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PURIFICATION AND PROPERTIES OF A MEMBRANE-BOUND ALDEHYDE DEHYDROGENASE FROM RAT LIVER MICROSOMES

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SUMMARY: A membrane-bound aldehyde dehydrogenase was solubilized from rat liver microsomes and purified about 150-fold by chromatography on ω-aminohexyl- and 5'-AMP-Sepharose columns with a recovery of about 40%. The purified enzyme was homogeneous upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its monomeric molecular weight was estimated to be 51,000. In aqueous solution, it existed as large, polymeric aggregates. Its activity towards straight-chain aliphatic aldehydes increased as their carbon chain length was increased at least up to dodecanal, whereas aldehyde dehydrogenase in the cytosolic fraction of rat liver was most active with hexanal as substrate.

Aldehyde dehydrogenase (ALDH) activity of mammalian livers can be detected not only in the cytosolic fraction but also in subcellular organelles (1-6). For example, Koivula and Koivusalo (4) reported that the cytosolic, mitochondrial,and microsomal fractions of rat liver contained 10-15, 40-45, and 35-40 %, respectively, of the total hepatic ALDH activity. The soluble, cytosolic ALDH has been purified to homogeneity from horse and beef livers and characterized in detail (7-11). However, no purification studies have as yet been reported on the mitochondrial and microsomal enzymes, and neither their properties nor their relationships to the cytosolic ALDH are well understood. In this communication, we report the purification and some properties of a membrane-bound ALDH from rat liver microsomes and present evidence that it is different from the cytosolic counterpart.

MATERIALS AND METHODS

Bovine serum albumin, ovalbumin, beef liver glutamate dehydrogenase, yeast alcohol dehydrogenase, and beef liver catalase were purchased from Sigma, Sepharose 4B and 5'-AMP-Sepharose from Pharmacia, and aldehyde substrates from Nakarai Chemical Co., Kyoto. ω-Aminohexyl-Sepharose was prepared from Sepharose 4B and 1,6-diaminohexane as described (12), and hydroxyapatite by the method of Tiselius *et al.* (13). Cytochrome b_5 (14) and NADH-cytochrome b_5 reductase (15) were purified from rabbit liver microsomes by the published methods.

Male Sprague-Dawley rats, weighing 200-250 g, were killed by decapitation. The livers were perfused with cold 0.15 M KCl solution and homogenized with 4 vol. of

Abbreviations used are: ALDH, aldehyde dehydrogenase; $DodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

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the KCl solution. The homogenate was centrifuged at 10,000 x g for 10 min, and the supernate was again centrifuged at 78,000 x g for 90 min. The microsomes thus sedimented were washed once with 0.1 M potassium phosphate buffer (pH 7.5) containing 1mM DTT and 1mM EDTA, and resuspended in the same buffer. The supernate obtained by the 78,000 x g centrifugation was subjected to ammonium sulfate fractionation, and the precipitate formed between 30 and 70 % saturation was dissolved in a minimum amount of 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 1 mM EDTA, and the solution was dialyzed against the same buffer for 30 h with several changes of the buffer. The dialyzate was centrifuged at 20,000 x g for 30 min, and the clear supernate was used as the crude cytosolic ALDH preparation.

ALDH activity was measured spectrophotometrically by following the reduction of NAD^+ at 340 nm. To 2 ml of 0.1 M sodium pyrophosphate buffer (pH 9.0) containing 0.5 % (w/v) Triton X-100 were added 10 µl of 0.1 M decanal (or other aldehyde) solution in ethanol and 20 µl of 50 mM NAD^+ in this order, and the reaction was started by the addition of 10 µl of the enzyme. Triton X-100 was used to facilitate the dispersion of long-chain aldehydes; it inhibited the reaction slightly (about 5 %). One unit was defined as the amount reducing 1 µmole of NAD^+ per min. When the crude cytosolic ALDH preparation was used, 2 mM pyrazol was included in the reaction mixture to inhibit alcohol dehydrogenase, which was absent in microsomes.

Protein was determined by the method of Lowry *et al.* (16). $DodSO_4$-polyacrylamide gel electrophoresis was carried out in the presence of 0.1 % $Dod\text{-}SO_4$ as described by Hinman and Philips (17) by using 6 % cross-linked gels, and the gels were stained with Coomassie blue R-250 and scanned in a Joyce-Loebl Chromoscan 200 recording densitometer at 575 nm. For sedimentation studies, 50 µg of purified microsomal ALDH (freed from detergents) in 0.2 ml of 25 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 1 mM EDTA was layered over 5 ml of a linear sucrose concentration gradient from 5 to 40 % (w/v) containing 50 mM Tris-HCl buffer (pH 8.0), 1 mM DTT, and 1 mM EDTA. Centrifugation was performed at 50,000 rpm for 10 h in a Hitachi 55P centrifuge using an RPS 50-2 rotor. After centrifugation, 3-drop fractions were collected from the bottom of the tube and assayed for ALDH activity. Cytochrome b_5, catalase, and NADH-cytochrome b_5 reductase were also subjected to the density gradient centrifugation under the identical conditions. Cytochrome b_5 was measured from the intensity of its oxidized Soret peak at 413 nm, assuming a millimolar extinction coefficient of 117 (18). Catalase was assayed spectrophotometrically (19). NADH-cytochrome b_5 reductase was determined by measuring its NADH-ferricyanide reductase activity (15).

RESULTS AND DISCUSSION

The ALDH activity of rat liver microsomes could not be solubilized by sonication and by extensive washing with 0.5 M KCl, indicating that the enzyme is tightly bound to the microsomal membrane, as reported previously (4,5). To compare the properties of this membrane-bound enzyme with those of cytosolic ALDH, we attempted the solubilization and purification of the former enzyme and found that the following three-step procedure could yield a homogeneous preparation of microsomal ALDH.

All the purification steps were conducted at 0-4°. Potassium phosphate buffers (pH 7.5) containing 1 mM DTT and 1 mM EDTA were used throughtout; they are simply

Table I. A summary of purification of ALDH from rat liver microsomes

Purification step	Protein (mg)	Total activity (unit)	Specific activity (units/mg prot.)	Purification (-fold)	Yield (%)
Microsomes	550	75.4	0.14	1.0	100
Solubilized supernate	275	69.2	0.25	1.8	92
Aminohexyl-Sepharose eluate	43	56.1	1.30	9.5	74
AMP-Sepharose eluate	1.6	32.5	20.29	145	43

called 0.1 M buffer, etc. Washed liver microsomes (0.5-0.6 g protein) were suspended (to 6-8 mg protein/ml) in 0.1 M buffer containing 20 % (v/v) glycerol and 0.5 % (w/v) sodium cholate. The suspension was gently stirred for 1 h, and then centrifuged at 78,000 x g for 2 h. The supernate ("solubilized supernate") was applied to an ω-aminohexyl-Sepharose column (1.8 x 14 cm) which had been equilibrated with the solubilizing buffer. The column was washed with three times the bed volume of the same buffer, and elution was conducted slowly (15 ml/h) with the same buffer in which the cholate concentration was reduced to 0.4 % and 0.1 % (w/v) Triton X-100 was included. The eluates containing ALDH activity were combined ("aminohexyl-Sepharose eluate") and directly applied to a 5'-AMP-Sepharose column (2 x 5 cm) equilibrated with 0.1 M buffer containing 20 % glycerol and 0.2 % Triton X-100. The column was washed with the same buffer until a colored material passed through the column, then with 0.45 M buffer containing 0.2 % Triton X-100, and finally with the 25 mM buffer containing 0.2 % Triton X-100. Elution was then performed with the last-mentioned buffer containing 0.2 mM NAD^+; the ALDH was thereby eluted as a sharp band ("AMP-Sepharose eluate"). A summary of a typical purification experiment is shown in Table 1. As can be seen, this procedure resulted in about 150-fold purification of ALDH over microsomes with a

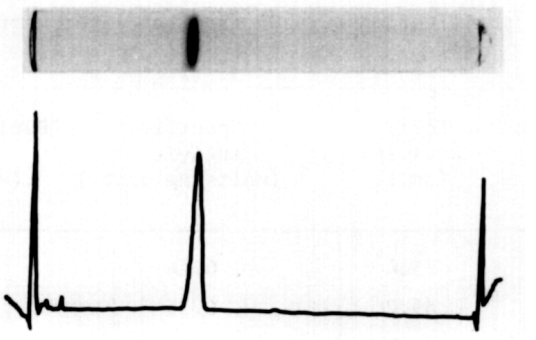
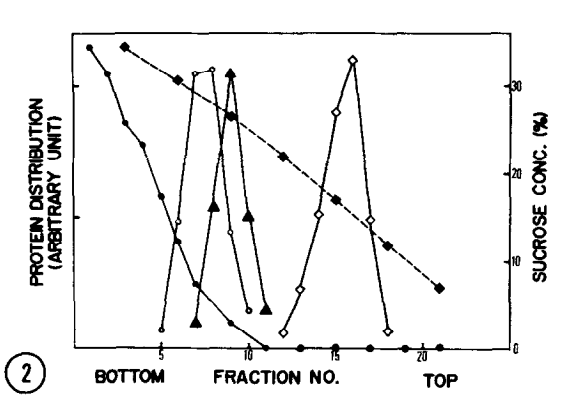


Fig. 1. $DodSO_4$-polyacrylamide gel electrophoresis of purified microsomal ALDH.

recovery of about 40 %. The final preparation could be stored at -20° for 3 weeks with a 20 % loss of the activity. Although this preparation contained Triton X-100, the detergent could be removed as follows. The preparation was applied to a hydroxyapatite column (2 x 5 cm), equilibrated with 25 mM buffer, and the column was extensively washed until no absorption at 276 nm due to Triton X-100 became detectable in the eluate. ALDH was then eluted with 0.45 M buffer, and the active eluate was dialyzed against 25 mM buffer and finally concentrated by means of a collodion bag.

$DodSO_4$-polyacrylamide gel electrophoresis of the purified ALDH preparation gave only a single protein band, indicating that it was homogeneous (Fig. 1). The detergent-free ALDH preparation showed only absorption band at 278 nm in the wavelength region from 260 to 650 nm (data not shown), indicating that it contained no colored prosthetic groups such as heme and flavin. The molecular weight of the protein band seen in the gel electrophoregram (Fig.1) was estimated to be about 51,000 by comparing its electrophoretic mobility with those of bovine serum albumin (M_r, 68,000), liver glutamate dehydrogenase (subunit M_r, 53,000) ovalbumin (M_r, 43,000), and yeast alcohol dehydrogenase (subunit M_r, 37,000) (data not shown). It has been reported that cytosolic ALDH purified from horse liver has a molecular weight of 220,000-240,000 and is composed of 4 identical subunits of 52,000-55,000 daltons (9,10), whereas the corresponding enzyme from rat hepatomas



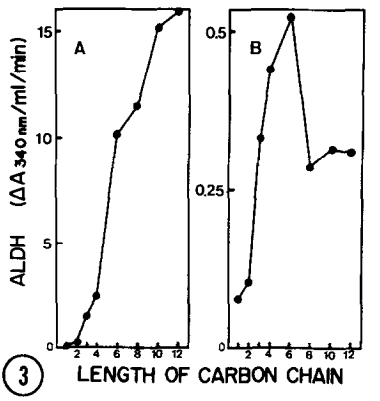


Fig. 2. Sucrose density gradient centrifugation of the detergent-free preparation of microsomal ALDH (●—●), cytochrome b_5 (◇—◇), catalase (▲—▲), and NADH-cytochrome b_5 reductase (○—○). The four proteins were centrifuged separately under the centrifugal conditions described in Materials and Methods. The distributions of the proteins are depicted in arbitrary units. Sucrose concentration (●···●) was determined by an Abbe refractometer.

Fig. 3. Reactivities of purified microsomal ALDH (A) and crude cytosolic ALDH (B) towards straight-chain aliphatic aldehydes. All the aldehydes were added to the reaction mixture at a final concentration of 1 mM. The amounts of purified microsomal ALDH and crude cytosolic ALDH used were 2 and 200 μg, respectively, per cuvette. The rate of NAD^+ reduction, expressed as ΔA at 340 nm/min, was plotted against the length of carbon chain of aldehyde substrates.

is a dimer consisting of 2 subunits having a molecular weight of 53,000 (11). The molecular weight of 51,000 estimated for rat liver microsomal ALDH is therefore similar to those of subunits of the cytosolic counterparts.

However, sucrose density gradient centrifugation of the detergent-free preparation of microsomal ALDH revealed that its dispersion state in aqueous solution was quite different from those of the cytosolic ALDHs. As shown in Fig.2, under the centrifugal conditions employed, a large portion of ALDH reached the bottom of the centrifuge tube, although some was still in the process of sedimentation. On the other hand, cytochrome b_5 (6.5 S (20)), catalase (11.2 S (21)), and NADH-cytochrome b_5 reductase (13.2 S (15)) formed sharp bands in the order of their sedimentation coefficient values. It was thus concluded that the purified ALDH existed in aqueous solution as large, polymeric aggregates, most of which having apparent molecular weight much higher than that of NADH-cytochrome b_5 reductase which has been shown to exist as an aggregate of about 360,000 daltons (15). The

capacity of microsomal ALDH to form aggregates in aqueous media suggested that it is an integral membrane protein, because many of such proteins are known to form aggregates because of their amphiphilic characters.

It was further found that microsomal ALDH was different from the cytosolic counterpart also in substrate specificity. As shown in Fig. 3A, microsomal ALDH showed practically no activities towards formaldehyde and acetaldehyde and its activity towards straight-chain aliphatic aldehydes increased as their carbon chain length was increased at least up to dodecanal (C_{12}). Fig. 3B showes, on the other hand, that the crude cytosolic ALDH preparation from rat liver possessed measurable activities on formaldehyde and acetaldehyde, and it showed a highest activity with hexanal (C_6) as substrate; octanal (C_8), decanal (C_{10}), and dodecanal (C_{12}) were much less effective than hexanal as substrates.

From these observations it is clear that rat liver microsomes contain a membrane-bound ALDH which is different from the enzyme present in the cytosolic fraction. Although the ALDH in liver mitochondria has not yet been isolated, there is evidence that this ALDH is localized in the mitochondrial matrix as a soluble protein (4). If this is so, then it is also different from the microsomal enzyme which is tightly bound to membranes. At present nothing is known of the significance of the occurrence of different types of ALDH in the three different fractions of hepatocytes. However, since aldehydes are usually toxic to living cells because of their high reactivities, it is likely that these ALDHs are functioning in their detoxication. Microsomes contain a number of enzymes which participate in lipid metabolism and some of them are known to produce long-chain aldehydes. For example, fatty aldehydes are formed by the reaction catalyzed by the microsomal alkyl glyceryl ether cleavage enzyme system (22). Microsomal ALDH is expected to catalyze the oxidation of such water-insoluble aldehydes produced on the surface of the microsomal membrane. This enzyme may also be involved in the conversion of fatty acids to α,ω-dicarboxylic acids in which microsomal fatty acid ω-hydroxylase is also takes a part.

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