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IDENTIFICATION OF UBIQUINONE BINDING PROTEINS IN UBIQUINOL-CYTOCHROME C REDUCTASE BY ARYLAZIDO UBIQUINONE DERIVATIVE

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SUMMARY--A functionally active arylazido-1-[^{14}C]-β-alanine ubiquinone derivative has been synthesized for the identification of the ubiquinone binding protein in ubiquinol-cytochrome c reductase. After photolysis, the ^{14}C activity was found to be specifically associated to proteins with mobilities relative to cytochrome c of 0.841 and 0.475 in the sodium dodecylsulfate polyacrylamide gel electrophoresis of the Weber and Osborn system. These two proteins have previously been identified as b cytochromes. The ^{14}C activity distribution pattern was observed to be identical in the presence or absence of phospholipids during the photolysis. Antimycin A also produces no change in the ^{14}C activity distribution among the proteins of this enzyme complex.

Although the participation of ubiquinone (Q)[1] in the mitochondrial respiratory chain has long been recognized (1, 2), the involvement of specific proteins in the redox reaction of Q has only recently been observed. Until the recent discovery of Q-binding proteins (3-6), Q was generally thought to exist as a mobile electron carrier, which shuttles the electron (proton) between the electron transfer complexes. The successful isolation of a protein (QPs) which binds Q and is able to convert soluble, purified succinate dehydrogenase into succinate-Q reductase emphasized the importance of the participation of protein(s) in the redox reaction of Q, as soluble succinate dehydrogenase is unable to reduce Q directly.

The detection of high concentrations of ubisemiquinone radical (4) in the cytochrome b-c_1 III complex (a highly purified ubiquinol-cytochrome c reductase) (7) during the reduction of the complex by catalytic amounts of succinate-Q reductase and succinate has further suggested the existence of a different Q

[1] Abbreviations used: PL, phospholipids; Q, ubiquinone; QPs, ubiquinone protein in succinate-ubiquinone reductase; QPc, ubiquinone binding proteins in the cytochrome b-c_1 region; SCR, succinate-cytochrome c reductase; SDS, sodium dodecylsulfate.

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binding protein (QPc) in the cytochrome b-c_1 region (4). The ubisemiquinone radical is closely associated with the b cytochromes as not only is the appearance of the ubisemiquinone radical concurrent with the reduction of cytochrome b but upon treatment with thenolytrifluoroacetone the ubisemiquinone radical is diminished simultaneously with re-oxidation of reduced cytochrome b (4). These facts indicate that Q is bound to cytochrome b or in the vicinity of cytochrome b or is in redox equilibrium with cytochrome b.

When antimycin A was added to the cytochrome b-c_1III complex prior to the addition of substrate, no ubisemiquinone radical was detected. The reduction of cytochrome b proceeded normally, with a slightly higher reduction rate. Antimycin A also diminishes the preformed ubisemiquinone radical, but has little effect on the re-oxidation of reduced cytochrome b. These results indicate that the binding of Q is perturbed by the presence of antimycin A either directly through competition at the binding site or indirectly by a conformational change resulting from an interaction between antimycin A and a specific protein (15) in the cytochrome b-c_1III complex. These indirect lines of evidence do not identify the protein or proteins responsible for Q binding in the cytochrome b-c_1 region. Recently we have employed a photoaffinity labelling technique using a functionally active ^{14}C labelled Q derivative to identify the Q binding protein (QPc) in the cytochrome b-c_1III complex, without awaiting the development of an isolation procedure. In this report we wish to present the results of the identification of QPc by photoaffinity labelling and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis techniques.

MATERIALS AND METHODS

The cytochrome b-c_1III complex, a highly purified ubiquinol-cytochrome c reductase, was prepared and assayed according to the reported methods (7). The phospholipid (PL)- and Q-depleted cytochrome b-c_1III complex was prepared by repeated ammonium sulfate precipitation in the presence of 0.5% sodium cholate and 20% glycerol (8), and the final product was dissolved in 50 mM Na/K phosphate buffer, pH 7.4, containing 10% glycerol to a protein concentration of approximately 5 mg/ml. The depleted complex was used within 30 min after preparation.

Photoaffinity labelled Q derivative, $Q_0C_{10}NAPA$, was prepared by the esterification of $Q_0(CH_2)_{10}OH$ and arylazido-β-alanine (9). The radioactive

derivative was made with ^{14}C label at the carboxyl group of β-alanine (9). The detailed synthetic procedure will be reported elsewhere. The specific radioactivity of $Q_0C_{10}NAPA$ was 2000 cpm/nmole. ^{14}C radioactivity counting was done in a Beckman liquid scintillation system, model SL-100, which has a counting efficiency of 64%.

Illumination of the sample was carried out using a spotting light equipped with a 300 quartzline lamp. The samples (0.5 ml) were placed in quartz cuvettes (2 x 9 x 44 mm) and immersed in water in a quartz windowed Dewar. A Truner filter No. 110-811 (7-60) was placed on the window. The water temperature in the Dewar was kept at around 8°. The sample was located 10 cm from the lamp. Two 10 min illumination peroids were used. The samples were illuminated, diluted to 8 ml with 50 mM Na/K phosphate buffer, and the proteins were recovered in the sedimented pellet after centrifugation at 45,000 rpm for 90 min in a Beckman centrifuge, model L, rotor 50. The pellets were suspended in 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.0, and incubated with 5 mg SDS per mg protein in the presence of 1% β-mercaptoethanol at 55° for 2 hours. Ten μl aliquots were placed on the gel columns (0.5 x 8 cm) and electrophoresis, staining and destaining, were carried out according to Weber-Osborn (10) and Swank-Munkres (11). Recovery of radioactivity from the electrophoretic gel column of each protein band was done by direct hydrolysis of the stained protein bands sliced from the gel columns according to the reported procedure (12). The hydrolysis was done with 5 N HCl at 120° for 10 hours. The hydrolysates were dried at around 100° and redissolved in 0.4 ml of water before admixing with 10 ml of Budget-Solve, complete counting cocktail, for radioactivity counting.

RESULTS AND DISCUSSION

Effectiveness of Arylazido Q Derivative ($Q_0C_{10}NAPA$) in the Electron Transfer Reaction--Table I shows the comparison of the effectiveness of $Q_0C_{10}NAPA$ (see Fig. 1) and Q_2 in the electron transfer reaction in succinate-cytochrome c reductase. When freshly prepared PL- and Q-depleted succinate-cytochrome c reductase was mixed first with Q_2 then with PL, succinate-cytochrome c reductase activity was restored. When $Q_0C_{10}NAPA$ was used to replace Q_2 in the system, the activity restored was equal to that observed when Q_2 was used. These results suggest that $Q_0C_{10}NAPA$ is functionally active both as an electron acceptor for succinate-Q reductase and as an electron donor for ubiquinol-cytochrome c reductase. Indeed, direct reduction of $Q_0C_{10}NAPA$ by succinate-Q reductase proceeded at the same rate as the reduction of Q_2. Oxidation of reduced $Q_0C_{10}NAPA$ by the cytochrome b-c_1 III complex was also observed, as predicted.

Identification of Ubiquinone Binding Protein (QPc) in the Cytochrome b-c_1 III Complex--For the purpose of identifying the Q binding protein, the $Q_0C_{10}NAPA$ was prepared with ^{14}C label at the carboxyl group of the β-alanine moiety, When

Table I. Comparison of the Effectiveness of Q_0C_{10}NAPA and Q_2 in the Electron Transfer Reaction in Succinate-Cytochrome c Reductase (SCR)

Treatment*	Activity
	%
SCR	100
SCR + Q_2	102
SCR + Q_0C_{10}NAPA	101
Depleted SCR	2
Depleted SCR + Q_2 + PL	100
Depleted SCR + Q_0C_{10}NAPA + PL	99

* 0.1 ml aliquots of the PL- and Q-depleted succinate-cytochrome c reductase (dSCR), 10 mg/ml, in 50 mM Na/K phosphate buffer, pH 7.4, were mixed with, (in order), 1 ul of Q_2 or Q_0C_{10}NAPA (3.67 mM in 95% ethanol), 0.06 ml of asolectin (10 mg/ml) and 0.4 ml of 50 mM Na/K phosphate buffer, pH 7.4. The mixture was incubated at 0° for one hour before the activity was assayed.

the ^{14}C labelled Q_0C_{10}NAPA was mixed with freshly prepared PL- and Q-depleted cytochrome b-c_1 III complex and illuminated for 20 min with a 300 Watt spot light at approximately 8°, Q_0C_{10}NAPA became covalently linked to the cytochrome b-c_1 III complex. The distribution of radioactivity among the proteins of the complex was revealed by SDS-polyacrylamide gel electrophoresis. Fig. 2 shows the protein and ^{14}C distributions of the cytochrome b-c_1 III complex on an electrophoretic gel column. Two distinct radioactivity peaks were observed.

To avoid the confusion resulting from variations in experimental conditions and the different investigator's preference on the assignment of molecular weights to protein bands in the SDS-polyacrylamide gel column, we have

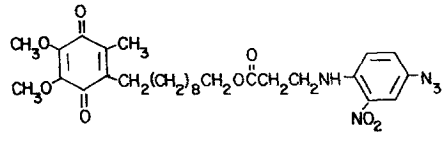

Fig. 1. Structure of Q_0C_{10}NAPA.

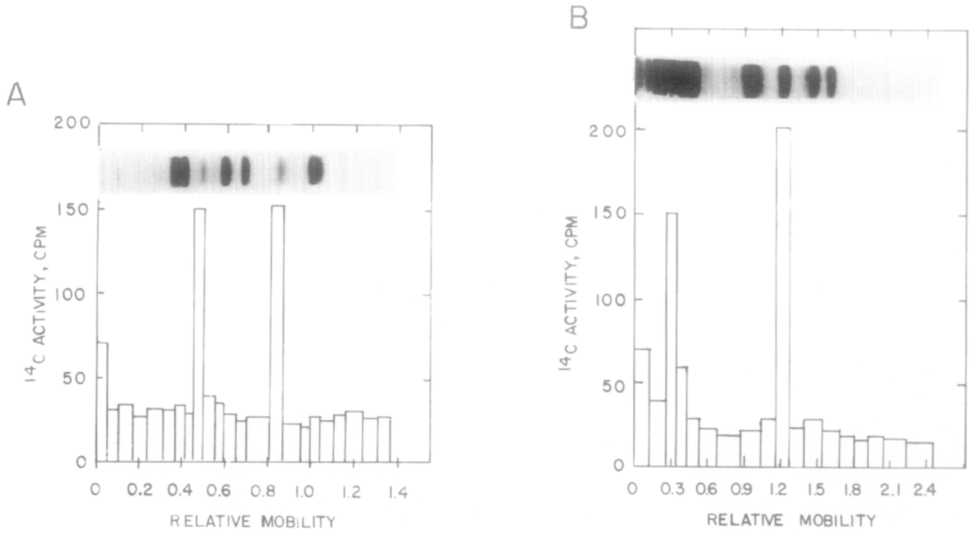

Fig. 2. Distribution of ^{14}C radioactivity among the proteins of the cytochrome b-c$_1$ III complex. (A), in the Weber and Osborn system, and (B), in the Swank and Munkres system. Twenty three stained gel columns (0.5 x 8 cm) were sliced into fractions according to the protein band location. Each fraction was pooled and weighed before being mixed with two weight volumes of 7.5 N HCl. The samples were sealed and hydrolyzed at 120° for 8 hours. The hydrolysates were cooled, the gels removed, and dried at 100°. The residues were redissolved in 0.4 ml of water, mixed with 10 ml of complete counting cocktail, Budget Solve, and the ^{14}C activity of each fraction was determined. The relative mobility is expressed using that of cytochrome c as one.

reported here the mobility relative to a known protein such as cytochrome c, in addition to the apparent molecular weight, which we assigned before (12). For example, R_c^{wo} and R_c^{sm} stand for the mobility relative to cytochrome c in the Weber-Osborn and the Swank-Munkres gel systems, respectively. One of the radioactivity peaks was located in the protein with R_c^{wo} of 0.841 and R_c^{sm} of 1.220 which has an apparent molecular weight of 17,000 (12) or 15,000 (13), depending on the investigator. This protein has been identified as one of the heme b associated proteins (14). The second radioactivity peak was located at R_c^{wo} of 0.475 and R_c^{sm} of 0.32. This protein has been identified to be a cytochrome b with a reported molecular weight of 37,000 (14). Since the distribution of the radioactivity is very specific, it is safe to assume that the proteins with relative mobilities, R_c^{wo} of 0.841 and 0.475 or R_c^{sm} of 1.22 and 0.32 are responsible for Q binding (QPc). Whether the Q is bound to both proteins, is

sandwiched between them, or is bound to one of the two proteins and the binding site is physically close to the other one, can not be asserted definitely. Furthermore, from the fact that a close association and redox equilibrium exists between the ubisemiquinone radical and cytochrome b, it is also highly possible that Q serves as a heme ligand of one or both of the cytochromes b or is even sandwiched between them. More experimental work is needed in order to definitely assign the mode of protein-Q interaction.

Effect of Antimycin A on Q Binding in the Cytochrome b-c_1 III Complex-- When the PL-and Q- depleted cytochrome b-c_1 III complex was treated with antimycin A prior to the addition of Q_0C_{10}NAPA and PL, the radioactivity covalently bound to the protein after photolysis was not significantly different from that observed without antimycin A treatment. No significant change in the radioactivity distribution pattern was found in the protein bands with R_c^{wo} of 0.475 and 0.841. These results indicate that antimycin A does not bind to the same site as Q. This is, in fact, consistent with the report (15) that the antimycin A binding protein is a small molecular weight protein present in Complex III. According to DasGupta and Rieske (15), the molecular weight of the antimycin A binding protein is 11,000, which is much smaller than that of a protein with R_c^{wo} of 0.841.

Effect of Phospholipids on Q Binding in the Cytochrome b-c_1 III Complex-- The reason for use of the PL- and Q-depleted preparation in studies of Q binding is to avoid the complications imposed by PL, because Q is a hydrophobic molecule and associates with PL. Since PL are functionally and structurally (8) essential in the native complex, studies of specific Q binding in the absence of PL may undermine the significance of the results. Therefore, we have also carried out the illumination of the cytochrome b-c_1 III complex in the presence of PL and determined the distribution of radioactivity among the proteins of this enzyme complex. A slightly lower (10%) recovery of radioactivity in the proteins was obtained. The distribution pattern, however, is the same as that obtained in the absence of PL. These results indicate that the Q binding to a

specific protein is not altered by the presence of PL. This result is consistent with the fact that addition of Q to the depleted cytochrome b-c_1 III complex must be made prior to the addition of PL in order to restore the full activity (16). The slight decrease in radioactivity recovery could be attributed either to a lower photoreaction efficiency in the presence of PL or, less likely, competition for the photoactivated nitrene between PL and protein. Since the decrease in the amount of radioactivity recovered in the SDS-polyacrylamide gel column is so small, its significance is questionable.

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