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A PHOSPHOLIPID-DEPENDENT NADH-COENZYME Q REDUCTASE FROM LIVER PLASMA MEMBRANE

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SUMMARY: A 34 kDa coenzyme Q reductase has been solubilized and purified from pig liver plasma membranes. The solubilized enzyme reduced coenzyme Q_0 with NADH. Ubiquinones with longer isoprenoid side chain such as Q_2 and Q_{10} were also reduced when the quinones and the enzyme were reconstituted into phospholipid liposomes. N-terminal sequencing of an internal peptide showed identity to bovine NADH-cytochrome b_5 reductase. Biochemical characterization of the purified enzyme indicated that the coenzyme Q reductase corresponds to an unusual form of NADH-cytochrome b_5 reductase.

Coenzyme Q (CoQ, ubiquinone) in the plasma membrane is available to act as an antioxidant (1) or to participate in transplasma membrane electron transport (2) which can be related to control of cell growth or transport (3). NADH can reduce CoQ in the plasma membrane (2), Golgi apparatus (4) or microsomes (5) although the nature of the reductase in these membranes has not been fully established. The reduction of the lipophilic CoQ would provide a means for electrons to traverse the membrane analogous to lipophilic quinone function in mitochondria, chloroplast and microbial plasma membranes. The reduced quinone can also provide electrons for antioxidant protection of phospholipids in the membrane. An enzyme which can reduce CoQ_{10} with NADH in a lipid environment has been purified from liver plasma membranes. The properties of this enzyme indicate that is unusual form of NADH-cytochrome b_5 reductase.

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Materials and Methods

Protein purification

The NADH-CoQ reductase was extracted from purified pig liver plasma membranes (6) with the non-denaturing zwitterionic detergent 3-[(cholamidopropyl) dimethyl-ammonio] 1-propanesulfonate (CHAPS) at a concentration of 2.5%. Before the detergent extraction extrinsic proteins were removed from the membranes by treating them with 0.5 M KCl in 25 mM Tris/HCl, pH 7.6 containing 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT and 10% glycerol. The protein extract was separated from the membrane residue by centrifugation at 105,000 g for 1 h at 4°C. Then, the NADH-CoQ reductase was purified by size exclusion chromatography on Sephacryl S-300 HR, ion exchange chromatography on DEAE-Sepharose 6B CL and affinity chromatography on 5'ADP agarose followed by elution with NADH as described in detail elsewhere (7).

Reductase activities

NADH-dependent reductase activities were measured in 50 mM Tris/HCl or phosphate buffer, pH 7.6. For NADH-CoQ reductase 0.2 mM NADH, 0.3 mM CoQ_0 and 2-5 μg of purified protein were present and absorbance was measured at 410 nm. An extinction coefficient of 0.7 $mM^{-1}cm^{-1}$ was used in calculations of specific activities. NADH-ferricyanide reductase activity was measured in the same reaction medium but substituting 0.4 mM potassium ferricyanide for CoQ_0. In this case assays contained about 1 μg of purified enzyme and absorbance was monitored at 420 nm. An extinction coefficient of 1 $mM^{-1}cm^{-1}$ was used in calculations of specific activities.

Reconstitution in liposomes

Purifed phospholipids were separated from a crude mixture of egg yolk phospholipids (Sigma) by acetone-precipitation of the ether-soluble fraction. The purified phospholipids were then resuspended in 20 mM Tris-HCl, pH 7.6, 1 mM EDTA. The oxygen in the phospholipid suspension was eliminated by sparging with a stream of nitrogen. The resulting turbid phospholipid suspension (10 mg/ml) was clarified by sonication under a nitrogen atmosphere. Lipophilic ubiquinones (CoQ_2 and CoQ_{10}) in ethanol were added to the phospholipid liposomes to a final quinone concentration of 50 μM. Then the mixture was incubated for 3 min at 37°C to allow for incorporation of the quinones. The CHAPS was removed from the purifed enzyme preparations by dialysis and then, the protein (3 μg) was reconstituted into phospholipid liposomes by freeze-thaw-sonication.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Samples were suspended in SDS-dithiothreitol loading buffer and heated for 15-20 min at 42°C before electrophoresis on a 10% polyacrylamide slab gels (8). The gels were stained with Coomassie blue. In another series of experiments the gels were blotted onto either nitrocellulose for immunostaining or PVDF membranes (Immobilon) for peptide digestion and N-terminal sequencing. The blots were stained with Ponceau S to visualize the protein.

Antibody preparation

A purified fraction of the NADH-CoQ reductase was run on a preparative SDS-gel and the 34 kDa band was excised. The excised band was used as inmunogen for preparation of a polyclonal antiserum following routine procedures for rabbit inmunization and serum processing. Immunostaining with anti p34 antibody in western blots was carried out with a secondary antibody conjugated to alkaline phosphatase.

Results

Affinity chromatography utilizing 5'ADP agarose resulted in the purification to homogeneity of a 34 kDa redox protein (p34, Fig. 1). The purified enzyme displayed NADH-ferricyanide and -CoQ_0 reductase activities. The two acceptors were reduced at rates of about

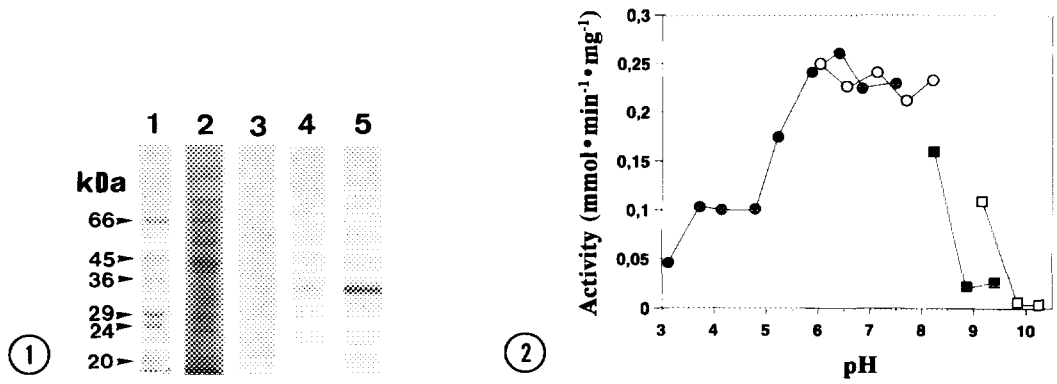

Fig. 1. SDS-polyacrilamide gel electrophoresis. Denatured samples were separated by electrophoresis and transferred to nitrocellulose sheets. Lanes 1-3: Ponceau-S staining, lanes 4-5: immunostaining with anti p34. Lane 1: molecular weight standards, lanes 2 and 4: whole plasma membranes (30 μg), lanes 3 and 5: purified 34 kDa CoQ reductase (0.5 μg).

Fig. 2. pH curve of the purified reductase. Activity measured was NADH-CoQ_0 reductase. (●): 40 mM citrate-phosphate buffer. (○): 40 mM phosphate buffer. (■): 40 mM Tris-HCl buffer. (□): 40 mM glycine-NaOH buffer.

0.7 mmoles mg^{-1} min^{-1} and 250 μmoles mg^{-1} min^{-1}, respectively. However, p34 was inactive with CoQ analogs with isoprenoid side chains such as CoQ_2 and CoQ_{10}. p34 had no significant activity with cytochrome *c* or ascorbate free radical, which do act as substrates for reduction by NADH in the isolated plasma membanes. p34 was highly specific for NADH as electron donor, no significant activity being obtained with NADPH. When p34 was incorporated into phospholipid liposomes it was active with both CoQ_2 and CoQ_{10}. Both quinones at 50 μM were reduced at a rate of about 2 μmoles mg^{-1} min^{-1}, a value that is probably limited by the quinone incorpotated into liposomes.

The purified p34 had absorption maxima at 460 and 490 nm which was decreased by NADH and restored by oxidation with persulfate. The 460-to-490 optical density ratio varied among different preparations of purified enzyme. When the enzyme was precipitated with trichloroacetic acid, the supernatant contained a flavin with absorption maximum at 445 nm. The amount of flavin extracted from the enzyme represented about 0.4 mol flavin per 34,000. An internal peptide originated by digestion of p34 with cyanogen bromide showed the following sequence: KLFQRSTPAITLENPD. This sequence is identical with amino acids # 24 to # 39 in bovine microsomal NADH-cytochrome b_5 reductase. NADH-CoQ_0 reductase activity showed a broad pH optimum between 6 and 8 with phosphate buffer (Fig. 2). The Km for NADH with CoQ_0 as acceptor was about 6 μM and the Km for CoQ_0 was about 625 μM.

The effect of a number of ions on both NADH-ferricyanide and -CoQ_0 reductase activities of purified p34 was tested (Table I). The various ions tested caused different effects on the enzyme depending upon the activity being measured. Zn^{2+} was a strong inhibitor of

Table I. Effect of different ions on NADH-ferricyanide and -CoQ_0 reductase activities of purified plasma membrane cytochrome b_5 reductase (p34). Values represent differences in activity *versus* no addition (in %). Positive data mean activation whereas negative data mean inhibition. Values are the mean from three separate experiments. Standard deviations were less than 5% of the mean. NE: no significant effect.

Addition	Acceptor	
	Ferricyanide	CoQ_0
1 mM NaCl	- 14.07	NE
1 mM KCl	NE	NE
1 mM $MgCl_2$	+ 19.28	NE
1 mM $CaCl_2$	NE	- 13.74
1 mM $MnCl_2$	+ 14.37	- 74.05
2 μM $ZnSO_4$	- 67.31	NE
20 μM $ZnSO_4$	- 80.44	NE

NADH-ferricyanide reductase but had little effect on the CoQ_0 reductase activity. Mn^{2+} inhibited the CoQ_0 reductase but had no effect on ferricyanide reductase. A polyclonal antibody raised against purified p34 reacted with the purified enzyme on a western blot and stained only one band of 34 kDa in whole plasma membranes (Fig. 1). However, incubation of purified p34 with this specific antiserum at several dilutions did not affect NADH-ferricyanide nor -CoQ_0 reductase activities.

Discussion

In this work we have shown that a 34 kDa electron transport flavoprotein (p34), purified from highly enriched plasma membranes, is able to catalyze the NADH-dependent reduction of CoQ_{10} in a lipophilic environment. The amino acid sequence of an internal peptide originated by digestion of the purified CoQ reductase showed that p34 is closely related (or identical) to NADH-cytochrome b_5 reductase. The similar molecular weight and specificity to pyridine nucleotides for the b_5 reductase and this enzyme are also consistant with a close relationship (9).

However, a number of biochemical properties of p34 appear to be different from those reported previously for the NADH-cytochrome b_5 reductase. For instance, p34 shows a 100-fold lower sensitivity to the thiol reagent *p*-hydroxymercuribenzoate (PHMB) than NADH-cytochrome b_5 reductase (7,9). NADH-cytochrome *c* reductase, an activity that represents *cis* electron transport through the cytochrome b_5 reductase-cytochrome b_5 system, is also much less sensitive to PHMB in the plasma membrane than in endomembranes (10). Also, the pH profile of the NADH-ferricyanide reductase activity assayed with p34 differs from that obtained previously for NADH-cytochrome b_5 reductase and spectral properties of p34 are different from those reported for NADH-cytochrome b_5 reductase (7,9). The absorption peak

at 490 nm might be a result of the interaction between the flavin and the protein, since the peak of the acid-extracted flavin shifts to 445 nm.

On the other hand, the ability to reduce CoQ_{10} has not been recognized to date as a function of cytochrome b_5 reductase. The requirement for phospholipids for this activity is an unexpected aspect of a protein like cytochrome b_5 reductase. Primary activities associated with cytochrome b_5 reductase have been the reduction of cytochrome b_5 on the cytosolic surface of endomembranes as a link for electrons to acyl CoA desaturase (11) or the reduction of methemoglobin in red cells by an erythroid-specific soluble form of the b_5 reductase (9).

Different forms of NADH-cytochrome b_5 reductase have been recently recognized by molecular biology studies. A soluble form is produced without a myristoyl anchor for membrane attachment whereas the enzyme primarily studied as a cytochrome b_5 reductase from the endoplasmic reticulum and outer mitochondrial membrane has a myristoyl anchor. A third form has been recognized with an extended hydrophobic terminal aminoacid sequence appropriate for insertion into phospholipid bilayers. It has been suggested that this third form could be a basis for plasma membrane electron transport in erytroid cells (12). A new form of soluble NADH-cytochrome b_5 reductase with an unknown function in the mitochondrial intermembrane space has been very recently described (13).

The function for such an enzyme at the plasma membrane has not been well defined. In addition to its possible function related to *cis* electron transport on the cytosolic side of the plasma membrane, the possiblity that cytochrome b_5 reductase might be also related to transplasma membrane electron transport *via* its action on a transmembrane carrier (as CoQ) has not been discarded (3). The ability to reduce CoQ in a lipid environment indicates that p34 posses unexpected properties which would be consistant with a role in the transfer of electrons through the plasma membrane. Q-reductase activity may be the primary action which is the basis for reduction of ferricyanide or ascorbate free radical on the outside of cells. These transmembrane activities have been shown to require CoQ (2,7). Furthermore, adding extra p34 to CoQ_{10}-supplemented plasma membranes stimulates NADH-ascorbate free radical reductase, an activity that represents *trans* electron transport, but NADH-cytochrome *c* reductase (*cis* electron transport) is not affected (7).

Since the quinone moiety of CoQ is freely movable in the lipid bilayers, it can orientate itself towards the cytosolic medium (14). At this location CoQ might be accesible to the catalytic domain of NADH-cytochrome b_5 reductase similarly to the reaction between intramembrane CoQ and cytosolic myoglobin (15). The difference in ion effects between ferricyanide reduction and CoQ reduction by purified p34 indicate that different sites on the enzyme are involved in the two activities. The inhibition of ferricyanide reductase by Zn^{2+} is consistant with Zn^{2+} inhibition of ferricyanide reduction observed with Ehrlich cells plasma membranes, interpreted as the involvement of histidine residues in the electron transfer from NADH to ferricyanide (16). In addition to its participation in transplasma membrane electron transport as an electron carrier, reduced CoQ (dihydroCoQ) has been proposed as a general

antioxidant in membranes due to either its activity as a free radical scavenger or to its role in the regeneration of other antioxidants as tocopherols (17). Also, dihydroCoQ may be consumed in membranes by reaction with cytosolic acceptors as the hypervalent states of myoglobin (15). From our studies it becomes apparent that dihydroCoQ in the plasma membrane can be regenerated *in situ* by a NADH-cytochrome b_5 reductase accepting electrons from cytosolic NADH.

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