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Microsomal Aldehyde Dehydrogenase or Its Cross-Reacting Protein Exists in Outer Mitochondrial Membranes and Peroxisomal Membranes in Rat Liver

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ABSTRACT. **We investigated the subcellular distribution of microsomal aldehyde dehydrogenase (msALDH) in rat liver and revealed by the immunoblotting method that msALDH or a cross-reacting 54-kDa protein(s) exists in the outer mitochondrial membranes and peroxisomes. Anti-msALDH antibody markedly inhibited the decanal aldehyde dehydrogenase activity of the outer mitochondrial membranes as well as that of the microsomes. Immunogold electron microscopic observations showed that gold particles are localized over the ER, outer mitochondrial membranes and peroxisomal membranes. These results suggest that msALDH or its cross-reacting related protein is distributed not only in the ER membranes but also in the mitochondrial outer membranes and peroxisomal membranes.**

Aldehyde dehydrogenases (ALDHs) (E.C.1.2.1.3) are a group of enzymes that catalyze the oxidation of wide varieties of aliphatic and aromatic aldehydes to the corresponding acids via a pyridine nucleotide-dependent reaction. These enzymes exist in most organs such as liver, brain, lung and stomach with the highest activity localized in liver, and may play a pivotal role in rapidly metabolizing cytotoxic aldehydes (7, 18, 29). Rat liver ALDHs are found in the various subcellular compartments such as mitochondria (matrix and outer membrane) and microsomes (8, 9, 26), peroxisomes (2) and cytosol (4).

Several ALDH isozymes have been identified in humans and other mammals (7, 18, 29). They were purified and characterized especially from livers of human and mammalian species. cDNAs and genes for the major liver ALDH isozymes such as class 1, class 2, and class 3 ALDH have been characterized and amino acid sequences of these ALDH isozymes have been deduced. In rat livers, each ALDH isozyme is distributed in a particular cytoplasmic compartment; phenobarbital-induced ALDH (variant class 1) and tumor-associated ALDH (class 3) in the cytosol and class 2 ALDH in the mitochondrial matrix. In normal rat liver, ALDH activity is localized primarily to the mitochondrial and microsomal fractions (8, 9, 26). Rat liver microsomal ALDH (msALDH) was purified by Nakayasu *et al.* (15) as a ~51 kDa protein. Takagi *et al.* (23) have shown that it is exclusively synthesized on the free polysomes and incorporated randomly into the rough and smooth endoplasmic reticulum (ER). Recently we have isolated and sequenced a cDNA clone for rat liver microsomal ALDH (msALDH) and deduced the amino acid sequence (14). Rat msALDH shared ~25% homology with rat class 1 and class 2 ALDH and 65.6% homology with rat class 3 ALDH (tumor-associated ALDH). Thus msALDH is clearly different from the other classes of ALDH. We therefore proposed to classify msALDH as a new class 4 ALDH (14). It consists of 484 amino acid residues with a hydrophobic sequence at the carboxy-terminal region, which is probably important for post-translational insertion of this enzyme into the ER membranes.

It has been reported that ALDH exists in rat liver outer mitochondrial membranes (26) and also in the peroxisomes (2). ALDH in these compartments, however, has not yet been characterized thoroughly. In this report we have shown that msALDH itself or a cross-reacting protein exists in both the outer mitochondrial membranes and peroxisomal membranes.

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Abbreviations: ALDH, aldehyde dehydrogenase; BSA, bovine serum albumin; ER, endoplasmic reticulum; IMS, intermembrane space; MP, mitoplast; msALDH, microsomal aldehyde dehydrogenase; MT, mitochondria; OMM, outer mitochondrial membrane; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Chemicals. Decanal, digitonin, nicotinamide, cytochrome *c* from horse heart, sodium succinate, sodium sulfite and glycylglycine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NAD and NADPH were from Oriental Yeast Co., Ltd. (Tokyo, Japan). Pyrazole, rotenone, l-glutamic acid, benzylamine and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO, USA). Goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate was from Tago, Inc. (Burlingame, CA, USA) and nitrocellulose membrane (GVHP) was from Millipore Kogyo K.K. (Yonezawa, Japan). Other reagents used were all purchased from commercial sources.

Purification of msALDH and preparation of its antibody. msALDH was purified from fresh rat livers according to the method of Nakayasu *et al.* (15). The purified enzyme exhibited a single band with an apparant molecular mass of 54 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified msALDH (100–200 μg) in Freund's complete adjuvant was injected into rabbits and the antibody was prepared as described previously (14).

Preparation of mitochondria, microsomes and peroxisomes and subfractionation of mitochondria. Preparation of rat liver mitochondria (MT) and microsomes and subfractionation of the mitochondria into outer mitochondrial membrane (OMM), mitoplast (MP) and intermembrane space (IMS) fractions were carried out according to the method of Schnaitman and Greenawalt (20).

The peroxisomes were prepared from rat liver homogenate as described by Leighton *et al.* (11), and were ~95% pure, contaminated with microsomes (3–4%) and mitochondria (1–2%), as judged by the marker enzyme assay as reported by Fujiki *et al.* (6).

Protein concentration was determined by the Bio Rad protein assay kit (Bio Rad Laboratories, CA, USA).

Enzyme activity determination. ALDH (EC1.2.1.3) was assayed using the method previously described by Nakayasu *et al.* (15), with decanal as substrate. For OMM marker, monoamine oxidase (EC1.4.3.4; MAO) was assayed using the method previously described by Schnaitman *et al.* (19). For mitochondrial matrix marker, glutamate dehydrogenase (EC1.4.1.3; GDH) was assayed according to the method of Leighton *et al.* (11). For mitochondrial inner membrane marker, succinate-cytochrome *c* reductase (EC1.3.99.1; SCR) was assayed according to the method described by Sottocasa *et al.* (22). For microsome marker, NADPH-cytochrome *c* reductase (EC1.6.2.4; FP2) was assayed based on the method of Sottocasa *et al.* (22). For mitochondrial intermembrane space marker, sulfite-cytochrome *c* reductase (EC1.8.3.1; SUL) was assayed using the method of by Wattiaux and Wattiaux (28).

SDS-PAGE, immunoblotting and quantitative immunoblot analysis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was run under reducing condition (0.1 M dithiothreitol) in 7.5% or 15% acrylamide gels according to the method of Laemmli (10). Immunotransfer onto nitrocellulose membranes was carried out according to the method of Burnette (3). Gels of SDS-PAGE were reacted with anti-human $ALDH_2$ IgG (1) and anti-msALDH IgG (14). They were also reacted with anti-porin IgG (16) as a marker of OMM, anti-NADPH cytochrome *c* reductase IgG (13) as a marker of microsomes and anti-catalase IgG (27) as a marker of peroxisomes. The antigen-IgG complexes were visualized with ECL (Enhanced Chemiluminescence) System (Amersham Co., CA, USA). In order to estimate the amount of msALDH in the microsomes and peroxisomes, mitochondria and submitochondrial fractions, these fractions were immunoblotted with anti-msALDH antibody at various protein concentrations as described above and the bands corresponding to msALDH were quantitatively analyzed by an UltroScan XL laser densitometer (Pharmacia LKB Biotechnology., Bromma, Sweden). The amount of msALDH was estimated from the gradient of the linear region of two curves as described previously (24). The amounts of porin and catalase in these fractions were also estimated in a similar manner.

Inhibition of the ALDH activities by antibody. Microsomes (1 mg protein) were incubated with various concentrations of anti-msALDH IgG (0.053 mg–5 mg) and mitochondrial and submitochondrial proteins (1 mg) were incubated with 2 mg of anti-msALDH IgG for 45 min at 25°C. Control assay was carried out with normal rabbit IgG. ALDH activity was assayed as described in "Enzyme Activity Determination".

Cryoimmunogold electron microscopy. Frozen ultramicrotomy of rat liver was performed according to the method of Tokuyasu (25), and immunogold electron microscopy was carried out based on the method described previously (14) using anti-msALDH antibody.

Pre-embedding immunogold labeling of agarose-embedded microsomes and mitochondria. Microsomes and mitochondria were fixed for 10 min by addition of equal volumes of 8% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and embedded in agarose as described by De Camilli *et al.* (5). The agarose gel was incubated for 30 min in PBS containing 10 mM glycine, then resuspended in PBS containing 0.5% BSA for 30 min, reacted with antiserum against msALDH and subsequently with IgG-gold complex (5 nm in diameter) according to the method of Otsu *et al.* (17).

RESULTS

In order to investigate whether rat liver msALDH exists also in the mitochondria (MT) and peroxisomes and to study in which compartment of the mitochondria this protein is localized, we prepared these organelles and subfractionated mitochondria as described in Materials and Methods.

Table I shows specific activities of various marker enzymes in rat liver mitochondria, submitochondrial fractions and microsomes. NADPH-cytochrome *c* reductase activity of the mitochondrial fraction suggests that

Table I. DISTRIBUTION AND SPECIFIC ACTIVITY OF VARIOUS ENZYMES IN RAT LIVER MITOCHONDRIA, SUBMITOCHONDRIAL FRACTIONS AND MICROSOMES.

	Mitochondria	Outer membrane	Mitoplast	Intermembrane space	Microsomes
MAO	109.3±7.6	339.9±48.7	20.8±7.6	114.4±11.4	ND
GDH	118.3±14.1	17.4±4.0	132.4±10.8	59.3±26.3	3.5
SCR	150.1±39.1	56.5±2.9	170.6±33.1	ND	0.41±0.3
SUL	47.9±15.5	2.0±0.8	0.77±0.38	86.1±14.0	ND
FP2	10.8±1.1 (5.1%)	34.4±11.2 (16.3%)	8.5±2.51 (4.0%)	4.65±0.93 (2.2%)	211.2±16.8 (100%)
ALDH	81.0±22.6	60.3±13.0	62.4±15.2	66.6±13.3	128.4±34.0

MAO: monoamine oxidase GDH: glutamate dehydrogenase SCR: succinate-cytochrome *c* reductase
SUL: sulfite-cytochrome *c* reductase FP2: NADPH-cytochrome *c* reductase ALDH: aldehyde dehydrogenase
Specific activity: mμmoles/min/mg protein as described in Materials and Methods. ND: not detected.

contamination of the microsomes in the mitochondrial fraction is ~5%. Marker enzyme assay of the submitochondrial fractions indicates that the subfractionation of the mitochondria was effectively carried out. Especially, monoamine oxidase, a marker enzyme of OMM, was 3.1-fold more concentrated in the OMM fractions than in the whole mitochondrial fractions.

In marked contrast, ALDH activity of the mitochondria with decanal as the substrate is distributed rather evenly in all the submitochondrial fractions (Table I). This result is expected if two or more ALDH isozymes with unique but broad substrate specificity exist in both the mitochondrial matrix and outer membrane.

By immunoblot analysis of the mitochondria and peroxisomes with anti-msALDH antibody, we detected a 54 kDa band which presumably corresponds to the 54 kDa msALDH of the microsomes (Fig. 1). This result suggests that msALDH itself or a cross-reacting protein is localized in both the mitochondria and peroxisomes.

Since the purity of the peroxisomes is ~95% and the 54 kDa band of the peroxisomes is as dense as that of the microsomes, it is ascertained that msALDH or the cross-reacting protein exists in the peroxisomes.

As described above, the purity of the mitochondria is ~92%, contaminated with the microsomes (5.1%) (Table I) and peroxisomes (2.9%) as shown later (Table III). Since the 54 kDa band of mitochondria is less dense than that of microsomes, it is not certain at this stage of analysis whether msALDH or the cross-reacting protein exists in mitochondria, or that the 54 kDa band of the mitochondria is exclusively due to msALDH of the microsomes and peroxisomes contaminating the mitochondrial fractions.

To compare the immunochemical properties of the microsomal and mitochondrial ALDH isozymes, we examined whether the ALDH activity with decanal as a substrate in microsomal fractions can be inhibited with anti-msALDH antibody. Fig. 2 shows that anti-msALDH IgG can effectively inhibit the ALDH activity (81%). Next we examined whether ALDH activity of the submitochondrial fractions can be inhibited by the antibody. As shown in Table II, the ALDH activity of the OMM fraction was effectively inhibited (71.2%), whereas that of the unfractionated MT fraction and the other two submitochondrial fractions (MP and IMS) were only slightly inhibited (10–20%). This result suggests that the OMM fraction contains msALDH or the cross-reacting protein which possesses ALDH activity similar to msALDH.

To confirm the finding that msALDH or the cross-reacting protein is indeed distributed in the OMM fraction, immunoblot analysis of the various submitochondrial fractions was carried out using anti-msALDH antibody. Fig. 3A shows that a band with apparent molecular mass of 54 kDa was found in all the submitochondri-

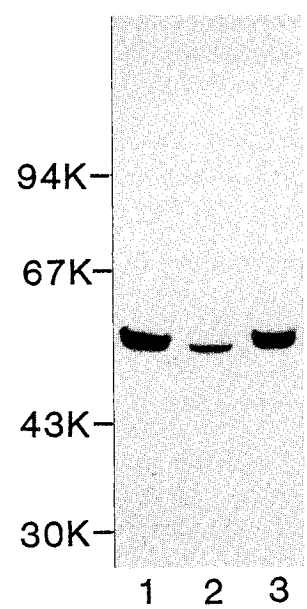

Fig. 1. Immunoblot analysis of rat liver microsomes, mitochondria and peroxisomes with anti msALDH antibody.
Lane 1. Microsomes (5 μg-protein); 2. Mitochondria (5 μg protein); 3. Peroxisomes (5 μg protein).

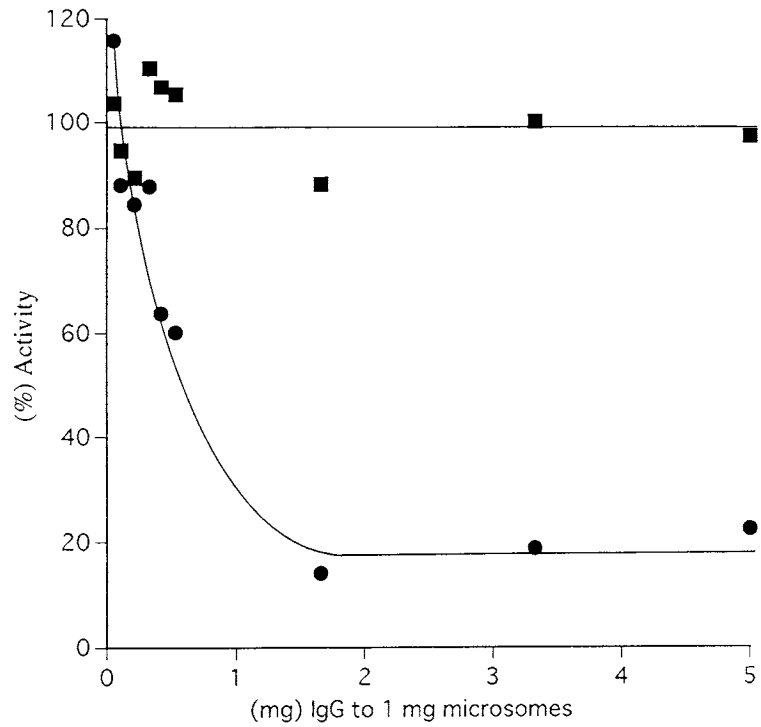

Fig. 2. Inhibition of ALDH activity of the microsomes to decanal by anti-msALDH antibody. Rat liver microsomes containing 1 mg of microsomal protein were incubated with increasing amounts of antibody (●). IgG prepared from non-immunized rabbit was used as a control (■).

al fractions with the highest reactivity in the OMM fraction and the lowest in the MP fraction. The immuno-reactivity of the OMM fraction was higher than that of the unfractionated mitochondria.

Cross-contamination of the other fractions to the OMM fraction was assayed by immunoblot analysis using antibodies against several marker proteins. Fig. 3B shows that the distribution of the 32 kD band of porin in the submitochondrial fractions is similar to that of msALDH with the most dense band in the OMM fraction. This result indicates that msALDH or the cross-reacting protein is indeed concentrated in the OMM fraction.

Using anti-NADPH-cytochrome *c* reductase IgG, contamination of microsomes in the submitochondrial fractions was estimated. As shown in Fig. 3C, the OMM fraction shows only a faint band and the other submitochondrial fractions shows no bands at all, indicating that the OMM fraction is barely contaminated with microsomes.

Table II. INHIBITION OF ALDH ACTIVITY TO DECANAL BY ANTI-MSALDH ANTIBODY.

Inhibition test

Fraction	(%) inhibition
Microsomes	81.6%
Outer mitochondrial membranes	71.2%
Mitochondria	26.5%
Mitoplast	11.6%
Intermembrane space	21.4%

We determined the amounts of msALDH, porin and catalase in the various fractions by quantitative immunoblot analyses as described in Materials and Methods. Fig. 4 shows quantitative analysis of msALDH in the microsomes and mitochondria using anti-msALDH antibody. Similar analyses were carried out for the submitochondrial fractions. The same quantitative immunoblot analyses were also performed using anti-porin and anti-catalase antibodies.

Table III summarizes the relative amounts of these proteins in each fraction assuming that the concentrations of ALDH in the microsomes, porin in the mitochondria and catalase in the peroxisomes are 100%, respectively. The concentrations of msALDH in the OMM, MP and IMS fractions were 3.6, 0.19 and 0.91 times that in unfractionated MT, respectively. These results were in good agreement with the distribution of the specific activity of MAO in these fractions. The concentration of msALDH or the cross-reacting protein in the peroxisome was similar to that in the microsomes. Distribution of porin in the OMM was 2.93-fold higher as compared to that in the unfractionated MT and this

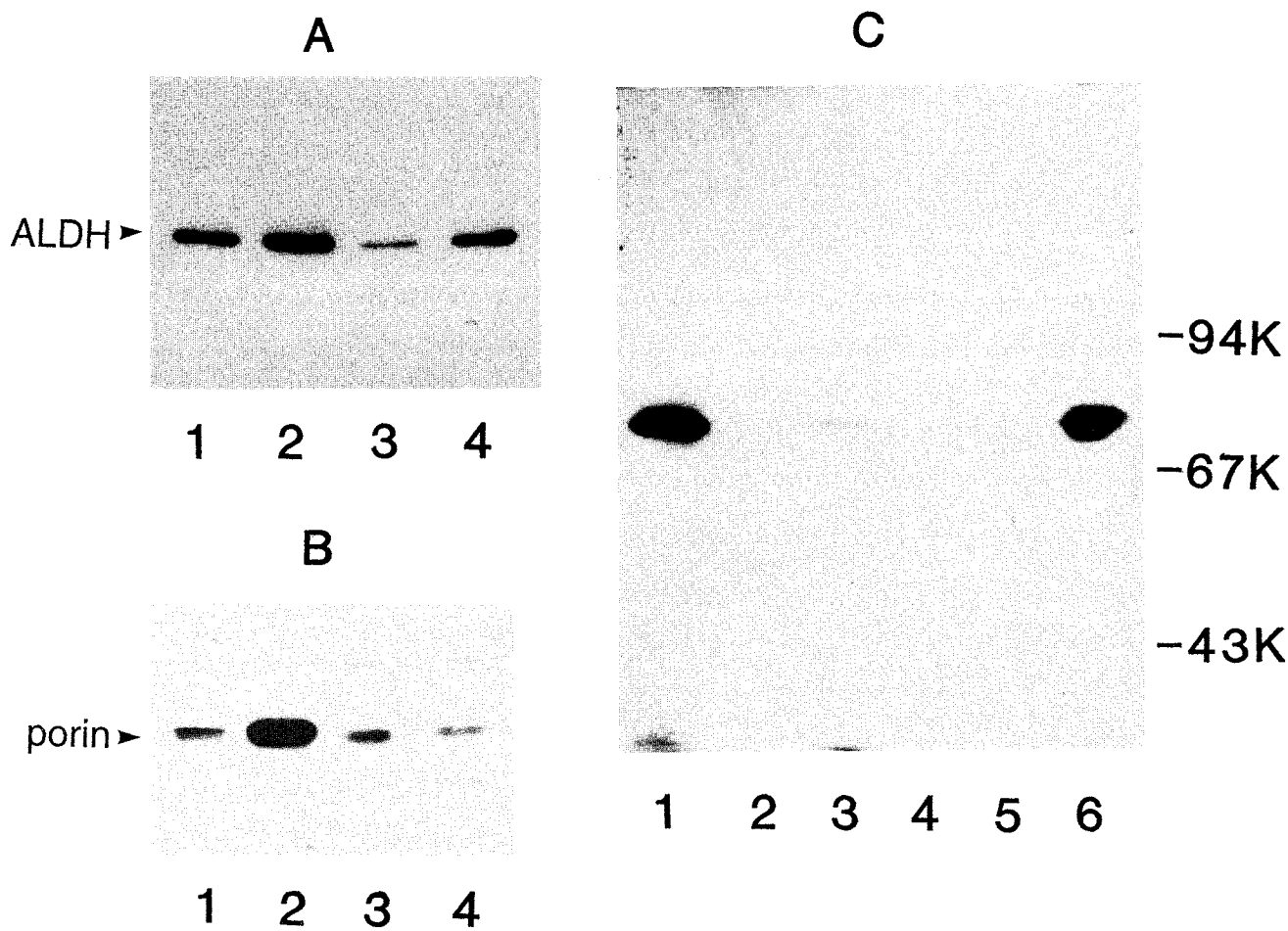

Fig. 3. Immunoblot analysis of the submitochondrial fractions with antibody against msALDH (A), porin (B), and NADPH-cytochrome *c* reductase (C), respectively.

A. Immunoblot analysis of msALDH. Each fraction (5 μg protein) was subjected to SDS-PAGE (7.5% acrylamide) followed by immunoblot analysis with anti-msALDH antibody. Lane 1, mitochondria; lane 2, outer mitochondrial membranes; lane 3, mitoplast; lane 4, intermembrane space.

B. Immunoblot analysis of porin. Each fraction (5 μg protein) was subjected to SDS-PAGE (15% acrylamide), and the transferred proteins were immunostained with anti-porin antibody. Lane 1, mitochondria; lane 2, outer mitochondrial membranes; lane 3, mitoplast; lane 4, intermembrane space. The band corresponding to porin is indicated by an arrowhead.

C. Immunoblot analysis of NADPH-cytochrome *c* reductase. Each fraction (microsomes 5 μg protein, the other fractions 20 μg protein) was subjected to SDS-PAGE (7.5% acrylamide), and the transferred proteins were immunostained with anti-NADPH-cytochrome *c* reductase antibody. Lanes 1 and 6, microsomes; lane 2, mitochondria; lane 3, outer mitochondrial membranes; lane 4, mitoplast; lane 5, intermembrane space.

result was similar to the distributions of MAO. The contamination of the peroxisomes in the mitochondrial fraction was 2.9%.

These results clearly indicate that msALDH and/or an immunologically cross-reacting protein does exist in the outer mitochondrial membranes and in the peroxisomes.

Fig. 5 shows cryoimmunogold electron micrographs of rat hepatocytes. Gold particles were mostly distributed over the cytoplasmic surface of the ER, outer mitochondrial membranes and peroxisomal membranes. However, they were barely distributed over the mitochondrial matrix, peroxisomal matrix or the cisternal space of the ER. Similarly, gold particles were bare-

Fig. 4. Estimation of the amounts of msALDH in rat liver microsomes and mitochondrial fractions by quantitative immunoblot analysis. (A) Immunoblot analysis of rat liver microsomes (MS) and mitochondria (MT) with anti-msALDH antibody. The increasing amounts of msALDH in microsomes and mitochondria were subjected to SDS-PAGE (7.5% acrylamide) followed by immunoblot analysis with anti-msALDH antibody. (B) The density of the bands in (A) was analyzed by an UltroScan XL laser densitometer and the densities in arbitrary units of the microsomes (○) and mitochondria (●) were plotted. The relative amount of msALDH was estimated from the gradient of the linear regions.

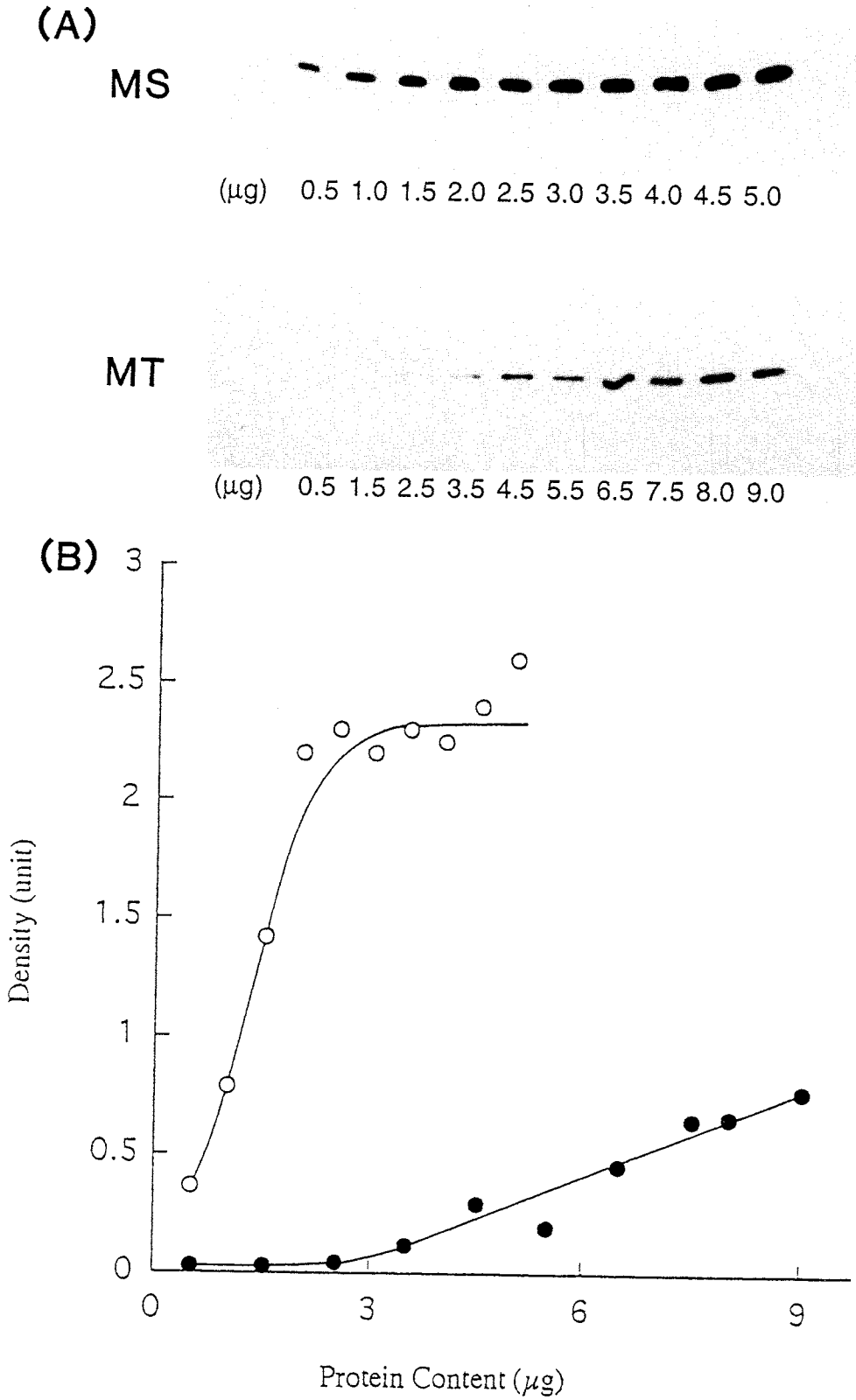
(A)
MS
(μg) 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0
MT
(μg) 0.5 1.5 2.5 3.5 4.5 5.5 6.5 7.5 8.0 9.0
(B)
Density (unit)
Protein Content (μg)
0
0.5
1
1.5
2
2.5
3
0
3
6
9

Fig. 4.

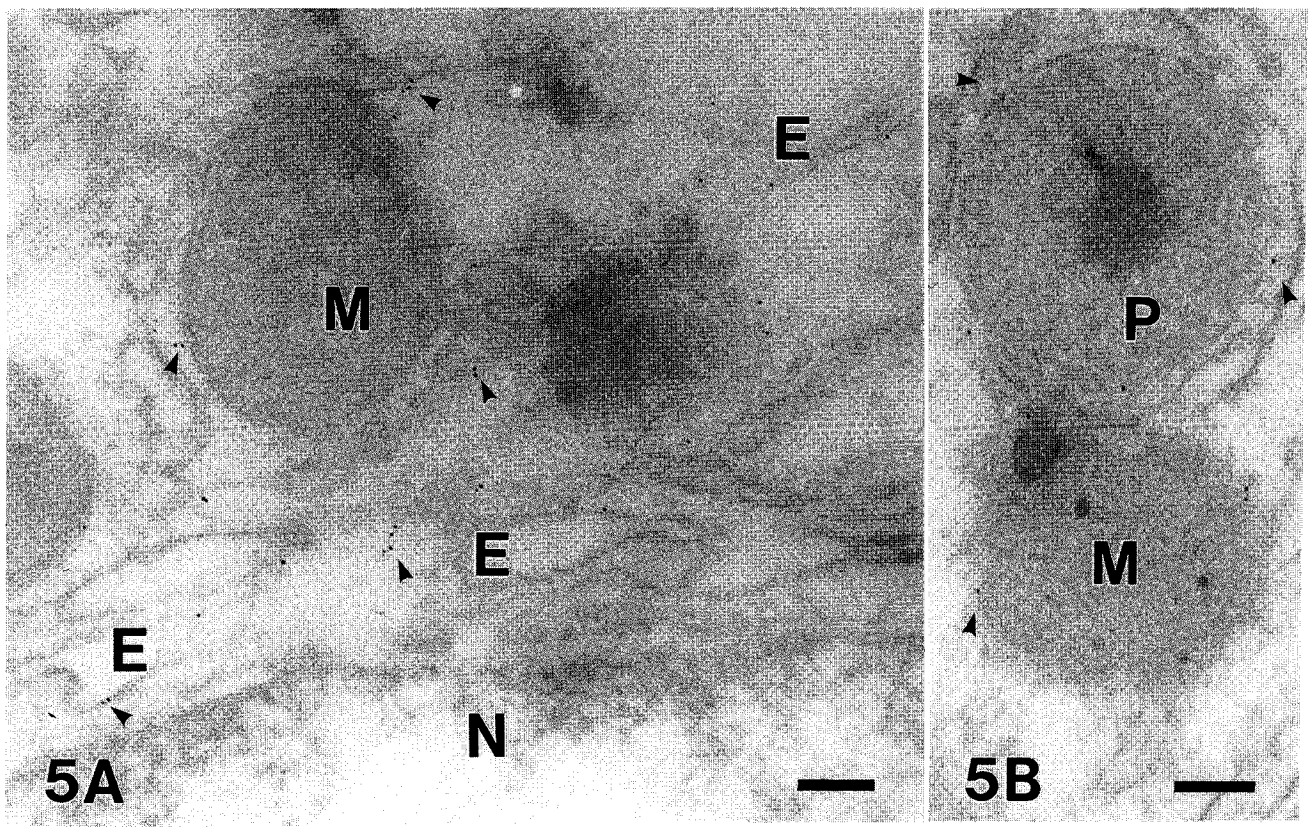

Fig. 5. Cryoimmunogold electron microscopy of msALDH in rat hepatocytes. Cryoultrathin sections of rat liver were stained by immunogold method and observed under an electron microscope. The cytoplasmic surfaces of the ER membranes (5A), outer mitochondrial membranes (5A and 5B) and peroxisomal membranes (5B) are labeled with gold particles (arrowheads). E, endoplasmic reticulum; M, mitochondria; N, nucleus; P, peroxisomes. ×54,000 Bar=0.20 μm

ly found in the nuclear matrix or the cytosolic space.

Fig. 6 shows agarose-embedded rat liver microsomes (A) and mitochondria (B) which were labeled with gold particles by pre-embedding immunogold electron microscopy. It is evident that the cytoplasmic surfaces of not only the microsomes but also the outer mitochondrial membranes are stained with gold particles. The degree of labeling in Fig. 6 was similar to that in Fig. 5.

Table III. DISTRIBUTION OF msALDH, PORIN AND CATALASE IN THE MITOCHONDRIA, SUBMITOCHONDRIAL FRACTIONS, MICROSOMES AND PEROXISOMES AS DETERMINED BY IMMUNOBLOT ANALYSIS.

	msALDH	Porin	Catalase
Mitochondria	11.0	100.0	2.9
Outer membrane	37.2	293.0	ND
Mitoplast	2.1	68.3	ND
Intermembrane space	10.1	17.1	ND
Microsomes	100.0	ND	ND
Peroxisomes	109.3	ND	100.0

The concentration of msALDH, porin and catalase in the microsomes, mitochondria and peroxisomes was assumed to be 100%, respectively. ND: not determined.

DISCUSSION

Smith and Packer (21) suggested two locations for aldehyde oxidation in rat liver mitochondria, one associated with the respiratory chain and one probably with the outer membrane. Tottmar *et al.* (26) also suggested existence of at least two different NAD^+-dependent ALDHs in rat liver; one with low Km for acetaldehyde was exclusively localized in the mitochondria and another with high Km for acetaldehyde in both the mitochondria and the microsomal fractions. Koivula and Koivusalo (9) reported that the msALDH had a high Km for aldehydes and had properties similar to the mitochondrial high-Km enzymes. Lindahl and Evces (12) isolated and characterized two mitochondrial and two microsomal ALDH isozymes with high Km values and showed that the major mitochondrial and microsomal isozymes with high Km were similar in some properties, but they were clearly distinguishable from each other by their response to inhibitors, their pH velocity profiles

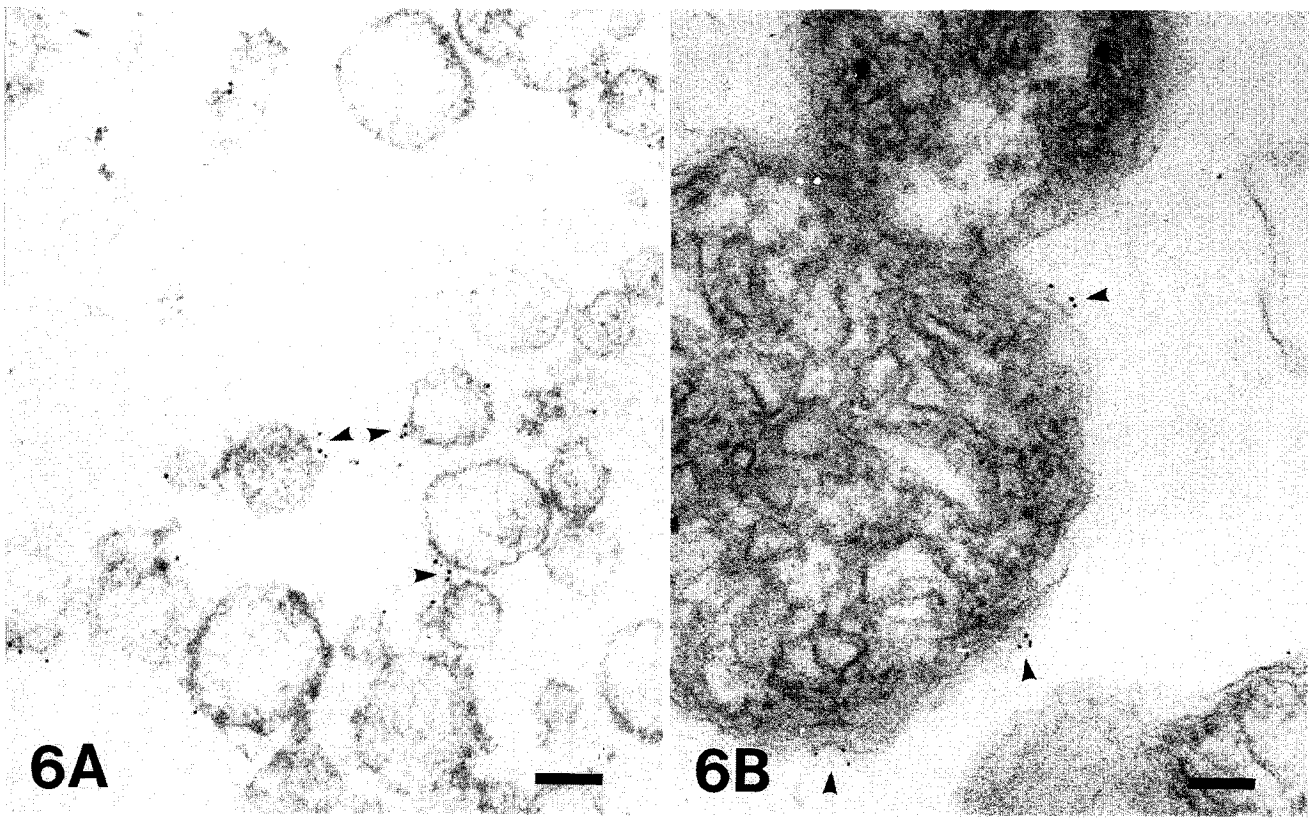

Fig. 6. Pre-embedding immunogold labeling with anti-msALDH antibody of agarose-embedded rat liver microsomes and mitochondria. The cytoplasmic surfaces of the microsomes (6A) and of the outer mitochondrial membranes (6B) are labeled with gold particles (arrowheads). ×87,000 Bar=0.10 μm

and their thermal stability.

Existence of ALDH in the peroxisomes was first proposed by Antonenkov *et al.* (2). They showed that clofibrate treatment caused a 2–3-fold increase in rat liver ALDH activity. The induced enzyme has a high Km for acetaldehyde and is located in the peroxisomal membrane.

From these reports, it is strongly suggested that msALDH and/or similar ALDH with high Km for acetaldehyde exists in the ER membranes, outer mitochondrial membrane and peroxisomal membranes. In order to clarify such a possibility, we utilized cell fractionation and the immunochemical technique to detect msALDH or the cross-reacting protein in the mitochondria, peroxisomes and the submitochondrial fractions. We also investigated the distribution of msALDH in rat hepatocytes by immunogold electron microscopy.

Our results clearly indicated that msALDH and/or the immunologically cross-reacting protein exists in both the outer mitochondrial membranes and peroxisomal membranes. The apparent subunit molecular mass of the msALDH in the microsomes, outer mitochondrial membranes and peroxisomes was similar (54 kDa).

In the present immunochemical and immunocytological experiments, we assumed that the anti-msALDH antibody does not cross-react with class-2 ALDH. Since purified rat liver class 2 ALDH was not available, we could not show this experimentally. However, comparison of Fig. 3A lane 3 with Fig. 3B lane 3 clearly indicates that the faint 54 kDa band of the former can be explained by the contamination of the outer mitochondrial membranes in the mitoplast fraction. This conclusion was supported by the very low homology in the amino acid sequence of rat msALDH with that of class 2 ALDH (only ~25%). We also examined the cross-reactivity of anti-human $ALDH_2$ antibody with rat msALDH. No 54 kDa band was detected in immunoblot analysis with rat liver microsomes (data not shown).

As reported by Takagi *et al.* (23), msALDH is exclusively synthesized on free polysomes and distributed randomly into the ER membranes. It is highly probable, therefore, that msALDH or the cross-reacting protein on the OMM and peroxisomal membranes is also synthesized on the free polysomes and distributed into the

OMM and peroxisomal membranes. Our *in vivo* transfection experiment with msALDH cDNA indicated that the msALDH molecules are distributed neither in the OMM nor the peroxisomal membranes but exclusively in the ER membranes (Masaki *et al.*, manuscript in preparation). It is strongly suggested, therefore, that the targeting signal in the carboxy-terminal region of msALDH is unique for each ALDH. The targeting signal of the cross-reacting protein in the OMM (OMM-ALDH) and peroxisomal membranes (P-ALDH) may be slightly different from that of the msALDH. In order to solve this problem, it is necessary to determine their primary sequences.

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