Reconstitution of ubiquinone cytochrome c reductase system contains 55 mg of the b-$c_1$-IV complex and of QP-C (fraction no. 68) as indicated in a total volume of 200 µl Tris 50 mM pH 8.0 The mixture was incubated at 35° for 1 hour. After incubation, it was made up to volume 500 µl with 0.05 M Tris 0.25 M sucrose buffer, pH 8.0. 10 µl aliquots were drawn and $QH_2$-c reductase activity was assayed in 1 ml assay mixture in presence of 70 M $Q_2H_2$ at about 23°.

references cited) in the protonmotive Q cycle may exist in the isolated product. The orientation in artificial vesicle may not be uniformly one-directioned so that these two QP-C's are interchangeable in activity.

It was found that addition of QP-C to the b-$c_1$-IV complex also restores the ubisemiquinone EPR signal of the b-$c_1$-III complex at room temperature. This observation rules out any possibility that the signal is due to any paramagnetic species other than QP-C, because the b-$c_1$-IV complex contains no flavin and the Rieske protein recently isolated in the undenatured form (11) does not show signal at room temperature. The Rieske protein (11) can show EPR signal only at low temperatures.

Finally, the isolation of QP-C eliminates questions (e.g., 12, 13) regarding the essentiality of QP in the respiratory chain. QP's constitute a separate class of Q-bound proteins comparable to heme proteins and flavoproteins. At this point, we have succeeded in isolating and purifying two of the at least three QP's.

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