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TRANSAMINASES OF BRANCHED CHAIN AMINO ACIDS

IV. PURIFICATION AND PROPERTIES OF TWO ENZYMES FROM RAT LIVER

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SUMMARY

Two transaminases for the branched chain amino acids (valine, leucine and isoleucine) with α-ketoglutarate were isolated from normal rat liver and purified by DEAE-cellulose, hydroxylapatite and Sephadex column chromatographies. One enzyme (enzyme I) was eluted by 0.02 M phosphate buffer from a DEAE-cellulose column and catalyzed the transamination of all three amino acids. The other enzyme (enzyme II) was eluted by 0.18 M buffer and was specific for leucine. Half the total activity of enzyme I was found in the supernatant, while only one quarter of the activity of enzyme II was localized in the supernatant. The rest of the total activity remained in the mitochondrial fraction. The optimal pH of enzyme I was 8.2 and that of enzyme II was 8.7. The K_m value for leucine of enzyme I was $7.5 \cdot 10^{-4}$ M and that for enzyme II was $2.5 \cdot 10^{-2}$ M. Enzyme I was activated by 2-mercaptoethanol but enzyme II was not. Anti-serum against hog heart transaminase, which has similar properties to rat liver enzyme I, inhibited the activity of enzyme I but not that of enzyme II. The two enzymes were compared and their physiological significance discussed.

INTRODUCTION

It is known that in higher animals branched chain amino acids (valine, leucine and isoleucine) are transaminated fairly rapidly in various tissues[1,2] and it has been suggested that transamination, rather than oxidative deamination, is the primary reaction in the metabolism of these amino acids[3,4]. However, no detailed characterization of this transamination was worked out until recently. In 1966, ICHIHARA AND KOYAMA[5] and TAYLOR AND JENKINS[6] independently reported that an enzyme (EC 2.6.1.6) from hog heart specifically catalyzes the transamination of these three amino acids. Subsequently, AKI *et al.*[7] reported that the enzyme is localized in both the

Abbreviation: PCMB, *p*-chloromercuribenzoate.

supernatant and the mitochondrial fractions and that the enzymes in the two fractions are isozymes.

In rat liver the activities for these amino acids are very low compared with those in other tissues[1-3,5], but the activity in the supernatant of the liver could be induced by various treatments, such as hydrocortisone injection or a high protein diet and the induced activity was specific for leucine[8]. These results suggested that in rat liver there may be two transaminases for these amino acids, one of which is active for all branched chain amino acids, as described before, while the other is specific for leucine.

The present report shows that there are indeed two transaminases in rat liver, which can be separated by DEAE-cellulose column chromatography. One is specific for leucine and the other is active for the three amino acids. Various other properties of the two enzymes are compared and the possible physiological significance of the enzymes is discussed.

Preliminary work on this subject has been reported[9].

METHODS

Enzyme assays

The reaction mixture contained (in μmoles): pyrophosphate buffer (pH 8.2), 50; L-amino acid, 30; α-ketoglutarate, 5; pyridoxal phosphate, 0.1; 2-mercaptoethanol, 20; and enzyme in a total volume of 1.5 ml. The incubation was carried out for 1 h at 37°. The keto acid formed was determined as reported previously[5]. The activity is expressed as μmoles of keto acid formed per 10 min and the specific activity as the activity per mg protein. Protein was measured by the method of LOWRY *et al.*[10]. In some cases the activity was expressed as change of absorbance at 440 mμ. Since the K_m value of leucine for the leucine-specific enzyme (enzyme II) is very high ($2.5 \cdot 10^{-2}$ M), it is difficult to add sufficient leucine to saturate the enzyme and the activity of enzyme II was calculated from the activity obtained at a concentration of 30 μmoles by extrapolating to the activity at the concentration of leucine which saturates enzyme II.

When pyruvic acid was examined as an amino acceptor, the activity was determined by the backward reaction[11]. The activity was measured by the method of BÜCHER *et al.*[12].

Preparation of anti-serum against hog heart enzyme

The hog heart supernatant enzyme was purified and the anti-serum was prepared as reported previously[7]. The anti-serum thus prepared showed a single precipitin line with the hog heart supernatant enzyme on an agar plate (Fig. 1).

RESULTS

Purification of enzyme I and II

Separation of two enzymes

Male Wistar rats, weighing 150–200 g, were maintained on laboratory chow. After sacrifice, their livers were removed and homogenized and fractionated in 0.25 M sucrose solution by the method of SCHNEIDER AND HOGEBOOM[13]. The supernatant fraction (A) from about 30 livers was fractionated with solid $(NH_4)_2SO_4$ in the presence

of $1 \cdot 10^{-3}$ M EDTA. This concentration of EDTA was added at all steps of purification. $5 \cdot 10^{-3}$ M 2-mercaptoethanol and $1 \cdot 10^{-5}$ M pyridoxal phosphate were also added to dialysis buffer and 2-mercaptoethanol was added to elution buffer for chromatography. The precipitate between 30 and 75% saturation of $(NH_4)_2SO_4$ was dissolved in $5 \cdot 10^{-3}$ M phosphate buffer (pH 7.8) and dialyzed against the same buffer. The dialyzed enzyme (B) was applied on a DEAE-cellulose column (1.2 cm × 30 cm) which had been equilibrated with the same buffer and enzyme was eluted with a linear concentration gradient of phosphate buffer (pH 7.8) from $5 \cdot 10^{-3}$ M to $3 \cdot 10^{-1}$ M. The activity for leucine appeared in the non-adsorbed fraction, at a concentration of about 0.02 M buffer and also at 0.18 M buffer, as shown in Fig. 2. The non-adsorbed fraction had very little activity and so was not studied in detail. The enzyme activity eluted with 0.02 M buffer is referred to hereafter as "enzyme I" and the one eluted with 0.18 M buffer as "enzyme II". Each fraction was then purified as follows.

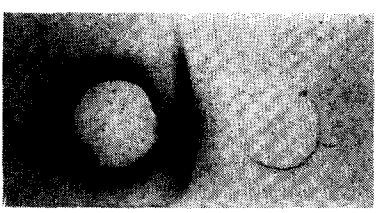

Fig. 1. Immunological double diffusion of hog heart supernatant enzyme. The right well contained the supernatant enzyme and the left contained anti-serum. The precipitin line was stained by Amido black 10B.

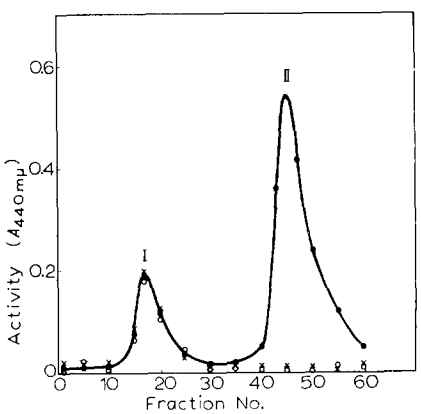

Fig. 2. DEAE-cellulose column chromatography of the two transaminases for branched chain amino acids. Fractions of 10 ml were collected. Activities for each amino acid are expressed as: ○, for valine; ●, for leucine; ×, for isoleucine.

Purification of enzyme I

The active fraction eluted from the DEAE-cellulose column with 0.02 M buffer was concentrated by precipitation with 75% satd. $(NH_4)_2SO_4$ and dialyzed against $1 \cdot 10^{-2}$ M phosphate buffer (pH 6.8). The dialyzed enzyme (C) was applied to a hydroxylapatite column which had been equilibrated with the buffer described above and the enzyme was eluted with a linear concentration gradient of phosphate buffer (pH 7.8) from $1 \cdot 10^{-2}$ M to $2 \cdot 10^{-1}$ M. The active fraction appeared at a concentration of about 0.06 M and this was concentrated in the same way as described above by $(NH_4)_2SO_4$ and dissolved in $5 \cdot 10^{-2}$ M pyrophosphate buffer (pH 8.2). This solution (D) was passed through a Sephadex G-200 column (2.3 cm × 100 cm) which had been equilibrated with $5 \cdot 10^{-2}$ M pyrophosphate buffer (pH 8.2), and the active fraction which had been sieved in this way was concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed. The specific activity of the dialyzed enzyme (E) was 186 as shown in Table I.

TABLE I

PURIFICATION OF ENZYME I

Procedure	*Total protein (mg)*	*Specific activity (μmole/ 10 min per mg protein) × 10⁻³*	*Total activity (μmoles/ 10 min)*	*Yield (%)*
A. Crude extract	12 069	2.9	35	100
B. $(NH_4)_2SO_4$	7 250	4.0	29	83
C. DEAE-cellulose	1720	9.9	17	49
D. Hydroxylapatite	235	21.3	5	14
E. Sephadex G 200	11	185.5	2	6

Purification of enzyme II

The active fraction obtained by DEAE-cellulose column chromatography was purified in the same manner as enzyme I but elution from the hydroxylapatite column was achieved at a higher concentration of phosphate buffer, *e.g.* from 0.2 M to 1.0 M, and activity appearing at a concentration of about 0.4 M. Sephadex G-200 was used for molecular sieving. The specific activity of the purified enzyme was 10 333 (Table II).

Properties of the two enzymes

Chromatographic properties

As shown in Fig. 2, two peaks of activity for transamination of leucine were clearly separated by DEAE-cellulose column chromatography. Enzyme I was eluted at a concentration of 0.02 M phosphate buffer, while enzyme II appeared with 0.18 M buffer. On hydroxylapatite column chromatography, enzyme I appeared with 0.06 M phosphate buffer while enzyme II was eluted with 0.4 M buffer.

Substrate specificity

Enzyme I was active for all three branched chain amino acids, but enzyme II was active only for leucine, as shown in Table III. No other amino acid would serve as

TABLE II

PURIFICATION OF ENZYME II

Procedure	*Total protein (mg)*	*Specific activity (μmoles/ 10 min per mg protein) × 10³*	*Total activity (μmoles/ 10 min)*	*Yield (%)*
A*. Crude extract	18 250	17	310	100
B*. $(NH_4)_2SO_4$	4 700	50	235	76
C. DEAE-cellulose	380	463	176	57
D. Hydroxylapatite	27	3 630	98	32
E. Sephadex G-200	3	10 333	31	10

* For A and B activities were calculated by subtracting the activity for valine or isoleucine from that for leucine.

TABLE III

SUBSTRATE SPECIFICITIES OF ENZYMES I AND II

Activities are expressed as percentages of that of leucine when α-ketoglutarate was added as an amino acceptor.

Substrate	*Relative activities*	
	Enzyme I	*Enzyme II*
Leucine	100	100
Valine	92	0
Isoleucine	105	0
α-Ketoglutarate		100
Oxaloacetate		37
Pyruvate		0

an amino donor. In view of the similarity in the inductions of tyrosine and leucine transaminases, special care was taken to distinguish between these two enzymes. Purified enzyme II was still contaminated with a considerable amount of tyrosine transaminase (EC 2.6.1.5) but these two enzymes were almost, though not completely, separated by sucrose density centrifugation, as shown in Fig. 3. From this and other findings, which will be discussed in a later section, it was concluded that tyrosine transaminase and enzyme II are different enzymes.

As amino acceptor of enzyme II, oxaloacetate was about 37% as active as α-ketoglutarate, but pyruvate did not act as an acceptor at all. The specificity of the amino acceptor for enzyme I was not examined, because TAYLOR AND JENKINS[6] reported that hog heart enzyme, which is very similar to enzyme I in liver, did not show any activity with these keto acids.

K_m values for substrates and cofactor

As shown in Table IV, the K_m of enzyme II for leucine is 30 times higher than that of enzyme I. This, together with inducibility of this enzyme II, may be significant

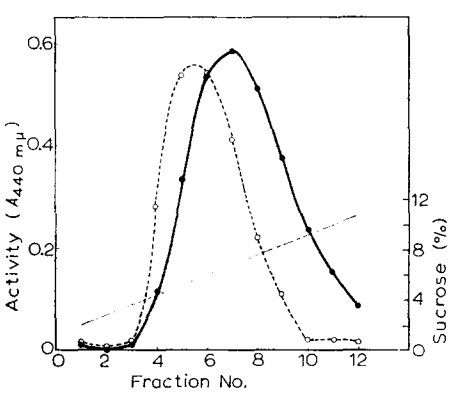

Fig. 3. Patterns of tyrosine transaminase and enzyme II on sucrose density gradient centrifugation. 106 μg of purified enzyme II were layered onto sucrose solution with a density gradient of 2–12% .Centrifugation was at 36 000 rev./min for 15 h. Fractions from the top of the tube are from the left of the figure. ○, activity with tyrosine; ●, with leucine.

TABLE IV

K_m VALUES FOR SUBSTRATES AND PYRIDOXAL PHOSPHATE

For measurement of K_m values for pyridoxal phosphate purified enzymes were dialyzed against dialysis buffer (pH 6.8) containing various additions (see text) with $1 \cdot 10^{-3}$ M hydroxylamine instead of added pyridoxal phosphate for overnight and then redialyzed against similar buffer without added hydroylamine and pyridoxal phosphate.

Substrate and cofactor	*K_m value (mM)*	
	Enzyme I	*Enzyme II*
Valine	4.3	
Leucine	0.75	25.0
Isoleucine	0.84	
α-Ketoglutarate	1.0	0.065
Pyridoxal phosphate	0.025	0.004

in connection with regulation of leucine metabolism. It should be added that the K_m value of valine of enzyme I is considerably higher than those of leucine and isoleucine, which are less than valine and about the same as each other. The relative K_m values of these three amino acids are very much like those of the hog heart enzyme[5,7]. The K_m value of enzyme I for α-ketoglutarate is 20 times higher than that of enzyme II.

Effect of 2-mercaptoethanol

The activities of the two enzymes in the crude homogenate were not affected by addition of 2-mercaptoethanol, but with purified preparations enzyme I was markedly activated by this reagent, as shown in Fig. 4, while enzyme II was slightly inhibited by it. However, enzyme II was inhibited completely by addition of $1 \cdot 10^{-3}$ M *p*-chloromercuribenzoate (PCMB), indicating that it is also an SH enzyme.

Optimal pH

The optimal pH of enzyme I was 8.2, while that of enzyme II was pH 8.7, as shown in Fig. 5.

Neutralization of the activity of enzyme I by anti-serum against hog heart enzyme

All the above results indicate that enzymes I and II are quite different trans-

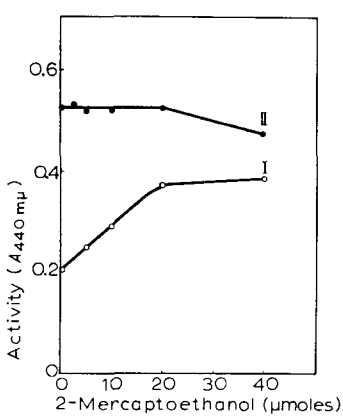

Fig. 4. Effect of 2-mercaptoethanol on enzymes I and II.

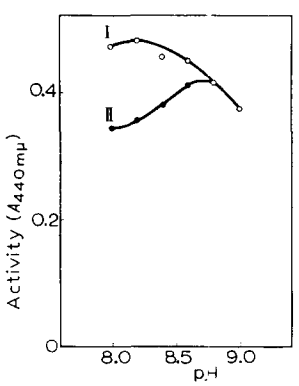

Fig. 5. Optimal pH values for enzymes I and II.

TABLE V

INHIBITION OF ENZYME I BY ANTI-SERUM AGAINST HOG HEART ENZYME

Anti-serum was preincubated with enzyme before addition of substrate.

Enzyme	*Substrate*	*Activity (mμmoles/10 min)*		*Inhibition (%)*
		− Anti-serum	*+ Anti-serum*	
I	Valine	60	29	52
	Leucine	67	30	55
	Isoleucine	69	26	62
II	Leucine	33	33	0

aminases and that enzyme I is very much like the heart enzyme reported previously[5]. Indeed, anti-serum against hog heart supernatant enzyme, which transaminates all branched chain amino acids, could neutralize the activity of enzyme I, but not that of enzyme II (Table V). This indicates clearly that enzyme I in rat liver is the same type of enzyme as that found in the heart. Increase of the anti-serum volume raised the inhibition of activity to 86%. This suggests that there may be about 14% contamination with unneutralizable enzyme with very similar enzyme properties. However this possibility is very unlikely, since the enzyme preparation used in the present study

TABLE VI

INTRACELLULAR DISTRIBUTION OF ENZYMES I AND II IN RAT LIVER

Activity of enzyme I is expressed as that for valine or isoleucine and activity of enzyme II is calculated by subtracting the activity of enzyme I from that for leucine.

Subcellular fraction	*Activity (%)*	
	Enzyme I	*Enzyme II*
Homogenate	100	100
Nuclei	9	4
Mitochondria	22	64
Microsomes	13	1
Supernatant	51	26

was considerably purified and the rate of inhibition was constant during the purification. Another possibility is that steric hindrance caused by anti-serum fixation is not sufficient to inhibit enzyme activity completely.

Subcellular localization of the liver enzymes

It has been reported that, in hog heart, the branched chain amino acid transaminase is localized both in the supernatant and the mitochondrial fractions and that the enzymes in these two fractions are isozymes[5,7]. The liver enzymes are also found in the both fractions (Table VI), but the distribution of the two enzymes in the fractions is quite different. Thus enzyme I is located mainly in the supernatant, while enzyme II is found predominantly in the mitochondria.

DISCUSSION

It is known from metabolic studies that valine is glycogenic, leucine ketogenic

and isoleucine has both properties[14]. It has also been shown that leucine is metabolized to hydroxymethylglutaryl-CoA and hence to cholesterol[15,16]. It is interesting that the carbons of leucine are more efficient precursors for cholesterol synthesis than those of acetate. These facts suggest that the specific transaminase for leucine described in this paper may play an important role in regulation of leucine metabolism and hence of cholesterogenesis and ketogenesis. It was reported from this laboratory that the transaminase activity for leucine, but not for valine and isoleucine, was induced specifically under various conditions[8]. Recently, we found that it is the activity of enzyme II which is induced[9]. It was also found that enzyme I is the only transaminase for branched chain amino acids present in fetal rat liver, but after birth the activity of enzyme II appeared and increased rapidly[17]. The details of these results will be reported elsewhere.

It should be mentioned that the induction of enzyme II under various conditions is very similar to that of tyrosine transaminase[18] and that enzyme II after partial purification still contained considerable tyrosine transaminase activity. These facts suggest the possible identity of the two enzymes. However, there are many results which indicate that they are in fact different enzymes: (1) the two activities can be separated by sucrose density centrifugation, though not completely, (2) purified tyrosine transaminase did not show any activity for leucine[19], (3) the K_m values for α-ketoglutarate and for pyridoxal phosphate of the two enzymes are significantly different—*i.e.* for α-ketoglutarate the K_m value of enzyme II is one-tenth of that of tyrosine transaminase and for pyridoxal phosphate the reverse is true[19], (4) diabetes or starvation induced tyrosine transaminase[18], but not enzyme II[8] and (5) tyrosine transaminase is localized almost exclusively in the supernatant[19], while enzyme II is found in both fractions, but especially in the mitochondrial fraction.

MEISTER *et al.*[20] and BRAUNSTEIN AND SENG[21] reported that glutamine transaminase (EC 2.6.1.15) showed non-specific activity with many keto acids including α-ketoisocaproate. However, it was reported recently that purified glutamine transaminase from rat liver did not show any activity with α-ketoisocaproate[22]. Moreover, the purified enzyme, which was kindly given by Dr. N KATUNUMA of this Institute, showed no activity when leucine and α-ketoglutarate were used as substrates. It was reported that in liver there is a transaminase (EC 2.6.1.12) which is active with pyruvate and many amino acids including leucine[2,4,11,23]. But purified enzyme II in the present work did not show any activity with pyruvate. The difference of enzyme I from other known transaminases was discussed in a previous paper[5].

It is interesting that the induction of the enzyme for leucine was limited to that in the supernatant and not in the mitochondria[8] and this is one reason why the present study was carried out mainly on the enzymes in the supernatant. Since the enzymes in the two fractions of hog heart were identified as isozymes[7], it is possible that the enzymes of liver which are also localized in the two fractions are isozymes.

The properties of enzyme I were very similar to those of the hog heart enzyme[5,7]. Thus, the two enzymes were similar in substrate specificity, their relative K_m values for the three amino acids (valine $>$ leucine $=$ isoleucine), their chromatographic behaviors on DEAE-cellulose, their activation by 2-mercaptoethanol, their subcellular localization and most important their immunological cross reaction. These similarities clearly indicate that enzyme I in liver is the same type of enzyme as that found in heart. Recent observations in this laboratory indicate that this type of enzyme is widely

distributed in various tissues of rat, but enzyme II is confined exclusively to liver[9]. Enzyme I in kidney, but not in liver, was shown to be induced by diabetic conditions[8].

The relation of the two enzymes described in the present paper somewhat resembles the relation of hexokinase (EC 2.7.1.1) to glucokinase (EC 2.7.1.2). The former is widely distributed in various tissues, has a low substrate specificity for sugar, a low K_m for glucose and is non-inducible, while glucokinase is highly specific for glucose, is only present in liver, is inducible by insulin and has a very high K_m for glucose[24,25].

The name leucine transaminase has been widely used for the activity for all branched chain amino acids, but from results of the present work it appears to be unsuitable and confusing. This was also pointed out by TAYLOR AND JENKINS[6]. It seems better to call enzyme I, which transaminates valine, leucine and isoleucine, "branched chain amino acid transaminase" and name enzyme II, which is specific for leucine, "leucine transaminase".

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