L-Threonine aldolase is not a genuine enzyme in rat liver

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Activity of L-threonine aldolase in rat liver cytosolic extract was not affected by the omission of alcohol dehydrogenase in a previously established NADPH-linked alcohol dehydrogenase-coupled assay. The liver extract was able to catalyse the dehydrogenation of NADPH with either acetaldehyde (a product of L-threonine aldolase action) or 2-oxobutyrate (a product of L-threonine dehydratase action). When the liver extract was chromatographed on a Sephacryl S-200 column, no threonine aldolase activity was detected in the eluate. However, activity of threonine aldolase re-appeared when the fractions with highest activity of lactate dehydrogenase and threonine dehydratase were mixed. Activity of threonine aldolase could also be abolished by removing threonine dehydratase from the liver extract with a specific antibody. Hence L-threonine aldolase should not be a genuine enzyme in the rat liver, and the apparent enzyme activity may result from a combined effect of threonine dehydratase and lactate dehydrogenase (or an oxo acid-linked NADPH dehydrogenase) in the liver cytosolic extract.

INTRODUCTION

L-Threonine dehydratase (EC 4.2.1.16) and L-threonine aldolase (EC 4.1.2.5) in cytosol, and L-threonine dehydrogenase (EC 1.1.1.103) in mitochondria, have been recognized as the major enzymes in mammalian liver for the degradation of L-threonine (Hetenyi *et al.*, 1984). The highly inducible nature of the dehydratase and its dramatic changes in enzyme activity during development in rats (Cihak *et al.*, 1975; Yeung & Yeung, 1980a) strongly suggest it is an important, if not the sole, enzyme for the degradation of this hydroxyamino acid.

Meltzer & Sprinson (1952) had investigated the metabolism of threonine in [4-¹⁴C]- and [¹⁵N]-DL-threonine-fed rats, and concluded that the major pathway for the degradation of threonine should be the aldol cleavage of the molecule to glycine and a C₂ fragment, presumably acetaldehyde. Attention has then been focused on the enzyme responsible for the aldol cleavage of L-threonine and its significance in the degradation of this amino acid in mammalian liver. Karasek & Greenberg (1957), with sheep liver, and Lin & Greenberg (1954), Malkin & Greenberg (1964) and Riario-Sforza *et al.* (1969) with rat liver, substantiated the deduction by Meltzer & Sprinson (1952) by the identification and characterization of an aldolase which acted on L-threonine. However, more recently Bird & Nunn (1979, 1983) showed that the commonly used NADH-linked alcohol dehydrogenase (ADH)-coupled assay for threonine aldolase over-estimated the enzyme activity, owing to interference by the co-existing threonine dehydratase and lactate dehydrogenase (LDH) in the liver extract. As an alternative, these authors introduced an NADPH-linked ADH-coupled assay which would eliminate the contaminating effect of LDH and threonine dehydratase in the estimation of the aldolase activity, because activity of threonine aldolase in the new assay was not inhibited by the LDH inhibitor, oxamic acid. The present study provides evidence to show that threonine dehydratase and LDH (or an oxo

acid-linked NADPH dehydrogenase) were responsible for activity of 'threonine aldolase' in the NADPH-linked assay of Bird & Nunn (1979), and there is no threonine aldolase in the rat liver.

EXPERIMENTAL

Materials

L-Threonine (allo-free) was obtained from Fluka AG, Buchs, Switzerland. DL-Allothreonine was the product of Koch-Light Laboratories, Colnbrook, Bucks., U.K. Sodium 2-oxobutyrate, sodium pyruvate, glyoxylic acid, oxamic acid, DL-dithiothreitol, 2-mercaptoethanol, pyridoxal 5-phosphate, NADPH (tetrasodium salt, type I), NADH (disodium salt, grade III), ADH (from bakers' yeast, crystallized and freeze-dried), LDH [rabbit muscle, type II; crystalline suspension in 2.1 M-(NH₄)₂SO₄], and Protein A-agarose were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acetaldehyde was obtained from Merck, Darmstadt, West Germany. Other reagents used were of analytical grade, obtained from either Merck or BDH Chemicals, Poole, Dorset, U.K.

Animals

Male Sprague-Dawley rats weighing 100–120 g, supplied by the Laboratory Animal Unit, University of Hong Kong, and housed at 24–28 °C with a 12 h-light/12 h-dark cycle were used in all experiments. Tap water and laboratory chow (Autoclavable rodent laboratory chow 5010; Ralston Purina Co., St. Louis, MO, U.S.A.) were given *ad lib.*, unless stated otherwise. Starved rats were given water only.

Preparation of rat liver extracts

Liver was homogenized in 3 vol. of a 0.1 M-potassium phosphate buffer, pH 7.3, containing 0.25 M-sucrose at 4 °C. The homogenate was centrifuged at 100 000 g (*r*_{av}, 5.85 cm) for 1 h at 4 °C. The lipid-free clear supernatant was used in the assays described below.

Abbreviations used: ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase.

Enzyme assays

L-Threonine aldolase and DL-allothreonine aldolase. The assay system was essentially that reported by Bird & Nunn (1979). The reaction mixture contained, in a final volume of 1 ml, 0.1 mmol of KH_2PO_4 (adjusted to pH 7.3 with KOH), 1 μmol of dithiothreitol, 10 nmol of pyridoxal phosphate, 0.15 μl of NADPH, 45 units of ADH, 50 μmol of substrate and 100 μl of rat liver supernatant. The above assay mixture except the substrate (in a volume of 100 μl) was equilibrated at 37 °C for 2 min in a black-sided semi-micro quartz cuvette, and the decrease in A_{340} was recorded with a Cary 17D spectrophotometer (0.1 mm slit width, 0.2 nm spectral band width) at 37 °C for 15 min. Reaction was started by the addition of substrate, and the decrease in A_{340} was measured for at least a further 20 min. The reference cuvette contained the same concentration of ingredients except NADPH and the substrate.

L-Threonine dehydratase. The assay was identical with that reported previously (Yeung & Yeung, 1980b).

Lactate dehydrogenase. The enzyme was assayed by the method of Meister (1950), except that the buffer used was 0.1 M-potassium phosphate, pH 7.3, and the final volume of the assay mixture was 1 ml.

2-Oxobutyrates-, pyruvate-, acetaldehyde- and glyoxylate-dependent NADPH dehydrogenases. The assay was identical with that for threonine aldolase, except that ADH, dithiothreitol and pyridoxal phosphate were omitted.

Gel-filtration chromatography of liver cytosol extract

A column (138 cm $\times$ 2.6 cm diam.) of Sephacryl S-200 (superfine grade; Pharmacia, Uppsala, Sweden) was packed by the standard procedure at 4 °C in 0.1 M-potassium phosphate buffer, pH 7.3, containing 0.14 M-NaCl, 1 mM-2-mercaptoethanol and 10 μM -pyridoxal phosphate. At the same temperature, 8 ml of liver extract was applied and eluted at a flow rate of 30 ml/min; 5 ml fractions were collected, and enzyme activities of LDH, threonine dehydratase, threonine aldolase, allothreonine aldolase and 2-oxobutyrates-dependent NADPH dehydrogenase in each fraction were determined. Protein concentration was monitored at 280 nm.

Purification of L-threonine dehydratase and preparation of anti-(L-threonine dehydratase) IgG

Purification of threonine dehydratase and the production of rabbit antiserum were carried out as described previously (Yeung & Yeung, 1980a). IgG from the antiserum was purified by Protein A-agarose affinity chromatography by the method of Goding (1976).

Immunoprecipitation of threonine dehydratase

This was achieved by incubating 100 μl (0.5 mg in 0.1 M-potassium phosphate buffer, pH 7.3) of IgG with 500 μl of liver supernatant at 37 °C for 5 min, and then for 2 h at 0 °C. The resulting mixture was centrifuged in a Microfuge for 10 min at 12000 g, at 4 °C, and the clear supernatant was kept for assay of enzyme activity.

RESULTS AND DISCUSSION

The aldol cleavage of threonine by the action of a specific aldolase was recognized to be important in the degradation of L-threonine in mammalian liver since the

Table 1. Apparent K_m and V_{max} values for rat liver cytosol on the dehydrogenation of NADPH with acetaldehyde, pyruvate, 2-oxobutyrates and glyoxylate

Enzyme activities at different substrate concentrations were assayed as described in the text. K_m and V_{max} were determined by the Lineweaver-Burk plot. Each value is the mean $\pm$ S.E.M. for three different determinations.

	K_m (mM)	V_{max} (μmol of NADPH transformed/min per g of liver)
Acetaldehyde	0.56 ± 0.03	1.28 ± 0.05
Pyruvate	1.66 ± 0.08	1.67 ± 0.10
2-Oxobutyrates	28.57 ± 0.62	0.67 ± 0.03
Glyoxylate	0.12 ± 0.02	19.29 ± 0.93

work of Meltzer & Sprinson (1952), Lin & Greenberg (1954), Karasek & Greenberg (1957), Malkin & Greenberg (1964) and Riario-Sforza *et al.* (1969). Many reference and text books in biochemistry have then emphasized the role of either the enzyme threonine aldolase (Neuberger, 1981) or the aldol cleavage (Lehninger, 1975; Devlin, 1982) in the catabolism of this amino acid in mammals. Bird & Nunn (1979) were the first to cast doubt on the NADH-linked ADH-coupled threonine aldolase assay of Malkin & Greenberg (1964). Using the NADPH-linked assay that they developed, Bird & Nunn (1979, 1983) showed that the activity of L-threonine aldolase was uniformly low in liver of rats subjected to various gluconeogenic states, and concluded that the aldolase, though present in liver, could not be an important enzyme for L-threonine degradation.

In the present study, it was found that enzyme(s) in the liver cytosolic extract was able to catalyse the dehydrogenation of NADPH with acetaldehyde, pyruvate, 2-oxobutyrates and glyoxylate efficiently. The apparent K_m and V_{max} (maximum velocity at infinite substrate concentration) values determined by the Lineweaver-Burk plot for the rat liver cytosol on these substrates are shown in Table 1. The liver extract had a relatively high K_m for 2-oxobutyrates (about 50 times that for acetaldehyde), but the V_{max} was only half of that for acetaldehyde. Hence dehydrogenation of NADPH with 2-oxobutyrates would be as efficient as with acetaldehyde and pyruvate when the concentration of the oxobutyrates is high and the dehydrogenase was present in large quantity. Although oxamate strongly inhibited (by about 70–80%) the 2-oxobutyrates-linked NADH dehydrogenase, it did not inhibit the 2-oxobutyrates-linked NADPH dehydrogenase (Table 2). Data in Table 2 also showed that 'threonine aldolase' activity was not affected to any significant extent by the inclusion of either oxamate or ADH in the assay mixture. Results thus suggested that Bird & Nunn (1979, 1983) had not eliminated the interference by the endogenous dehydrogenase and threonine dehydratase in their assay.

When the liver cytosolic extract was chromatographed on a column of Sephacryl S-200, activities of allothreonine aldolase, LDH and threonine dehydratase were resolved in different portions of the eluate (Fig. 1). Most of the activity of 2-oxobutyrates-dependent NADPH dehydrog-

Table 2. Effects of oxamate and ADH on the enzyme activities of 2-oxobutyrate-dependent NADH and NADPH dehydrogenases and 'threonine aldolase' in liver extracts of 48 h-starved rats

Enzyme activities were assayed as described in the text (except that ADH was omitted in the assay for threonine aldolase unless specified) and expressed in μmol of NADH or NADPH transformed/h per g of liver. The concentrations of oxamate and ADH in the assays were $3 \mu\text{mol/ml}$ and 45 units/ml respectively.

Rat	Addition...	Enzyme activity						
		Oxobutyrate-dependent NADH dehydrogenase ($\times 10^{-4}$)		Oxobutyrate-dependent NADPH dehydrogenase		Threonine aldolase		
		None	Oxamate	None	Oxamate	None	Oxamate	ADH
1		4.05	0.87	42.13	47.84	2.00	1.96	2.01
2		3.86	1.16	32.84	34.81	1.55	1.58	1.69

Fig. 1. Enzyme profiles of Sephacryl S-200 molecular-sieving chromatography of rat liver cytosolic extract

Liver cytosolic extract was prepared from rats which had been starved for 48 h as described in the text. Protein concentration (·····) was expressed as percentage transmittance at 280 nm. LDH (—) and threonine dehydratase (----) were assayed as described in the text and are expressed as μmol of NAD^+ reduced/min per fraction ($\times 10^{-1}$) and μmol of 2-oxobutyrate formed/min per fraction respectively. Allothreonine aldolase (-·-·-·-), threonine aldolase and 2-oxobutyrate-dependent NADPH dehydrogenase (- - - - -) were assayed as described in the text, except that a 0.8 ml portion of each fraction was used, instead of buffer, in a final assay volume of 1 ml, and enzyme activities were expressed as μmol of NADPH transformed/h per fraction.

enase was superimposed on that of LDH. Recoveries of the activities of LDH, threonine dehydratase, allothreonine aldolase and 2-oxobutyrate-dependent NADPH dehydrogenase were 61, 65, 53 and 40% respectively. No activity of threonine aldolase was detected in fractions between the void volume and the total internal volume. Results thus suggested that activity of 'threonine aldolase' would not be caused by a single species of protein in the liver cytosol.

Table 3. Activities of 'L-threonine aldolase' in mixtures of LDH and threonine dehydratase

(A) LDH from fraction 70 and threonine dehydratase from fraction 81 of Fig. 1, and (B) purified rabbit muscle LDH and rat threonine dehydratase, were mixed and assayed as described for threonine aldolase activity, except that ADH was omitted. Enzyme activities were expressed as μmol of 2-oxobutyrate formed or NADH or NADPH transformed/h per ml of assay mixture.

Mixture	Enzyme activity			
	LDH	Threonine dehydratase (TDH)	Threonine aldolase	LDH/TDH
(A) 1	222.7	20.4	0.020	10.9
2	167.1	25.5	0.018	6.6
3	111.4	30.6	0.016	3.6
(B) 1	463.0	37.0	0.031	12.5
2	926.0	37.0	0.099	25.0
3	926.0	14.8	0.086	62.6
4	926.0	7.4	0.012	125.1
5	1924.0	14.8	0.127	130.0

'Threonine aldolase' activity re-appeared when the fractions containing the highest activities of LDH and threonine dehydratase were mixed and assayed in the absence of ADH (Table 3). There was a lag period of about 15 min before the reconstructed 'threonine aldolase' reaction rate increased beyond the control value and reached a constant value. Decreasing the amount of LDH in the mixture resulted in a slight decrease in the enzyme activity. Previously, Meister (1950) had reported that LDH was able to use NADPH in the reduction of pyruvate and other oxo acids at a very slow rate (100-fold less than with NADH). In order to investigate the possible involvement of LDH, 'threonine aldolase' activity was assayed in mixtures with different concentrations of purified rabbit LDH and rat threonine dehydratase. The mixtures of purified enzymes did possess activities of 'threonine aldolase' comparable with those from the Sephacryl S-200 chromatography (Table

Table 4. Effect of anti-(threonine dehydratase) IgG treatment on the enzyme activity of L-threonine dehydratase, L-threonine aldolase and DL-allothreonine aldolase in liver extracts of normal and 48 h-starved rats

Immunoprecipitation was carried out as described in the text. Controls were treated in the same way as their counterparts except that antibody was not added. The control IgG was prepared from rabbit immunized with an emulsion of equal volumes of Freund's adjuvant (Calbiochem) and 0.9% NaCl as described in the text. Enzyme activities are means $\pm$ S.E.M. for the numbers of rats in parentheses, and are expressed as μ mol of 2-oxobutyrate formed or NADPH transformed/h per g of liver.

	Enzyme activity		
	L-Threonine dehydratase	L-Threonine aldolase	DL-Allothreonine aldolase
Normal control (7)	13.29 $\pm$ 0.81	0.41 $\pm$ 0.11	6.71 $\pm$ 0.31
Normal + control IgG (3)	12.94 $\pm$ 0.78	0.43 $\pm$ 0.08	6.68 $\pm$ 0.25
Normal + anti-dehydratase IgG (7)	<0.001	<0.001	6.44 $\pm$ 0.39
Starved control (7)	51.32 $\pm$ 4.93	1.68 $\pm$ 0.23	6.08 $\pm$ 0.47
Starved + control IgG (3)	50.84 $\pm$ 3.66	1.63 $\pm$ 0.19	6.11 $\pm$ 0.41
Starved + anti-dehydratase IgG (7)	<0.001	<0.001	5.62 $\pm$ 0.31

3). A similar lag period before the detection of the oxidation of NADPH also occurred. At higher concentrations of LDH, for the same amount of threonine dehydratase, a shorter lag period and higher 'aldolase' activity were detected. The presence of a lag phase in the assay of the reconstructed threonine aldolase, which is absent from assays with the original liver extract, may then be accounted for by the effect of a decrease in the relevant dehydrogenase. Success in the reconstruction of the activity of 'threonine aldolase' by mixing LDH and threonine dehydratase provides strong evidence to argue against a true threonine aldolase, and suggests that the apparent activity of the 'aldolase' in the NADH- and NADPH-linked assays is likely to result from a combined action of LDH (and/or an oxo acid-dependent NADPH dehydrogenase co-migrating with LDH) and threonine dehydratase.

Bird & Nunn (1983) had stated that threonine aldolase activity varied independently of threonine dehydratase activity. On close examination of their data, it was found that the enzyme activities of threonine aldolase in the rat liver did increase like that of threonine dehydratase, but with a much smaller magnitude. In the present study, a similar relationship of the activities of these two enzymes in the normal and starved rat liver to those reported by Bird & Nunn (1983) was demonstrated (Table 4). Allothreonine aldolase in rat liver was not sensitive to starvation. It follows that the 'aldolase' activity will always fluctuate relative to the dehydratase, but with a much lower magnitude only if the activity of 'threonine aldolase' results from a coupled reaction of threonine dehydratase and LDH (or a 2-oxobutyrate-dependent NADPH dehydrogenase, which occurs abundantly in liver cytosol) in the assay of Bird & Nunn (1979).

The most convincing evidence against the presence of a true 'threonine aldolase' in rat liver comes from the finding that threonine aldolase activities in cytosolic extracts prepared from normal and starved rat livers were abolished when threonine dehydratase was removed by immunoprecipitation with specific antibody (Table 4). Removal of the dehydratase did not affect the enzyme activity of allothreonine aldolase.

Results from this investigation clearly show that endogenous threonine dehydratase and an oxo acid-dependent NADPH dehydrogenase (probably LDH) in the cytosol extract are responsible for the apparent enzyme activity of 'threonine aldolase'. Thus 'threonine aldolase' is not a genuine enzyme in rat liver. Furthermore, data of the present study (Table 4) also confirmed the existence of an allothreonine-metabolizing enzyme, possibly its aldolase or serine hydroxymethyl-transferase (Palekar *et al.*, 1973), which will not act on L-threonine.

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