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PURIFICATION AND PARTIAL CHARACTERIZATION OF ALDEHYDE DEHYDROGENASE FROM HUMAN ERYTHROCYTES

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Summary

Human erythrocyte aldehyde dehydrogenase (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3) was purified to apparent homogeneity. The native enzyme has a molecular weight of about 210 000 as determined by gel filtration, and SDS-polyacrylamide gel electrophoresis of this enzyme yields a single protein band with a molecular weight of 51 500, suggesting that the native enzyme may be a tetramer. The enzyme has a relatively low K_m for NAD (15 μM) and a high sensitivity to disulfiram. Disulfiram inhibits the enzyme activity rapidly and this inhibition is apparently of a non-competitive nature. In kinetic characteristics and sensitivity to disulfiram, erythrocyte aldehyde dehydrogenase closely resembles the cytosolic aldehyde dehydrogenase found in the liver of various species of mammalians.

Introduction

Acetaldehyde, which is produced mainly in the liver primarily by the action of alcohol dehydrogenase, (rather than ethanol itself), appears to play multiple roles in mediating the pharmacological and biochemical effects of ethanol [1—3]. In recent years, the distribution and functions of aldehyde dehydrogenase (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3) are much better understood and there have been many sophisticated studies done on purification and characterization of isozymes of aldehyde dehydrogenase and subcellular localization of these isozymes in the mammalian liver [4—6].

Since the liver is the primary site of ethanol oxidation in vivo and has the highest activity of aldehyde dehydrogenase among the various organs [7,8], liver enzyme is considered to be of major importance in the regulation and

control of acetaldehyde metabolism. Taking into account the nonspecific distribution profiles of acetaldehyde in the entire body, however, further-extended research on acetaldehyde metabolism in the other tissues is warranted. In previous work [9], we found that human erythrocytes have aldehyde dehydrogenase activity which is strongly inhibited by a low concentration of disulfiram. We did not, however, determine if this enzyme might oxidize acetaldehyde significantly at a low concentration range observed in case of the ingestion of ethanol [10,11]. We report herein our purification and preliminary kinetic characterization studies of the enzyme, done in attempt to determine if aldehyde dehydrogenase in erythrocytes actually does play a role in acetaldehyde metabolism in vivo.

Materials and Methods

Chemicals. NAD, NADP and disulfiram were purchased from Sigma Chemical Company. CM-Sephadex C-50, DEAE-Sephadex A-50, 5'-AMP-Sepharose 4B and Sephadex G-200 were obtained from Pharmacia Fine Chemicals. All other chemicals were of reagent grade. Acetaldehyde and propionaldehyde were redistilled periodically before use and these distilled aldehydes were shown to be pure by the method of Cederbaum et al. [12].

Assay methods. Aldehyde dehydrogenase activity was measured spectrophotometrically at 22°C. The units of activity in μmol/min were calculated using a molar extinction coefficient of 6220 $M^{-1} \cdot cm^{-1}$ for NADH at 340 nm [13]. During enzyme purification, assay was performed in the medium consisting of 50 mM sodium pyrophosphate buffer, pH 8.6 (optimum pH), 1 mM NAD and 2 mM propionaldehyde. Further work on characterization of the purified enzyme was done in 100 mM sodium phosphate buffer, pH 7.4. Assays were initiated with the addition of enzyme to the reaction mixture. The enzyme was dialysed against glass-distilled, deionized water that was saturated with nitrogen gas before the kinetic and inhibition experiments.

Protein determination. The protein concentration was measured by the method of Lowry et al. [14].

Gel electrophoresis. Polyacrylamide gel electrophoresis (7.5% gel) was performed either in a pH 8.9 buffer system [15] or in a system of sodium phosphate buffer (100 mM for gel, 50 mM for electrode), pH 7.3. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [16].

Enzyme purification. (1) Hemolysis: Human blood obtained from ten healthy male donors who had not ingested alcohol within 48 h, was collected in heparinized tubes. The stroma-free hemolysate [9] obtained from 100 ml of packed erythrocytes was dialysed for 18 h against 30 vol. of 30 mM sodium phosphate buffer, pH 6.0 (buffer 1), with two changes of buffer. Throughout the purification experiments, buffer solution contained 0.1% 2-mercaptoethanol and 1 mM EDTA since activity of the erythrocyte enzyme was reduced by sulfhydryl blocking agents [9] and divalent or heavy metal ions (unpublished data). CM-Sephadex chromatography; after the recentrifugation of dialysed hemolysate, the supernatant obtained was applied to a CM-Sephadex column (20 × 8 cm) equilibrated with buffer 1. The eluate immediately

following the void volume contained the aldehyde dehydrogenase activity. *(2) $(NH_4)_2SO_4$ fractionation*: The pooled fractions of the active CM eluate were precipitated at 30 and 70% saturation $(NH_4)_2SO_4$ respectively and the latter precipitate retained. The resultant pellet was dissolved in 30 mM sodium phosphate buffer, pH 6.8 (buffer 2) and was exhaustively dialysed against the same buffer. *(3) DEAE-Sephadex chromatography*: The insoluble material was removed by centrifugation and the supernatant was then applied to a DEAE-Sephadex column (35 × 3 cm) equilibrated with buffer 2. The column was washed by approx. 800 ml of buffer 2 and was subsequently eluted with linear salt gradient formed from 1000 ml of buffer 2 and 1000 ml of buffer 2 containing 1 M NaCl. *(4) AMP-Sepharose chromatography*: The active fractions obtained from the above step were pooled and the pH was adjusted back to 6.0 with 1 M NaH_2PO_4. The sample was applied to a 5'-AMP-Sepharose column (15 × 1.2 cm) equilibrated with buffer 1, and washed by the same buffer until the extraneous proteins were removed. The column was then extracted with 100 mM phosphate buffer, pH 8.0. *(5) Gel filtration*: Fractions with enzyme activity were concentrated by ultrafiltration from a volume of 70 ml to 0.5 ml. The concentrated sample was applied to a Sephadex G-200 column (80 × 1.8 cm) equilibrated with buffer 1 and was eluted at 9.0 ml/h with the same buffer. For the estimation of the molecular weight of aldehyde dehydrogenase, bovine serum albumin, beef liver catalase, urease (Sigma) and alcohol dehydrogenase (Boehringer Mannheim) were also chromatographed as standards in the same manner.

Results

Purification

Results of typical puridication experiments are summarized in Table I. Through these steps, about 3 mg of enzyme can be obtained from 100 ml of packed erythrocytes and polyacrylamide gel electrophoresis of this enzyme shows a single protein band both in pH 8.9 buffer system [15] and pH 7.3 buffer system. The exact recovery and rate of purification cannot be calculated in comparison with hemolysate since it is impossible to estimate the aldehyde

TABLE I

PURIFICATION OF ALDEHYDE DEHYDROGENASE FROM HUMAN ERYTHROCYTES

	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol · min^{-1} · mg^{-1})	Protein concn. (mg/ml)	Yield (%)	Purification (-fold)
CM-Sephadex	1494	5.19	0.0035	1.78	100	1
$(NH_4)_2SO_4$ fractionation (30—70%)	802	4.18	0.0052	16.7	80.4	1.5
DEAE-Sephadex	43.4	2.25	0.0518	0.723	43.3	14.8
AMP-Sepharose	4.7	2.03	0.432	0.0671	39.2	123.4
Sephadex G-200	2.9	1.34	0.462	0.0725	25.9	132.0

* Before protein measurement, an aliquot of sample was dialysed against 30 mM sodium phosphate buffer (pH 6.0) to remove 2-mercaptoethanol and EDTA.

dehydrogenase activity of hemolysate spectrophotometrically. Using headspace gas chromatographic method [9], however, we examined the activity of hemolysate by measurement of substrate (propionaldehyde) decrease, and 0.15 nmol · min^{-1} · mg^{-1} protein as specific activity and 6360 nmol/min as total activity were obtained. From these values it is calculated that recovery is about 20% and purification is about 3000 fold at the final step of purification. The enzyme was stored in the presence of 20% glycerol at −20°C, and at these conditions, loss of enzyme activity is less than 15% per a month.

Molecular weight

The native molecular weight of aldehyde dehydrogenase was estimated to be 215 000 by gel filtration on a column of Sephadex G-200 calibrated with standards of known molecular weight. A single protein band with a molecular weight of 51 500 was observed by SDS-polyacrylamide gel electrophoresis of this protein, suggesting that the native enzyme may be a tetramer composed of subunits of similar molecular weight.

Substrate specificity

The specificity of aldehyde dehydrogenase for aldehydes and coenzymes is presented in Table II. These results show that this enzyme can oxidize a rather broad spectrum of both aliphatic and aromatic aldehydes and requires NAD more specifically as a coenzyme than NADP. When a wide range concentration of propionaldehyde is used, the double-reciprocal plots for the activity of this enzyme as a function of substrate concentrations exhibit two phases with abrupt transition at about 0.5 mM. Although clear point which separates two phases was not found, similar result was obtained in case of acetaldehyde. Therefore, K_m values for these two aldehydes are determined at high and low substrate concentration ranges. The double-reciprocal plots for the activity as a function of other tested aldehydes or coenzymes, however, are linear over a wide concentration range.

TABLE II

SUBSTRATE SPECIFICITY OF ALDEHYDE DEHYDROGENASE FROM HUMAN ERYTHROCYTES

In the determination of K_m values for aldehydes, NAD was kept at 1 mM and in determination for NAD or NADP, 8 mM propionaldehyde was used. V values are relative to propionaldehyde as substrate. V for propionaldehyde is 0.27 μmol NADH · min^{-1} · mg^{-1}.

Substrate *	K_m (μM)		Relative V
Acetaldehyde **	17	830	0.85
Propionaldehyde **	9	765	1
DL-Glyceraldehyde	205		0.53
Glycolaldehyde	180		0.36
Benzaldehyde	0.65		0.30
Cinnamaldehyde	0.55		0.22
NAD	15		
NADP	5200		

* Concentration of aldehyde was not corrected for hydration.

** For acetaldehyde and propionaldehyde, the double-reciprocal plots are not linear. K_m values listed on the left were determined at a low substrate concentration range (0—70 μM) and at a high concentration range (0.8—20 mM) on the right, respectively.

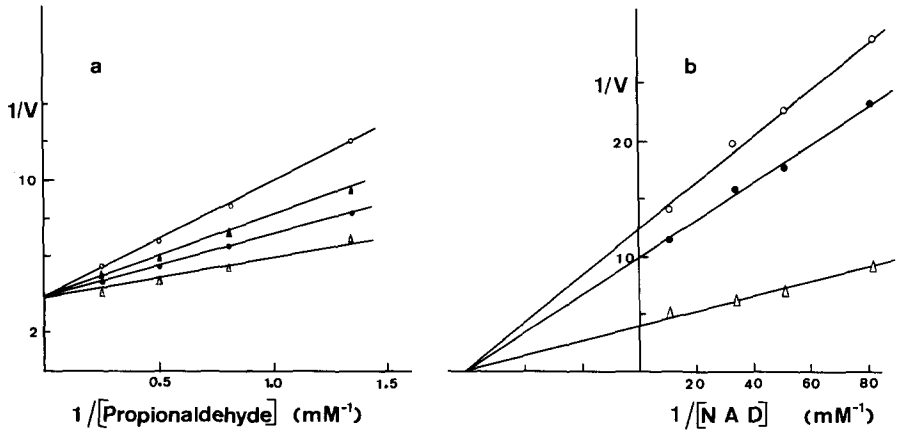

Fig. 1. Double-reciprocal plots showing the effect of chloral hydrate (a) and disulfiram (b) on aldehyde dehydrogenase activity. (a) Enzyme activity was measured at a fixed concentration of 1 mM NAD, and at various concentrations of propionaldehyde in the presence and absence of chloral hydrate. △, without chloral hydrate: ●, 100 μM: ▲, 150 μM: ○, 300 μM chloral hydrate. (b) Enzyme activity was measured at a fixed concentration of 20 mM propionaldehyde, and at various concentrations of NAD in the presence and absence of disulfiram. △, without disulfiram: ●, 0.25 μM: ○, 0.5 μM disulfiram. The enzyme concentration in the assay mixture was 0.13 μM (based on a molecular weight of 215 000).

Inhibition studies

The data in Fig. 1a show that inhibition of aldehyde dehydrogenase activity by chloral hydrate is competitive with aldehyde, and the K_i value derived from these plots is 170 μM. On the other hand, disulfiram inhibits the enzyme activity in a non-competitive fashion with NAD (Fig. 1b) as reported for sheep liver cytosolic enzyme [17]. This non-competitive nature is also observed with respect to propionaldehyde. The enzyme appears to be inactivated rapidly by disulfiram since preincubation of the enzyme with disulfiram for 10 min gives the same percent inhibition as our standard methods which are without preincubation of the enzyme with disulfiram.

Inhibition of the enzyme activity by a range of disulfiram concentrations are tested in the presence of 1 mM NAD, 20 mM propionaldehyde and 0.15 μM enzyme solution. Inhibition is proportional to the disulfiram concentration almost linearly to approx. 50% of the control rate, and gradual decline of the inhibition rate is observed with increasing concentration of disulfiram (50% inhibition at 0.17 μM and 90% at 1.15 μM, respectively). Although inhibition of the enzyme activity by disulfiram is not restored by dialysing the disulfiram-treated enzyme preparation, addition of a high concentration of 2-mercaptoethanol (0.2 M) restores the enzyme activity almost completely (90—100%).

Discussion

In recent years, two major isozymes of aldehyde dehydrogenase have been isolated from various mammalian livers [5,6,18]. One enzyme is apparently cytosolic and the other mitochondrial in origin. Between these two isozymes, there are marked differences in kinetic properties and sensitivities to disulfiram inhibition. Cytosolic enzyme has a high K_m for aldehydes, low K_m for NAD

and is strongly inhibited by disulfiram. The other enzyme which originates in mitochondria has a low K_m for aldehydes, high K_m for NAD and considerably lower sensitivity to disulfiram. Considering the affinity to coenzyme and sensitivity to inhibitor, it is suggested that erythrocyte enzyme is similar to liver cytosolic aldehyde dehydrogenase. This idea is also supported by the findings of others that in purified sheep liver [19] and human liver [6] cytosolic enzyme has a biphasic nature of the kinetics over a wide range concentration of acetaldehyde and propionaldehyde as substrate.

The inhibitory effect of disulfiram on aldehyde dehydrogenase activity has been given considerable attention as it has a significant physiological importance [17,20,21]. From the results of disulfiram inhibition experiments, it can be said that severe and rapid inactivation of the erythrocyte enzyme is caused by irreversible association, presumably covalent interaction between disulfiram and enzymic thiol group as reported with regard horse [5] and sheep [17] liver cytosolic enzyme.

Since there have been many evidence that treatment of disulfiram causes a marked accumulation of acetaldehyde after ethanol ingestion, disulfiram sensitive aldehyde dehydrogenase has been considered to be responsible for acetaldehyde oxidation in vivo. In addition, blood acetaldehyde level in a healthy subject, as the result of ingestion of ethanol, are reported to be a range of 10—100 μM [10,11]. Considering the K_m values for acetaldehyde and relatively low K_m for NAD, it is suggested that aldehyde dehydrogenase in erythrocyte as well as in liver enzyme can oxidize blood acetaldehyde significantly in vivo. Raskin [22] suggested that substantial amounts of ethanol-derived acetaldehyde appear to be oxidized at extrahepatic sites, presumably by ubiquitous aldehyde dehydrogenase [7,8] because the hepatic venous acetaldehyde levels are several times higher than the peripheral blood levels in laboratory animals [23]. Activity of the erythrocyte enzyme is rather low compared with that of the liver enzyme, and no doubt the liver is the major site of acetaldehyde metabolism. However, from the fact that considerably large amounts of erythrocytes are widely distributed throughout the entire body, it is reasonable to expect that these corpuscles contribute to the acetaldehyde oxidation in vivo.

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References

1 Walsh, M.J., Hollander, P.B. and Truitt, E.B. Jr. (1969) J. Pharmacol. Exp. Ther. 167, 173—186
2 Nakano, J. and Kessinger, J.M. (1972) Eur. J. Pharmacol. 17, 195—201
3 Erwin, V.G., Kim, J. and Anderson, D. (1975) Biochem. Pharmacol. 24, 2089—2095
4 Feldman, R.I. and Weiner, H. (1972) J. Biol. Chem. 247, 260—266
5 Eckfeldt, J., Mope, L., Takio, K. and Yonetani, T. (1976) J. Biol. Chem. 251, 236—240
6 Greenfield, N. and Pietruszko, R. (1977) Biochim. Biophys. Acta 483, 35—45
7 Deitrich, R.A. (1966) Biochem. Pharmacol. 15, 1911—1922
8 Raskin, N.H. and Sokoloff, L. (1972) J. Neurochem. 19, 273—282
9 Inoue, K. Ohbora, Y. and Yamasawa, K. (1978) Life Sci. 23, 179—184
10 Korsten, M.A., Matsuzaki, S., Feinman, L. and Lieber, C.S. (1975) N. Engl. J. Med. 292, 386—389

11 Tabakoff, B., Anderson, R.A. and Ritzmann, R.F. (1976) Biochem. Pharmacol. 25, 1305—1309
12 Cederbaum, A.I., Lieber, C.S. and Rubin, E. (1976) Arch. Biochem. Biophys. 176, 525—538
13 Kornberg, A. and Horecker, R.L. (1953) Biochem. Prep. 3, 24—28
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
16 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
17 Kitson, T.M. (1978) Biochem. J. 175, 83—90
18 Crow, K.E., Kitson, T.M., MacGibbon, A.K.H. and Batt, R.D. (1974) Biochim. Biophys. Acta 350, 121—128
19 MacGibbon, A.K.H., Blackwell, L.F. and Buckley, P.D. (1977) Eur. J. Biochem. 77, 93—100
20 Hart, G.J. and Dickinson, F.M. (1977) Biochem. J. 163, 261—267
21 Sanny, C.S. and Weiner, H. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 634
22 Raskin, N.H. (1975) N. Engl. J. Med. 292, 422—423
23 Forsander, O.A., Hillbom, M.E. and Lindros, K.O. (1969) Acta Pharmacol. Toxicol. 27, 410—416