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PURIFICATION AND CHARACTERIZATION OF 2-OXOALDEHYDE DEHYDROGENASE FROM RAT LIVER

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Summary

The partial purification (123-fold) of 2-oxoaldehyde dehydrogenase (2-oxoaldehyde : NAD(P)$^+$ oxidoreductase, 1.2.1.23) from rat liver was carried out using a purification procedure which involved $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex chromatography, Blue-Dextran affinity chromatography and CM-Sephadex chromatography. A single form of the enzyme was observed, mol. wt. approx. 50000 by gel chromatography. 2-Oxoaldehyde dehydrogenase appears to be highly specific for NADP$^+$ and methylglyoxal. No activity is observed in the absence of certain amines which have vicinal amino and hydroxyl groups. The only known amine which activates the enzyme at physiological pH is L-serine methyl ester, suggesting that the regulation of this enzyme in vivo may require a derivative of serine.

Introduction

The physiological functions of 2-oxoaldehydes, especially methylglyoxal, have been a source of speculation for many years, Early efforts to find a metabolic role for the glyoxalase system, which catalyzes the conversion of methylglyoxal to lactic acid [1,2], resulted in the suggestion that methylglyoxal was an intermediate in glycolysis [3]. When this was shown not to be the case [4,5], interest in 2-oxoaldehydes diminished until the 1960s when Szent-Gyorgyi suggested that 2-oxoaldehydes might regulate cell division [6—8]. In spite of this increased interest, the role of 2-oxoaldehydes is still unclear. Although the biosynthesis of methylglyoxal has been observed in certain microorganisms [9—11], no biosynthetic pathway for methylglyoxal has been found in mammalian systems.

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Considerable work has been reported on the oxidative metabolism of methylglyoxal which is converted by the glyoxalase system to lactic acid in an intramolecular redox reaction or is oxidized by 2-oxoaldehyde dehydrogenase (2-oxoaldehyde : $NAD(P)^+$ oxidoreductase, EC 1.2.1.23) to pyruvic acid. This dehydrogenase from sheep liver has been studied indetail [12,13]. The enzyme is specific for 2-oxoaldehydes, although not specific for methylglyoxal. In addition, an amine with a structure similar to Tris is required. The reaction with Tris is observed only at high pH values; no amine has been found which activates the enzyme at physiological pH. Sheep liver 2-oxoaldehyde dehydrogenase utilizes either NAD^+ or $NADP^+$ [12,13].

In an attempt to understand better the role that 2-oxoaldehyde dehydrogenase may have in the metabolism of methylglyoxal, we have studied the enzyme from rat liver in order to test whether this source of the enzyme might provide additional information about the nature of the physiologically important amine.

Materials and Methods

Chemicals

Commerical methylglyoxal, 40% aqueous solution (Aldrich) was purified by distillation. Kethoxal (β-ethoxy-α-ketobutyraldehyde) was a gift from the Upjohn Co., Kalamazoo, Michigan, U.S.A. Phenylglyoxal was prepared as described previously [14]. NAD^+, $NADP^+$, NADH, NADPH, lactate dehydrogenase, D,L-2-amino-1-propanol, L-serine methyl ester, D,L-serine methyl ester, D-serine, L-threonine, D,L-threonine methyl ester, galactosamine, glucosamine (Sigma), L-2-amino-1-propanol (Aldrich), Tris (Fisher), L-serine (Nutritional Biochemicals), 3-amino-1-propanol and ethanolamine (Eastman) were used as purchased.

Column materials

CM-Sephadex C50, DEAE-Sephadex A50, and Sephadex G150 (Pharmacia) were prepared according to the manufacturer's instructions. Blue Dextran affinity material was prepared according to the procedure of Ryan and Vestling [15] except that commercial cyanogen bromide-activated Sepharose 4B (Pharmacia) was used.

Enzyme assays

Routine measurements of 2-oxoaldehyde dehydrogenase activity in the various column chromatography procedures used in the purification of the enzyme were carried out in 0.1 M sodium pyrophosphate buffer, pH 9.3, containing 0.5 mM $NADP^+$, 4 mM methylglyoxal, and 16 mM D,L-2-amino-1-propanol in a 3-ml reaction volume. Background was monitored at 340 nm for 1 min prior to addition of enzyme sample. All reactions, at 25°C, were followed using a Gilford-modified Beckman DU with a circulating water bath for temperature control. Initial rates of conversion of $NADP^+$ to NADPH, corrected for the background reaction, were recorded. 1 unit of activity corresponds to the production of 1 μmol NADPH/min.

Protein determinations

Proteins were determined using the convenient Coomassie Blue dye-binding method of Bradford [16] which allows one to determine concentrations of protein in the presence of sulfhydryl reagents. The procedure was standardized with bovine serum albumin.

Results

Purification of 2-oxoaldehyde dehydrogenase from rat liver

Step 1. Livers from 24 male Sprague-Dawley rats, 150—175 g, which had been killed by decapitation, were cut into small pieces and were homogenized in a glass tissue homogenizer with a close-fitting Teflon pestle, in 0.04 M phosphate buffer, pH 7.2, containing 0.1 M sucrose, 0.05 M KCl, 0.03 M EDTA, and 0.01 M dithioerythritol in the ratio 3 ml buffer per g liver. The homogenate was centrifuged at 10000 × *g* for 30 min, and the supernatant was then centrifuged at 330000 × *g* for 15 min giving 270 ml of supernatant, total activity 393 units.

Step 2. Solid $(NH_4)_2SO_4$, 66 g, was added slowly at 0°C to the 270 ml of supernatant. The resulting 40% saturated solution was centrifuged at 10000 × *g* for 15 min. The supernatant was treated with 83 g of $(NH_4)_2SO_4$ to give an 80% saturated solution, and the solution was centrifuged at 10000 × *g* for 15 min. Most of the 2-oxoaldehyde dehydrogenase activity was in the 40—80% pellet which was dissolved in pH 7 phosphate buffer, $I = 0.02$, giving 52 ml of solution, total activity 332 units.

Step 3. After concentration of the sample by ultrafiltration, a portion of the 40—80% cut (110 units) was placed on a DEAE-Sephadex A50 column (2.6 × 28 cm) equilibrated at 4°C with 0.05 M pH 8 Tris. Protein was eluted with this buffer using a linear salt gradient from 0 to 0.15 M KCl over an elution volume of 300 ml. A single peak of activity was collected in a volume of 66 ml which had 45 units (44%) of activity. The sample was concentrated to 7.8 ml, 41 units of activity remaining.

Step 4. A sample of concentrate from step 3 (31 units) was placed on a Blue Dextran affinity column (1.5 × 26 cm) equilibrated with pH 7 phosphate buffer ($I = 0.02$). Proteins were eluted with this buffer until the effluent appeared free of protein. The column then was developed with a linear KCl gradient from 0.02 to 1.5 M over an 80-ml volume. 2-Oxoaldehyde dehydrogenase activity appeared as a single peak which was collected in a volume of 17.5 ml and concentrated to 7.2 ml, leaving 31 units (100%) of activity.

Step 5. A sample of concentrate from step 4 (8.7 units) was placed on a CM-Sephadex C50 column (0.9 × 25 cm) equilibrated with pH 6 phosphate buffer ($I = 0.1$). Protein was eluted using a linear KCl gradient from 0.1 to 0.4 M over a 30-ml volume. Activity appeared as a single peak which was collected in a volume of 18 ml containing 7.9 units (89%) of activity. Concentration of the sample to 3 ml left 7.4 units of activity. This sample was stored in 50% glycerol at −20°C. Activity slowly decreased, but the rate of decrease was slowed by addition of $NADP^+$. Conditions which would give long-term stability have not yet been found. The purification scheme is summarized in Table I.

TABLE I

PURIFICATION SCHEME FOR 2-OXOALDEHYDE DEHYDROGENASE FROM RAT LIVER

For each step, only a fraction of the material from the previous purification step was used; See Results section for details.

Step	Vol. (ml)	Units	Protein (mg/ml)	Sp. act. (IU)	% yield (Step)	Overall purification
1. 330 000 × *g* supernatant fraction	270	393	24.6	0.059	100	1
2. $(NH_4)_2SO_4$ fractionation 40–80%	52	332	75.1	0.085	84	1.4
3. DEAE-Sephadex A50 pH 8	7.8	41	5.0	1.05	44	18
4. Blue Dextran Affinity column	7.2	31	0.81	5.39	100	91
5. CM-Sephadex C50 pH 6	3.0	7.4	0.34	7.25	89	123

Gel chromatography of 2-oxoaldehyde dehydrogenase

A sample of activity from the Blue Dextran affinity column, 25 units, was placed on a Sephadex G150 column (2.6 × 100 cm). The sample was eluted with pH 7 phosphate buffer, $I = 0.02$, giving 19 units (75%) of activity (Fig. 1). The Sephadex G150 column was calibrated with bovine serum albumin, glyoxalase-I from rat erythrocytes, yeast glyoxalase-I and chymotrypsin. The elution

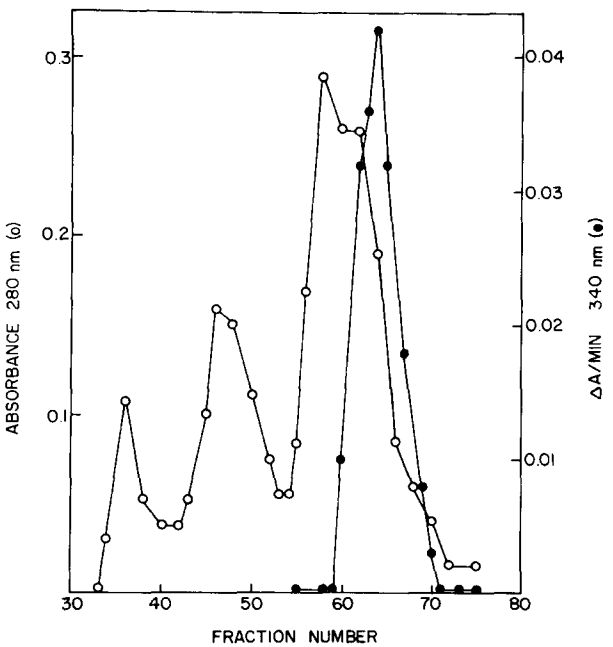

Fig. 1. Purification of 2-oxoaldehyde dehydrogenase on Sephadex G150, pH 7. Protein profile, 280 nm, (o); activity profile (●); 5 ml per fraction. Activity was measured using 100-μl aliquots.

volume of 2-oxoaldehyde dehydrogenase is identical to that of glyoxalase-I from rat erythrocytes indicating that the approximate molecular weight is 50000 [17].

Electrophoresis of 2-oxoaldehyde dehydrogenase

Electrophoresis of the enzyme sample, specific activity 7.25 (123-fold purification), on 7% polyacryamide, pH 8.9, followed by staining with Coomassie Blue revealed 3 main protein bands. Attempts to purify the enzyme further by chromatography on hydroxyapatite using a sodium phosphate gradient to elute the enzyme did not change the specific activity. However, the material eluted from the hydroxyapatite column showed several more bands on electrophoresis than the starting material. The presence of any sodium phosphate in the sample appears to cause the formation of multiple forms on electrophoresis. The sheep liver enzyme reportedly was homogeneous after a 90-fold purification. Further studies must be carried out to determine the extent of purity of the 123-fold purified enzyme.

Amine requirements of 2-oxoaldehyde dehydrogenase

2-Oxoaldehyde dehydrogenase from rat liver shows no activity in the absence of certain amines, similar to the amine requirements reported for the enzyme from sheep liver [13]. However, the specificity for the amines appears to be quite different for the two enzymes. In the case of the sheep live enzyme, amines with a structural resemblance to Tris generally were active, with the most effective amine being L-2-amino-1-propanol. All of the active amines had vicinal hydroxyl and amino groups. No amine was found which gave activity at physiological pH.

Table II shows the effects of a series of amines on the activity of 2-oxoaldehyde dehydrogenase from rat liver. This enzyme has higher specificity for amines than does the sheep liver enzyme. L-2-amino-1-propanol gives very little activity whereas the a D,L mixture of this amine gives the highest activity observed, suggesting that only the D-isomer is active. Tris and related amines also give very little activity. A number of biological compounds with vicinal hydroxyl and amino groups were tested. Only serine methyl ester was observed to give a high degree of activation, and, interestingly, both L-serine methyl ester and D,L-serine methyl ester were effective under the experimental conditions used.

Effects of pH on the 2-oxoaldehyde dehydrogenase reaction

D,L-2-amino-1-propanol and L-serine methyl ester were tested as activators of the enzyme reaction as a function of pH. The results are shown in Fig. 2. In both cases, activity falls abruptly above pH 9.3 suggesting that the enzyme may be denatured at these pH values. The pH vs. rate profiles below pH 9.3 suggest the involvement of groups with apparent pK values of 8.6 and 7.7 for the D,L-2-amino-1-propanol- and L-serine methyl ester activated reactions, respectively. The solid curves in Fig. 2 were calculated using these pK values. In the pH range examined, $NADP^+$ and methylglyoxal were saturating. The only difference between the two pH profiles (Fig. 2) is the nature of the activating amine. The apparent inflection points agree quite well with the reported pK values for

TABLE II

ACTIVATION OF 2-OXOALDEHYDE DEHYDROGENASE BY AMINES

All initial rates were recorded at 340 nm, pH 9.3, pyrophosphate buffer (0.1 M), 25°C, with 5 mM methylglyoxal, 0.5 mM $NADP^+$, 16 mM amine and a constant amount of 2-oxoaldehyde dehydrogenase.

Amine	ΔA/min
D,L-2-amino-1-propanol	0.032
L-2-amino-1-propanol	0.006
L-serine methyl ester	0.031
D,L-serine methyl ester	0.028
L-serine	0.005
D-serine	0.005
Ethanolamine	0.0
Tris	0.003
2-Amino-2-methyl-1,3-propanediol	0.005
3-Amino-1-propanol	0.0
Galactosamine	0.0
Glucosamine	0.0
Pyridoxamine	0.0
L-threonine	0.006
D,L-threonine methyl ester	0.006

2-amino-1-propanol and serine methyl ester [19]. This suggests that the neutral form of the amine may be the active species. However, a more detailed analysis of the pH dependence of both K_m and V will be required in order to evaluate the role of the amine in this reaction. Although the enzyme reaction with D,L-2-amino-1-propanol shows very little activity at physiological pH, the reaction with L-serine methyl ester, which is optimal at pH 8.5, shows considerable activity at pH 7.4. This is the only known amine which gives any reasonable activation of the enzyme at physiological pH. This suggests that some serine

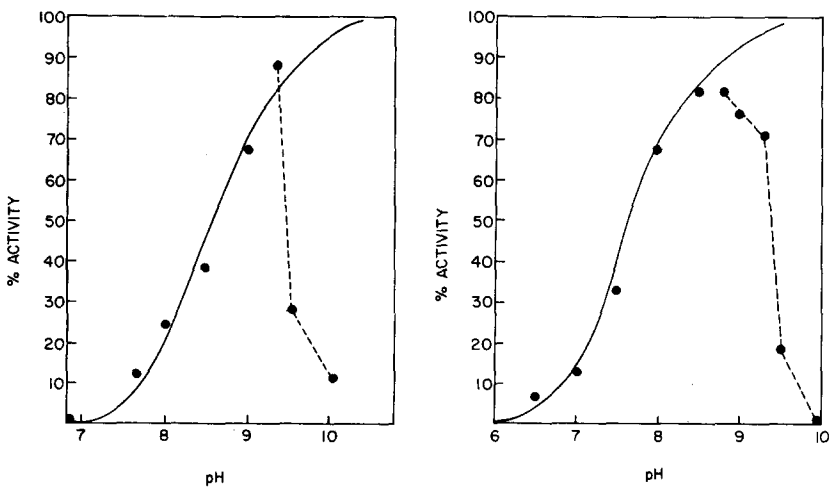

Fig. 2. pH vs. rate profile for 2-oxoaldehyde dehydrogenase using D,L-2-amino-1-propanol (left) and L-serine methyl ester (right). [$NADP^+$] = 0.5 mM, [methylglyoxal] = 5 mM, and [amine] = 16 mM. Activity is lost above pH 9.3 (dotted lines). Assay conditions were 25°C using sodium phosphate or sodium pyrophosphate buffers to adjust the pH.

derivative or similar compound may regulate the enzyme in vivo. The requirement for vicinal hydroxyl and amino groups on the activating amine appears to be an absolute requirement, both for the sheep liver enzyme and for the rat liver enzyme.

Nucleotide requirements for 2-oxoaldehyde dehydrogenase

2-Oxoaldehyde dehydrogenase from rat liver is active only with $NADP^+$; no activity was observed with NAD^+ up to 1 mM concentration, regardless of which amine was present. Addition of phosphate does not alter this behavior. This is in marked contrast to the enzyme from sheep liver which uses either NAD^+ or $NADP^+$ [12,13]. In addition, the enzyme from sheep liver is activated by phosphate if NAD^+ is the cofactor [18].

Substrate specificity of 2-oxoaldehyde dehydrogenase

The enzyme from sheep liver is specific for 2-oxoaldehydes, but not specific for methylglyoxal. Phenylglyoxal and hydroxypyruvaldehyde are also good substrates, showing similar kinetic parameters to methylglyoxal [12]. 2-oxoaldehyde dehydrogenase from rat liver has a greater specificity than the sheep liver enzyme. Neither phenylglyoxal nor kethoxal at 1 mM concentrations give any activity. The enzyme appears to be highly specific for methylglyoxal. The apparent Michaelis constants for methylglyoxal, $NADP^+$ and L-serine methyl ester are given in Table III.

Determination of the product of the 2-oxoaldehyde dehydrogenase reaction

The enzyme from sheep liver was shown to convert methylglyoxal to pyruvic acid [12]. Pyruvate also was measured using lactate dehydrogenase, but the amount of pyruvate observed never exceeded 65% of the theoretical amount. Nevertheless, the reaction could not be reversed [12]. To test 2-oxoaldehyde dehydrogenase from rat liver for reaction product and reversibility, the reaction was run at pH 8.5 with 0.17 mM $NADP^+$, 16 mM L-serine methyl ester and 2.6 mM methylglyoxal. After completion of the reaction, the pH was adjusted to 6.3, and lactate dehydrogenase was added. The change in absorbance at 340 nm indicated 100% of the calculated amount of pyruvic acid was present. The enzyme reaction at pH 8.5 was repeated with 0.32 mM NADPH, 16 mM L-serine methyl ester and 20 mM pyruvate. No evidence for any reverse reaction was observed. Thus, the $NADP^+$-dependent oxidation of methylglyoxal to pyruvic acid, catalyzed by rat liver 2-oxoaldehyde dehydrogenase, at pH 8.5 appears to proceed with a large negative free energy change.

TABLE III

APPARENT MICHAELIS CONSTANT FOR METHYLGLYOXAL, $NADP^+$, AND L-SERINE METHYL ESTER IN THE 2-OXOALDEHYDE DEHYDROGENASE REACTION AT pH 8.5

Reaction was in pyrophosphate buffer (0.1 M), 25°C.

[Methylglyoxal]	[$NADP^+$]	[Amine]	K_m (M)
variable	0.5 mM	16 mM	$8.0 \cdot 10^{-4}$
5 mM	variable	16 mM	$1.4 \cdot 10^{-4}$
5 mM	0.5 mM	variable	$8.0 \cdot 10^{-3}$

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