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COMPARISON OF ALDEHYDE DEHYDROGENASES FROM CYTOSOL AND MITOCHONDRIA OF RAT LIVER

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SUMMARY

Comparison of crude and partially purified aldehyde dehydrogenase preparations (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3) from the cytosolic compartment and from the mitochondria of the rat liver indicates that two different enzymes are involved. Each compartment has one main aldehyde dehydrogenase enzyme which differ in their response to P_i stimulation, in their specificity towards various aldehydes, and in their apparent K_m values. The enzymes show a molecular weight difference in gel filtration. Starch gel electrophoresis shows that both preparations have one active band with the same mobility. The existence of low-K_m and high-K_m enzymes in the rat liver is suggested and the work towards purification of the low-K_m enzymes is described.

INTRODUCTION

The studies on the localization of aldehyde oxidation in rat liver suggest that both the cytosolic and the mitochondrial compartments are involved (see ref. 1). The relative importance of these systems is far from established. One indication of the possible role each of these enzymes may play in the overall metabolism is their capacity to oxidize certain aldehydes in the *in vitro* systems. In this respect the results vary widely (see ref. 1). Too little attention has been paid to the possibilities of obtaining artefactual distribution of the activity as discussed by de Duve[2].

Previous studies indicated that the mitochondria account for 80% of the total aldehyde dehydrogenase activity (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3) in the rat liver[1]. The activity associated with the mitochondrial fraction could not be removed by repeated washings of the mitochondria. It could only be separated from the particles by sonication. A possibility, however, remained that the cytosolic activity resulted because of some damage to the mitochondria during the homogenization thus releasing part of the activity to the post-mitochondrial supernatant.

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To study this question it was decided to take a closer look on the properties of the activity in the two compartments.

Later on, it was felt necessary to make an effort to purify the enzymes involved in order to establish that the differences seen between the crude preparations genuinely represented the enzyme species and were not influenced by the crude environment where the activities were present. In this paper evidence is presented that, in the conditions employed, only one main enzyme species is involved in both compartments and that the mitochondrial and the cytosolic enzymes are, indeed, different entities.

MATERIALS AND METHODS

Preparation of the enzymes

Five male Wistar rats were used for each experiment. The rats were anaesthetized with Nembutal^R (5 mg/100 g body wt) and the livers perfused *in situ* with iso-osmotic NaCl. A 25% (w/v) homogenate was made in 0.25 M sucrose–1 mM EDTA–1 mM mercaptoethanol (pH 7.4) and centrifuged at 24 000 × *g* for 15 min (all values in this paper refer to g_{max}). The supernatant was further centrifuged at 105 000 × *g* for 60 min. The 24 000 × *g* sediment was resuspended in the precentrifugation volume and layered over an equal volume of 0.44 M sucrose–1 mM EDTA–1 mM mercaptoethanol (pH 7.4). The tubes were centrifuged at 700 × *g* for 5 min. The resulting supernatant was sonicated as described[1] and centrifuged at 105 000 × *g* for 60 min.

The clear supernatants obtained after ultracentrifugation are referred to as crude mitochondrial and cytosolic enzymes. At a subsequent stage the precipitates obtained between 35 and 55% $(NH_4)_2SO_4$ saturation (the cytosolic enzyme) and between 40 and 60% (the mitochondrial enzyme) at 0 °C were spun down at 38 000 × *g* for 20 min and the pellets dissolved in approx. 10 ml of distilled water. Both solutions were desalted by passage through a 16 cm × 2.6 cm Sephadex^R G-25 column equilibrated with 5 mM KH_2PO_4–1 mM EDTA–1 mM mercaptoethanol (pH 7.0). Pooled fractions were centrifuged for 15 min at 38 000 × *g*. The cytosolic preparation was applied to a 19 cm × 2.6 cm DEAE-cellulose column (Whatman DE-11) equilibrated with the buffer used for desalting. The same buffer was also used to elute the activity. The active fractions in the effluents were pooled. After the total of 3 bed volumes of the buffer, the mitochondrial fraction was applied on the same column. The column was again washed with 3 bed volumes of the buffer, and a linear P_i gradient was then built up with 500 ml of the buffer and 500 ml of 50 mM KH_2PO_4–1 mM EDTA–1 mM mercaptoethanol (pH 7.4). The activity maximum appeared at the beginning (at 8–10 mM P_i concentration as measured conductometrically). The cytosolic and mitochondrial enzyme preparations were applied to a 11 cm × 2.6 cm CM-cellulose column (CM 2100 from Mackerey, Nagel and Co.) equilibrated with 5 mM KH_2PO_4 buffer (pH 7.0), containing 1 mM EDTA and 1 mM mercaptoethanol as above. The column was washed with 3 bed volumes of the buffer between the two samples. The fractions in the effluents were pooled, reduced to a few ml by ultrafiltration and then lyophilized. Both preparations were dissolved in a small amount of distilled water, centrifuged for 15 min at 38 000 × *g*, and stored at −15 °C.

Assay of acetaldehyde dehydrogenase

The general conditions have been described before[1]. The reaction vessel contained 1 mM pyrazole, 2 mM NAD^+, 0.1–0.5 mM acetaldehyde and the enzyme preparation in a total volume of 0.5 ml of 0.1 M KH_2PO_4 buffer (pH 7.4). The rate of disappearance of acetaldehyde was measured with a Perkin–Elmer F 40 headspace gas-liquid chromatograph. In some experiments specified in the text the formation of NADH was followed at 340 nm. The reaction was performed in 1 ml silica cuvettes with a light path of 1 cm at 25 °C. The reference cuvette contained all the reagents except the aldehyde.

Proteins for the calculation of specific activities were measured by the method of Lowry *et al.*[3].

Gel filtration

A Sephadex^R G-150 column was calibrated with cytochrome *c* (Merck), ovalbumin, bovine serum albumin and catalase from Sigma Chemical Co., and with yeast alcohol dehydrogenase, pig heart malate dehydrogenase and rabbit muscle lactate dehydrogenase from Boehringer and Sons.

The molecular weights of the last three enzymes were obtained from the manufacturer. Each 1-ml sample was individually loaded on the top of a 26 cm × 1.2 column equilibrated with 5 mM KH_2PO_4 buffer (pH 7.4), and 0.5-ml fractions were collected. Void volumes were measured with Blue Dextran. Aldehyde dehydrogenase was localized enzymatically, cytochrome *c* at 410 nm, Blue Dextran at 260 nm, and other proteins at 280 nm.

Starch gel electrophoresis

The electrophoresis and subsequent localization of aldehyde dehydrogenase activity were essentially those originally introduced by Smithies[4] and Robbins[5], respectively. They were used as described by Pikkarainen[6]. The gels were stained for 2 h in the dark at room temperature in the presence of 5 mM acetaldehyde.

RESULTS

Purification

The homogenization medium used previously[1], namely 0.154 M KH_2PO_4–0.25 M sucrose–3 mM $MgCl_2$, was replaced by 0.25 M sucrose–1 mM EDTA–1 mM mercaptoethanol. This was based on the observation that in the presence of high P_i concentration and $MgCl_2$ the mitochondria tended to aggregate around the nuclei and consequently sedimented with the nuclear fraction. In the present system over 70% of the aldehyde dehydrogenase activity associated with the mitochondria was recovered in the mitochondrial fraction (700 × *g* supernatant). This change made it possible to use only a small number of animals as a starting material for the purification procedures.

Table I shows the purification steps in greater detail.

Comparison of some kinetic properties

When P_i stimulation was measured in crude preparations a phosphate solution, at pH 7.4, was added to the reaction mixture to give the required P_i concentrations,

TABLE I

PURIFICATION OF CRUDE ENZYME PREPARATIONS

Step	*Fraction**	*Protein (mg)*	*Activity (nmoles/min)*	*Spec. act. (nmoles/min/mg protein)*	*Yield (%)*	*Purification (-fold)*
105 000 × *g* supernatant	C	3150	9 300	2.95	100	1
	M	1390	21 500	15.5	100	1
$(NH_4)_2SO_4$ precipitate (desalted)	C	870	7 130	8.20	77	3
	M	372	11 000	38.7	51	2
DEAE-cellulose fraction	C	215	4 280	19.9	47	7
	M	38	4 940	130	23	8
CM-cellulose fraction	C	143	3 060	21.4	33	7
	M	29	4 190	143	19	9

* C stands for cytosolic, M for mitochondrial preparation.

and initial rates for acetaldehyde disappearance were measured. As shown in Fig. 1, the effect was confined to the mitochondrial preparation and was closely parallel to that found in the total homogenate[1]. The final preparations were first run through a Sephadex^R G-25 column. pH was adjusted to pH 7.4 with NaOH. The stimulatory effect again showed up in the mitochondrial fraction but was somewhat lower than in crude preparations, and in some cases a small increase was evident in the cytosolic preparation as well.

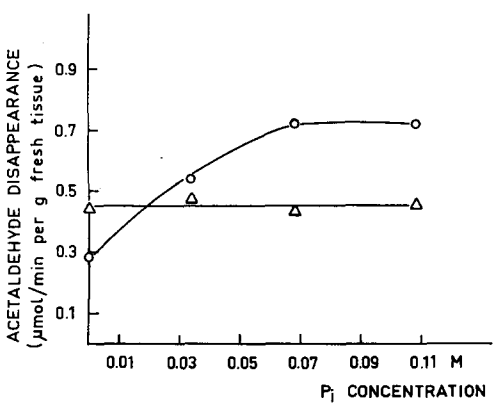

Fig. 1. Effect of P_i on aldehyde dehydrogenase activity. Crude enzyme preparations were incubated in the presence of an appropriate concentration of P_i as described in Materials and Methods. ○, mitochondrial fraction; △, cytosolic fraction.

Both crude and final mitochondrial preparations showed an apparent K_m value for acetaldehyde below 10^{-5} M, whether measured by gas–liquid chromatography or spectrophotometrically. On the other hand, with both methods the cytosolic enzyme showed a value higher than 10^{-5} M. Some inconsistency was encountered from one preparation to another in this fraction but the value generally fell close to $3 \cdot 10^{-5}$ M (Fig. 2).

pH optima for purified preparations were measured only in 0.1 M KH_2PO_4

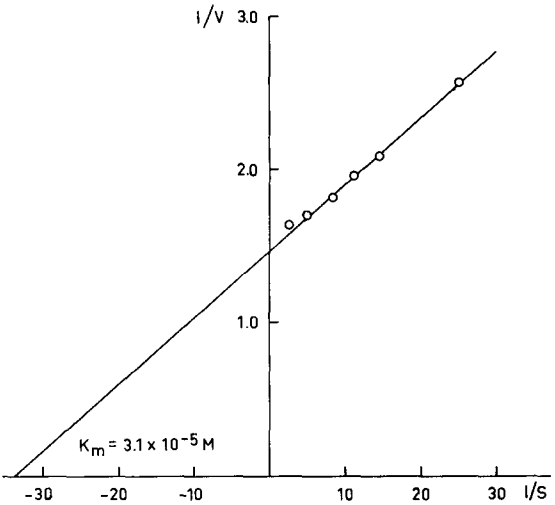

Fig. 2. Lineweaver–Burk plot for crude cytosolic enzyme preparation. Assay conditions as described in Materials and Methods.

buffer adjusted with NaOH over the pH range used. The maximal activity was not yet attained at pH 8.9 and the optimum seems to be around or about pH 9 for both fractions.

Purified preparations were tested for specific activity for several aldehydes. Table II shows the relative values. The same experiment was repeated with the gas–liquid chromatographic method. Only acet-, propion-, *n*-butyr-, isobutyr-, and isovaleraldehydes could be analyzed in the chromatograph. The difference between the two preparations was clear when measured in analogous conditions. However, the results cannot be compared with the spectrophotometric data because the calibration steps for quantitation were not done.

TABLE II

SUBSTRATE SPECIFICITY OF FINAL ENZYME PREPARATIONS

Enzyme activities were measured spectrophotometrically at 340 nm. Assay conditions as described for acetaldehyde in the section Materials and Methods. Each substrate had a final concentration of 1 mM. The activity with acetaldehyde was taken as one and other results compared to this value.

Substrate	*Increase in $A_{340\ nm}$ per unit time*	
	Cytosolic enzyme	*Mitochondrial enzyme*
Acetaldehyde	1.00	1.00
Formaldehyde	0.16	1.02
Propionaldehyde	0.79	0.68
n-Butyraldehyde	1.21	0.74
Isobutyraldehyde	0.58	0.69
Isovaleraldehyde	0	0.36
Benzaldehyde	0.79	0.17
Glyceraldehyde	0	0.03
Indolyl-3-acetaldehyde	0	0.19

Behaviour in gel filtration

The crude, $(NH_4)_2SO_4$-precipitated, and final preparations behaved similarly on gel filtration. The mitochondrial activity always emerged earlier, the difference in elution volumes being 1–1.4 ml in similar columns. One column was calibrated and the result is shown in Fig. 3. The molecular weights based on this single run are 180 000 for the mitochondrial enzyme and 110 000 for the cytosolic one.

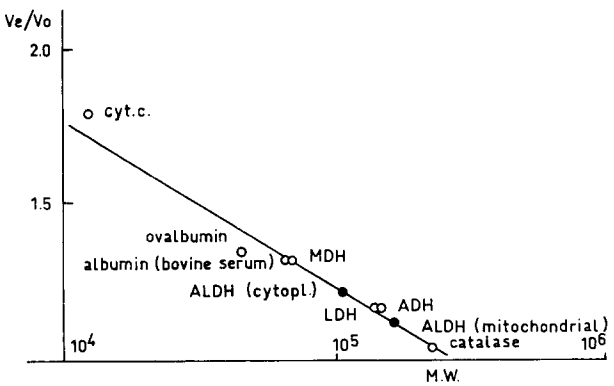

Fig. 3. Molecular weight determination of cytosolic and mitochondrial aldehyde dehydrogenase on Sephadex G-150. Preparations from Sephadex G-25 after $(NH_4)_2SO_4$ precipitation. The column was calibrated as described under Materials and Methods. MDH, malate dehydrogenase; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase.

Gel electrophoresis

Only one active band was developed in starch gel electrophoresis for either the mitochondrial or cytosolic preparation. The pattern was run for every intermediate purification step and the two bands always ran closely parallel. Some patterns showed two minor bands in the cytosolic preparation, as described earlier by Pikkarainen[6]. A cathodically moving diffuse band found in crude cytosolic preparations belongs to alcohol dehydrogenase[7]. A schematical drawing of the pattern is shown in Fig. 4.

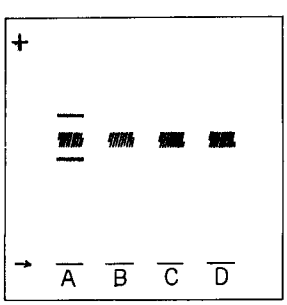

Fig. 4. Starch gel electrophoresis of crude and purified preparations from the cytosolic and mitochondrial compartments. Run at pH 7.8 as described[6]. Staining for aldehyde dehydrogenase activity in the presence of 5 mM acetaldehyde for 2 h (ref. 6). The arrow indicates the origin. A and B, crude cytosolic and mitochondrial preparations, respectively. C and D, final preparations.

DISCUSSION

Purification

Since the termination of the work towards purification, as reported here, two aldehyde dehydrogenase enzymes were resolved from the supernatant portion of the rat liver by DEAE-cellulose column chromatography[8]. Similar findings were reported from another laboratory[9]. In the present work preliminary binding experiments with DEAE-cellulose for the cytosolic preparation from pH 6.5 to pH 8.0 produced only one maximum. This activity did not bind to the column. No other peaks appeared when P_i gradient was established and extended to 0.5 M P_i concentration. The mitochondrial preparation bound loosely to the column and was eluted at a low P_i concentration. A shallow gradient resulted in a useful purification since some other proteins eluted at the same range but the activity was confined to a small number of fractions. The elution profiles were normal for both the cytosolic and the mitochondrial enzyme.

The fact that the resolution between the two reported cytosolic enzymes was not achieved may indicate that they emerged as a single peak. About a third of the activity was not recovered at DEAE-cellulose step of the cytosolic preparation. This could mean a specific inactivation of the other enzyme species. However, considering the differences between the cytosolic enzyme reported here and those referred to earlier[8,9], notably the higher K_m value, another possibility is suggested, namely the existence of a high-K_m and a low-K_m enzyme(s) in the rat liver. In the conditions employed in the present work (acetaldehyde 0.1–0.5 mM; 0.1 mM in the assay of ion-exchange cellulose column fractions) a high-K_m activity could go undetected.

Properties

The sensitivity of the methods used to measure K_m values did not allow reliable data below 10^{-5} M acetaldehyde concentration. At that concentration the mitochondrial enzyme catalyzed the reaction at a maximal rate. Therefore it was not possible to estimate the value for K_m in this preparation. On the other hand, lower than maximal rates were recorded in all cases for the cytosolic preparations at 10^{-5} M substrate concentration. The K_m value ranged from $1 \cdot 10^{-5}$ to $8 \cdot 10^{-5}$ M. The preparation in Fig. 2 ($3.1 \cdot 10^{-5}$ M) represents a typical figure. The measurement of the peak height differences representing the disappearance of acetaldehyde from the reaction mixture was less accurate in the case of the cytosolic enzyme at least partly because of a lower specific activity compared to the mitochondrial enzyme.

The K_m value for the cytosolic enzyme differs from those reported for similar enzymes from various sources. It is lower than the values assigned to the cytosolic enzymes from the rat liver by Shum and Blair[8] (1 and 4 mM) and by Deitrich[9] (0.4 mM with propionaldehyde). However, it is higher than the values reported for apparently cytosolic enzymes from several other liver tissues including human liver (0.75 μM, ref. 10; around 1 μM, ref. 11).

Unexpectedly, the pH optimum for both the cytosolic and the mitochondrial enzyme after the final purification step was above pH 8.9. This differs from the optimum around pH 7.6 for the crude homogenate[1]. The latter was assayed in the presence of 0.154 M KH_2PO_4–0.25 M sucrose–3 mM $MgCl_2$ instead of 0.1 M KH_2PO_4

buffer. The effect of this change is not known. Proteolytic digestion is a possibility that cannot be completely ignored due to a real time factor involved. However, it is known that non-enzymatic production of acetaldehyde takes place in certain conditions and seems to require a small molecular substance in the reaction mixture*. An enzymatic process activated at higher pH values is also a possibility. Such artifacts were ruled out during this work at pH 7.4 but the phenomena could be pH-dependent processes in which case the pH optimum obtained for the crude homogenate (pH 7.6) may be unreliable. However, the data furnished by Sippel[12] seems to rule out a non-enzymatic process, at least one that follows the mechanism outlined in his paper.

The presence of 5 mM acetaldehyde in the medium during the staining of the active bands on starch gels raises a possibility that the high-K_m enzyme(s) will also react. Although acetaldehyde is highly volatile at room temperature, it was found to be lost only slowly when as dilute solutions. So the effective concentration of acetaldehyde remains high for much of the 2-h staining period. The pattern for the cytosolic preparations (Fig. 4) suggests the presence of two other aldehyde oxidizing enzymes which may or may not be related to the two cytosolic enzymes reported earlier[8,9]. The minor bands together represented at most about 10% of the total intensity of the bands. They seemed to exist only in fresh preparations but were never seen beyond DEAE-cellulose stage. This suggests that the activities represented by the minor bands were labile and that the enzymes were lost during DEAE-cellulose fractionation because of their instability or due to their separation from the main enzyme fraction.

The experiments described here show a clear-cut difference between the aldehyde dehydrogenase preparations from the cytosol and the mitochondria of the rat liver. The properties of the purified preparations did not appreciably differ from those of corresponding crude preparations when the same property was studied in both cases. The suggestion that the activity in each compartment is due to one but not the same enzyme species is born out from the fact that the peaks from ion-exchange chromatography and gel filtration experiments were reasonably symmetrical. The main band in the electrophoresis, although wide, was sharp and never showed any sign of more than one active component. If more than one enzyme were present some part of the comparison is less meaningful, *e.g.* the specificity patterns. However, it seems to be reasonable to conclude that the main enzyme species in the cytosolic and the mitochondrial compartments are genuinely different entities.

A very recent report by Grunnet[13] describes a mitochondrial aldehyde dehydrogenase which in many respects resembles the one discussed above. It is NAD^+ dependent, has a similar pH optimum curve (only followed up to pH 8.9 in this study), has a broad specificity and shows a very low K_m value (below 0.1 μM). However, another K_m value at 1 mM was also reported thus suggesting that the high-K_m and low-K_m enzymes discussed above in connection with the cytosolic enzymes may also exist in the mitochondrial compartment. The instability during the purification steps was also similar and apparently even more pronounced as experienced during the present study.

* Personal communication from H. Sippel of this laboratory.

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