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PARTICIPATION OF A CYTOCHROME b_5-LIKE HEMOPROTEIN OF OUTER MITOCHONDRIAL MEMBRANE (OM CYTOCHROME b) IN NADH-SEMIDEHYDROASCORBIC ACID REDUCTASE ACTIVITY OF RAT LIVER

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SUMMARY: The participation of a cytochrome b_5-like hemoprotein of outer mitochondrial membrane (OM cytochrome b) in the NADH-semidehydroascorbate (SDA) reductase activity of rat liver was studied. NADH-SDA reductase activity was strongly inhibited by antibodies against OM cytochrome b and NADH-cytochrome b_5 reductase, whereas no inhibition was caused by anti-cytochrome b_5 antibody. NADH-SDA reductase exhibited the same distribution pattern as OM cytochrome b-mediated rotenone-insensitive NADH-cytochrome c reductase activity among various subcellular fractions and submitochondrial fractions. Both activities were localized in outer mitochondrial membrane. These observations suggest that OM cytochrome b-mediated rotenone-insensitive NADH-cytochrome c reductase system participates in the NADH-SDA reductase activity of rat liver.

The outer mitochondrial membrane of rat liver contains a b-type cytochrome which shows spectral characteristics similar to those of microsomal cytochrome b_5 (1,2). This novel b-type cytochrome was termed "OM cytochrome b" (2). As reported in our previous paper (2), OM cytochrome b was purified to homogeneity after solubilization with trypsin. Although OM cytochrome b was almost indistinguishable from microsomal cytochrome b_5 in molecular weight and electrophoretic behavior after trypsin solubilization, it was an entity clearly different from cytochrome b_5 in immunological and spectral properties as well as in the amino acid composition (2,3). This cytochrome, together with NADH-cytochrome b_5 reductase, constitute the rotenone-insensitive NADH-cytochrome c reductase of outer mitochondrial

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Abbreviation: SDA, semidehydroascorbate.

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membrane (4). However, nothing is known about the function of OM cytochrome b.

In the present study, we examined the participation of OM cytochrome b in the NADH-dependent reduction of semidehydroascorbate (SDA), which has been found in microsomes and mitochondria of rat liver, by use of an antibody specific to OM cytochrome b. SDA, which is produced by the oxidation of ascorbate in various ascorbate-requiring reactions (5-8), has been reported to be reduced again to regenerate ascorbate by NADH-SDA reductase system (9, 10). However, general agreement has not yet been made as to the nature of the electron transferring components involved in NADH-SDA reductase activity. The participation of NADH-cytochrome b_5 reductase and cytochrome b_5 was proposed by Iyanagi and Yamazaki (11), and Hara and Minakami (12), respectively, from their kinetic studies. On the other hand, Staudinger and his colleagues (13,14) suggested the presence of a NADH-SDA reductase system independent of cytochrome b_5 and its reductase based on their studies using an antibody against NADH-cytochrome b_5 reductase and some chemical inhibitors. However, our immunochemical study clearly indicated the involvement of OM cytochrome b in NADH-SDA reductase activity of rat liver mitochondria.

MATERIALS AND METHODS

Cell fractionation of rat liver was carried out as described previously (15). Subfractionation of liver mitochondria was performed by the method of Parsons and Williams (16) as described in a previous paper (4). OM cytochrome b and cytochrome b_5 were purified to homogeneity from rat liver mitochondria and microsomes, respectively, and rabbit antibodies against these cytochromes were prepared as described previously (2). The antibody against NADH-cytochrome b_5 reductase, which was purified from rat liver microsome by the method of Takesue and Omura (17), was generously supplied by Dr. M. Noshiro of this laboratory.

NADH-SDA reductase activity was assayed using ascorbate and ascorbate oxidase, which was purified from Japanese cucumber by the method of Tokuyama et al. (18), as the generating system of semidehydroascorbate. The reaction mixture contained 0.1 mM NADH, 2 mM ascorbate, 2 units of ascorbate oxidase, 1 μM rotenone, 100 mM potassium phosphate buffer (pH 7.0) and enzyme. The amount of ascorbate and its oxidase was adjusted to give maximum enzyme activity. NADH-cytochrome c and NADPH-cytochrome c reductase activities were measured as described by Takesue and Omura (17) and Omura and Takesue (19), respectively. Rotenone (final concentration; 1 μM) was used in the assay of rotenone-insensitive reductase activities. Inhibition of NADH-SDA and NADH-cytochrome c reductase activities by the antibodies were determined after preincubation of subcellular fractions with the antibodies for 10 min at 25 °C.

Succinate-cytochrome c reductase, monoamine oxidase, and sulfite oxidase were measured by the methods of Stotz (20), McEwen (21), and Cohen and Fridovich (22), respectively. Protein was determined by the method of Lowry et al. (25) using bovine serum albumin as the standard.

RESULTS

Inhibition of NADH-SDA reductase activity by antibodies. In our previous report (4), it was shown that the antibodies against OM cytochrome b and cytochrome b_5 were highly specific to each antigen and no cross reaction was observed between them. Fig. 1 shows the effect of these antibodies on the NADH-SDA reductase activity of rat liver mitochondria. About 70 % of the reductase activity was inhibited by anti-OM cytochrome b antibody, whereas no inhibition was caused by anti-cytochrome b_5 antibody even at an amount of the antibody which exhibited maximal inhibition of mitochondrial rotenone-insensitive NADH-cytochrome c reductase activity. The inhibition of the NADH-SDA reductase activity by anti-OM cytochrome b antibody was not complete, but 20 to 40 % of the reductase activity usually remained even after the treatment with excess amounts of the antibody. Specificity of the inhibition by anti-OM cytochrome b antibody was confirmed by the observation that the inhibitory effect of the antibody was completely abolished by the pretreatment of the antibody with purified OM cytochrome b (data not shown).

As reported previously (4), OM cytochrome b and NADH-cytochrome b_5 reductase constitute a rotenone-insensitive NADH-cytochrome c reductase system found in the outer mitochondrial membrane of rat liver. In order to examine the relation between the NADH-cytochrome c reductase system containing OM cytochrome b and NADH-SDA reductase activity, the effect of the antibody to NADH-cytochrome b_5 reductase on mitochondrial NADH-SDA reductase activity was tested. As can be seen in Fig. 2, the reductase activity was almost completely inhibited by this antibody, suggesting the participation of OM cytochrome b-mediated rotenone-insensitive NADH-cytochrome c reductase system in NADH-SDA reductase activity.

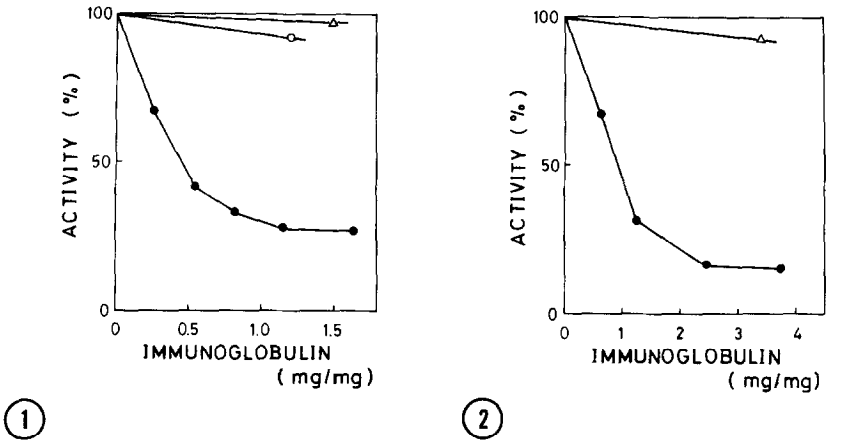

Fig. 1. Inhibition of Mitochondrial NADH-SDA Reductase Activity by Antibodies against OM Cytochrome b and Cytochrome b_5.
Mitochondria (1.5 mg protein) were preincubated with various amounts of the antibodies for 10 min at 25 °C, then NADH-SDA reductase activity was assayed. Control reductase activity was 70.8 nmoles NADH oxidized/min. ● , Anti-OM cytochrome b antibody; o , anti-cytochrome b_5 antibody; △, non-immune globulin(control).

Fig. 2. Inhibition of Mitochondrial NADH-SDA Reductase Activity by Anti-NADH-cytochrome b_5 Reductase Antibody.
Mitochondria (1.5 mg protein) were preincubated with various amounts of the antibody. Control reductase activity was 70.8 nmoles NADH oxidized/min. ● , Anti-NADH-cytochrome b_5 reductase antibody; △ , non-immune immunoglobulin (control).

Subcellular and submitochondrial distribution of the activity in rat liver.
The subcellular distribution of NADH-SDA reductase activity in rat liver was examined using appropriate enzyme markers for various subcellular fractions (Fig. 3). The reductase activity sensitive to anti-OM cytochrome b antibody is also shown in the figure (shaded areas in the figure). NADH-SDA reductase activity was found to be localized in the mitochondrial fraction and it showed a distribution pattern similar to succinate-cytochrome c reductase and monoamine oxidase, which are typical marker enzymes for inner and outer mitochondrial membranes, respectively. When NADH-SDA reductase was assayed in the presence of excess amounts of anti-OM cytochrome b antibody, about 70 % of the reductase activity of mitochondria was inhibited as described above. On the other hand, the activities found in other fractions were less sensitive to the antibody and about 30 % of the activities were inhibited.

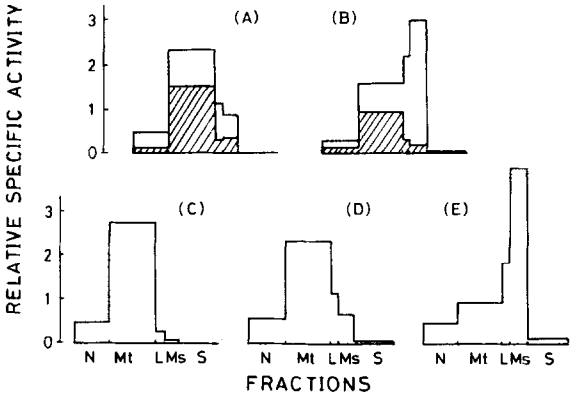

Fig. 3. Distribution of NADH-SDA Reductase and Marker Enzymes among Subcellular Fractions from Rat Liver.

Cell fractionation was carried out as described previously (15). (A) NADH-SDA reductase, (B) Rotenone-insensitive NADH-cytochrome c reductase, (C) Succinate-cytochrome c reductase, (D) Monoamine oxidase, (E) NADPH-cytochrome c reductase. N, Mt, L, Ms and S represent the nuclear, mitochondrial, lysosomal, microsomal and soluble fractions, respectively. Ordinate: relative specific activities of fractions (the percentage of activity recovered in each fraction over the percentage of protein in the same fraction). Abscissa: protein contents of subcellular fractions. Shaded area represents the activity sensitive to anti-OM cytochrome b antibody.

Although the highest activity of rotenone-insensitive NADH-cytochrome c reductase was found in the microsomal fraction, considerable amount of the reductase activity was also recovered in the mitochondrial fraction. The sensitivity of the activity to anti-OM cytochrome b antibody varied from fraction to fraction. Only 10 % of the activity of microsomes was sensitive to the antibody, whereas the activity of mitochondria was inhibited by about 60 %.

It is clear from Fig. 3 that both NADH-SDA and NADH-cytochrome c reductase activities sensitive to the antibody against OM cytochrome b exhibited almost the same distribution pattern suggesting that both activities share common components. This conclusion was further supported by their submitochondrial distributions (Table I). NADH-SDA reductase activity was localized in outer mitochondrial membrane and about 70 % of the activity was inhibited by anti-OM cytochrome b antibody. The distribu-

Table I. Submitochondrial Distribution of NADH-SDA Reductase Activity in Rat Liver

Fract.	Protein	SDH	Sul. ox.	MAO	NADH-c red.		NADH-SDA red.	
					total[1]	OM-b[2]	total[1]	OM-b[2]
	%	nmoles/min/mg protein						
Mit.	100	286	98	13.3	159	65	48	36
IM-Matrix	72	395	12	16.2	161	89	55	45
IMS	19	0	505	0.1	2	1	0	0
OM	9	294	17	26.3	621	178	147	96

1) activities in the absence of the antibody
2) activities sensitive to anti-OM cytochrome b antibody
Mit, Whole mitochondria; IM-Matrix, inner membrane-matrix fraction; IMS, inter membrane space fraction; OM, outer membrane fraction; SDH, succinate-cytochrome c reductase; Sul. ox., sulfite oxidase; MAO, monoamine oxidase, NADH-c red., rotenone-insensitive NADH-cytochrome c reductase.

tion of the activity sensitive to the antibody was again quite similar to that of the corresponding activity of NADH-cytochrome c reductase.

All of these findings described above were also confirmed by using a mixture of ascorbate and dehydroascorbate in stead of ascorbate and ascorbate oxidase as a SDA-generating system.

DISCUSSION

It was previously shown that cytochrome b_5 and OM cytochrome b share a common reductase, NADH-cytochrome b_5 reductase, and exhibit rotenone-insensitive NADH-cytochrome c reductase activity found in microsomes and mitochondria of various mammalian tissues (4). The cytochrome b_5-mediated NADH-cytochrome c reductase system is known to play important roles in the desaturation of fatty acyl-Co A's (24) and in the hydroxylation of some drugs (25,26). The present study clearly demonstrates that OM cytochrome b-mediated reductase system of rat liver participates in the NADH-dependent reduction of SDA to ascorbate, and that cytochrome b_5 appears to have no contribution to this activity. It is, however, still unknown whether the electron from OM cytochrome b is transferred directly to the ascorbate free radical, or a third component is required between OM cytochrome b and the

free radical, like the terminal desaturase in the fatty acid desaturation system involving cytochrome b_5.

Our results also indicate that NADH-SDA reductase activity in rat liver is mainly localized in mitochondria, and that the contribution of OM cytochrome b to this activity is about 60 % of the total. The rest of the activity is distributed widely among various subcellular fractions. Mitochondrial localization of NADH-SDA reductase activity has recently been reported for rat liver (6) and bovine adrenal medulla (8), and the reductase activities were suggested to play roles in the protection of membranes from lipid peroxidation and in the dopamine-β-hydroxylation by regenerating the reduced form of the cofactor, ascorbate, respectively. Although no information is available about the components involved in the NADH-SDA reductase described in these reports, OM cytochrome b possibly plays roles in these metabolic functions. It is also likely that OM cytochrome b-mediated reductase activities are coupled with some other basic functions of mitochondria in view of a wide distribution of this cytochrome in the outer mitochondrial membrane of various tissues (4).

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