Heterogeneity of Hepatic Tyrosine Aminotransferase. Separation of the Multiple Forms* from Rat and Frog Liver by Isoelectric Focussing and Hydroxylapatite Column Chromatography and Their Partial Characterization

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L-Tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5.; TAT) and other enzymes that transaminate tyrosine in rat liver cytosol have been separated into four fractions by isoelectric focussing. One of the forms is probably identical to mitochondrial L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1.; mASAT). The other three forms have pI 's of 4.72, 4.98 and 5.30 and K_m values of 1.3 and 0.3 mM for tyrosine and α -ketoglutarate. These heat stable forms have little or no ASAT activity. Rat liver TAT is also separated into three peaks by hydroxylapatite. Each fraction gives only one peak of activity when electrofocussed separately.

In the frog, three groups of peaks of TAT activity have been separated by hydroxylapatite column chromatography. The first group is connected with ASAT activity. These peaks (pI 's 6.35, 6.50 and 6.90) are heat stable and have a K_m value for tyrosine of 4 mM. These fractions probably represent cytoplasmic ASAT (sASAT). The second group of peaks has at least two subforms (pI 's 9.0 and 9.4, K_m for tyrosine 15 mM). These forms probably represent mASAT. The third group consists

of three forms that resemble the major forms of rat liver TAT.

These results indicate that heterogeneity is common to many aminotransferases and independent of regulation by glucocorticoids.

Tyrosine aminotransferase (TAT) is the first enzyme in the catabolic pathway of the amino acid tyrosine. It has been very widely studied because of its complicated regulation. Many agents such as corticosteroids,¹ glucagon, insulin,² dibutyryl-3',5'-cyclic AMP and adrenalin³ are able to induce the enzyme in the liver of the rat and in rat hepatoma cell culture. This is why the enzyme of the rat has been largely used as a model in studies of enzyme regulation.⁴ TAT from frog liver is known to have different hormonal regulation. It is unaffected by corticosteroids. Tyrosine and dibutyryl-3',5'-cyclic AMP enhance the activity, while glucose decreases it to less than half.⁵

It has been reported that TAT from rat liver can be separated into three or four multiple forms by hydroxylapatite column chromatography,^{6,7} electrophoresis in polyacrylamide gel⁸ and by CM-Sephadex.⁹

At first it was thought that the multiple forms are regulated separately by different effectors.⁶ However, it has been shown that the forms are interconverted in an orderly sequence in the homogenate.⁹ A recent report suggests that TAT is a phosphoprotein;¹⁰ it has been proposed that the heterogeneity results from different phosphate content. Johnson and

* It is essential that the reader be familiar with the following definitions:

"Multiple forms of TAT" refer to all proteins that can transaminate tyrosine; it will be shown that other enzymes than EC 2.6.1.5. can be detected as "multiple forms of TAT" due to their broad substrate spectra.

"Isoenzymes": ASAT is known to have two isoenzymes (sASAT and mASAT) that have different primary structures.

"Subforms": It will be shown in this paper that TAT, sASAT and mASAT can be divided into several subforms. The only known difference between the subforms is in their net charges.

Grossman have reported that the elution pattern of TAT activity is strongly affected by the concentration of phosphate in the original homogenization medium.¹¹ It has also been suggested that some of these multiple forms are in fact other aminotransferases such as ASAT.¹² Summing up, the physiological role of the multiple forms has remained obscure; we do not know whether they are degradation products or some other post-translational modifications or mere artifacts.

The aim of the present work was to obtain more information about the matters described above by separating the multiple forms by isoelectric focussing and correlating the fractions obtained by different techniques to each other. The enzyme from frog liver was studied and compared to that of the rat, because the former is known to have different and far less complicated hormonal regulation.⁵ We also regarded it necessary to investigate the interrelations of TAT, sASAT, mASAT and L-alanine:2-oxoglutarate aminotransferase (EC 2.6.1.2.; ALAT) to exclude any errors that might arise due to broad substrate spectra.¹¹ Further, we wanted to find out whether or not aminotransferases other than TAT consisted of subforms.

MATERIAL AND METHODS

Animals and tissue preparations. Male Wistar rats (average weight 200 g) of a strain bred in our laboratory were used. The animals were fed *ad libitum* with a standard diet (Hankkija, Helsinki). The frogs were provided by Porla Fisheries, Lohja, Finland. They were kept at +4 °C and "equilibrated" at +18 °C for 5 days before killing. Only male frogs were used; their average weight was about 30 g.

All the animals were killed between 1 and 2 p.m. to avoid the effects of the diurnal variations.¹³ The animals were decapitated and the livers were removed immediately, rinsed in ice-cold 100 mM potassium phosphate buffer, pH 6.8 (in hydroxylapatite column chromatography experiments) or 320 mM glycerol (in electrofocussing experiments) and homogenized in 2 volumes of the same buffer with a Potter-Elvehjem glass homogenizer using a tightly fitting Teflon pestle (10 strokes). The homogenates were centrifuged at 100 000 *g* for 60 min in a Spinco Model L-50 ultracentrifuge. The resulting supernatant fractions were immediately used in the experiments. These procedures were performed at 0–4 °C.

Hydroxylapatite column chromatography. Hydroxylapatite was prepared in our laboratory as described previously¹⁴ and equilibrated with excess 100 mM potassium phosphate buffer, pH 6.8. After packing the columns (1.5 cm × 12 cm) were washed with excess buffer. The sample was applied on top and the column was washed with the buffer until no more protein was eluted. The bound proteins were then eluted with a continuous potassium phosphate gradient 100–500 mM (pH 6.8). The total volume of the gradient was 1000 ml in all experiments. The flow rate was kept at about 0.5 ml/min. Fractions of about 5 ml were collected by an Isco fraction collector. Protein was estimated by following the absorbancy at 280 nm.

Isoelectric focussing. The isoelectric focussing apparatus (Model 8101) was purchased from LKB, Bromma, Sweden. A glycerol gradient 60–0 % v/v was used. The Ampholines were used at 1 % concentration and they were divided 1:3 between the light and dense gradient solutions. The gradient was mixed by a device manufactured by LKB, Bromma, for this purpose. The sample was divided equally between both mixing vials. The focussing was started (anode at top) with a voltage of 400 V. After 20 h the voltage was elevated to 500 V and after another 20 h the current was stopped and fractions (2 ml) were collected. The pH of every fraction was immediately measured by a Radiometer Titrator (Radiometer, Copenhagen). The enzyme activities were determined in the fractions directly, since preliminary experiments showed that the Ampholines do not appreciably disturb the assays.

When hydroxylapatite fractions were electrofocussed, the samples were dialyzed against water to remove excess ions so as to keep down the current.

Concentration of the fractions. This was performed with an Amicon ultrafiltrator under 3.5 atm pressure in nitrogen atmosphere using PM-10 membranes. The fractions from electrofocussing were diluted at least 1:10 with 100 mM potassium phosphate buffer, pH 6.8, concentrated, diluted again at least 1:10 with the same buffer and reconcentrated.

Estimation of heat stability. The heat stability of separated fractions was studied by heating them (in a volume of 300 μ l) to +60 °C for 5 min in the presence of 1 mg/ml of bovine serum albumin and 0.5 mM pyridoxal-5'-phosphate. The control tubes (containing equal amounts of the two stabilizers) were kept at +0 °C. After the incubation a normal tyrosine aminotransferase assay was performed adding the usual amount of pyridoxal-5'-phosphate.

Determination of kinetic constants. The K_m values for tyrosine and α -ketoglutarate were determined by the method of Lineweaver and Burk.¹⁵ The linear regression coefficients were calculated by computer using the least squares

method. The calculations are based on six to eight rate determinations.

Assays. Tyrosine aminotransferase was assayed by the following method. A mixture containing 80 volume units of 7 mM tyrosine in 125 mM potassium phosphate buffer, pH 7.6, 2 volume units of 0.5–0.05 M α -ketoglutarate and 1 volume unit 5 mM pyridoxal-5'-phosphate was prepared. The sample to be assayed (150 μ l) was pipetted in test tubes, and 1.245 ml of the mixture described above was added. After shaking, a portion of 465 μ l was taken out of the assay mixture and pipetted in a test tube containing 35 μ l of 10 M KOH; the tubes were kept on ice. The assay mixture was incubated at 37 °C for 30–45 min, after which another portion of 465 μ l was taken and handled similarly. After this the KOH-tubes were incubated at +37 °C for another 30 min. The absorbancy of each sample was read against its own zero time blank in 0.5 ml quartz cuvettes at 331 nm in a Gilford spectrophotometer. This method is a technical modification of that of Diamondstone.¹⁶

ASAT and ALAT activities were estimated with a previously described method¹⁷ using the assay sets of Boehringer Mannheim GmbH. In hydroxylapatite column chromatography, the protein elution profile was studied by following the absorbancy at 280 nm.

Reagents. The following reagents were used: L-tyrosine, pyridoxal-5'-phosphate and bovine serum albumin (Sigma), α -ketoglutaric acid, ethylenediamine, and glycerol (Merck). Ampholines were purchased from LKB, Bromma, Sweden.

RESULTS

Isoelectric focussing of rat liver cytosol. Fig. 1 shows the tyrosine aminotransferase activity profile in isoelectric focussing in the pH range 3.5–10. A broad peak was seen around pH 5. This peak is distinctly separate from those of aspartate and alanine aminotransferases. It was often divided into three fractions. Another peak was seen above pH 9. This peak also had aspartate aminotransferase activity. It is a minor form but was consistently found. A trace of activity was sometimes seen around pH 8, but due to its minute amount no characterization of this form was possible.

To obtain better separation, rat liver cytosol was then electrofocussed in a narrower pH range (3–6). The broad peak at about pH 5 was distinctly divided into three forms having pI's of 4.72, 4.98 and 5.30 (Fig. 2a).

In hydroxylapatite column chromatography, three distinctly separate peaks (designed 1, 2,

Fig. 1. Isoelectric focussing of rat cytosol in the pH range 3.5–10. Rat supernatant fraction (2 ml in 320 mM glycerol) was electrofocussed as described in the text. TAT was assayed with 0.5 mM α -ketoglutarate. ●, TAT; □, ASAT; ▲, ALAT; ..., pH. TAT activity is expressed as milliunits per fraction and ALAT and ASAT activities as units per fraction (the ASAT and ALAT peaks are somewhat smaller than in reality because the assay was performed so that the highest activities were in the unlinear region).

and 3) were resolved (Fig. 3). Most of the aspartate and alanine aminotransferase activities were eluted in the buffer front, but some aspartate aminotransferase activity was connected with the middle peak. The elution pattern varied so that the first peak sometimes dominated and only traces of the other two were seen.

The hydroxylapatite fractions were electrofocussed separately to correlate the fractions obtained by the two different techniques to each other. Each hydroxylapatite fraction gave only one peak in isoelectric focussing (Fig. 2 b–d). The fractions had pI's 5.2, 5.0 and 4.8 (fractions 1, 2 and 3, respectively). These pI's are in good agreement with those seen in Fig. 2a.

The multiple forms of hepatic TAT of Rana temporaria. Three groups of multiple forms of TAT were obtained from frog liver by hydroxylapatite column chromatography (Fig. 4). The

Fig. 2. a: Isoelectric focussing of rat cytosol in pH range 3–6. b–d: Isoelectric focussing of the three subforms of TAT obtained by hydroxylapatite column chromatography (b=1, c=2, and d=3 in Fig. 3). The unit of TAT activity is μmol of *p*-hydroxyphenylpyruvate formed/min. TAT was assayed by 10 mM α -ketoglutarate. ●, TAT; ..., pH.

first group (designated group A) travelled in the front as did aspartate and alanine aminotransferases. This peak was hardly discernible when the assay was performed using 10 mM α -ketoglutarate but it was observed that this peak was strongly inhibited at the unphysiologically high concentration of the substrate used in the standard assay. Another band of activity was eluted by 150 mM phosphate. This group (designated group B) was often divided into two peaks, though in the experiment shown in Fig. 4 this was not so clearly

seen. These fractions were consistently associated with ASAT activity that was often divided into two peaks. These two closely travelling peaks were only minor forms comprising not more than 5 % of total TAT activity.

In the middle region of the phosphate gradient a group of three peaks of TAT activity (group C) was eluted. Of these multiple forms, the two first ones (C1 and C2) were constantly obtained; the third, however, was generally smaller than the other two and was often ab-

Fig. 3. Hydroxylapatite column chromatography of rat cytosol. A 2.5 ml portion of rat liver cytosol (in 100 mM potassium phosphate buffer, pH 6.8) was applied on top of a hydroxylapatite column and eluted as described in Material and Methods. TAT activity was assayed using 0.5 mM α -ketoglutarate to avoid any substrate inhibition. The activities are expressed as μ mol of product formed/(fraction min). $\bullet$, TAT; $\square$, ASAT; - - -, absorbancy at $\lambda = 280$ nm; —, phosphate concentration. ALAT activity (not shown) was eluted in the front.

Fig. 4. Hydroxylapatite column chromatography of frog cytosol. The procedure as well as the symbols and units are as in Fig. 3. TAT was assayed with 0.5 mM (fractions 1–20) or 10 mM (fractions 20–110) α -ketoglutarate.

Fig. 5. a: Isoelectric focussing of frog liver cytosol (2 ml of supernatant focussed). b: Isoelectric focussing of fraction A obtained from frog liver by hydroxylapatite column chromatography. c: Electrofocussing of the combined fraction B from hydroxylapatite column chromatography. α -Ketoglutarate was used at 0.5 mM concentration in TAT assays. ASAT activity is expressed as μmol of product formed/(fraction min) and that of TAT as nmol /(fraction min). The symbols are as in Fig. 1.

sent. None of these three multiple forms had any ASAT or ALAT activity.

The isoelectric focussing profile showed that most activity was found around pH 5. When the analyses were performed at low α -ketoglutarate concentration this fraction seemed smaller compared to the others (Fig. 5a). Most often the peak was divided into two; three fractions were seldom seen. Another group of multiple forms was seen below pH 7. It also had aspartate aminotransferase activity. Both enzyme activities were divided into two or three fractions. Two peaks were found above pH 9; these multiple forms, again, had ASAT activity.

When group A from hydroxylapatite column chromatography was electrofocussed separately, three peaks of TAT activity were obtained (Fig. 5b). All three were accompanied by similar peaks of strong ASAT activity. The pI 's of these multiple forms were 6.35, 6.50 and 6.90. Sometimes, some activity was found at pH 7.2. We suggest that here the TAT and ASAT activities are due to the same enzyme, the cytoplasmic isoenzyme of ASAT, which, as can be seen in Fig. 5, has subforms as does TAT.

When group B from hydroxylapatite column chromatography was electrofocussed separately, two peaks with pI 's 9.0 and 9.4 were seen (Fig. 5c). This suggests that these fractions represent the mitochondrial isoenzyme of ASAT, which would thus also have subforms. A study of the subcellular localization of TAT activity (using 10 mM α -ketoglutarate in the assay) revealed that about one sixth of the activity is associated with mitochondria. This may be sufficient to give a contamination to the cytosol that could be responsible for the two peaks of activity; the possibility that this enzyme is partly cytoplasmic in nature must not, however, be overlooked.

These experiments show that even other aminotransferases have subforms and this is not a special property of TAT.

Characterization of the multiple forms from the two sources. The kinetic constants of the different multiple forms for tyrosine and α -ketoglutarate were determined (Table 1). The K_m -values for tyrosine of the three main fractions from rat liver were about 1 mM, as well as those of the three hydroxylapatite fractions of the frog proposed to be subforms of TAT (*i.e.*

Table 1. Summary of the properties of the multiple forms of TAT. The inhibition by α -ketoglutarate was calculated as percent of the highest activity measured, heat denaturation as percent of activity lost by heating to 60 °C for 5 min. The K_m -values for tyrosine were determined using 10 mM (rat HA 1-3, rat IF fractions and frog HA C 1, C 2 and C 3) or 0.5 mM (frog HA A and B) α -ketoglutarate.

Species	$K_m(\text{Tyr})$ mM ^d	$K_m(\alpha\text{-KG})$ mM ^d	Isoelectric point	ASAT activity	Heat denaturation/%	Inhibition by 10 mM α -KG at pH 7.6/%	Suggested proper name
Rat, HA 1 ^e	1.4	0.4	5.2	-	0	None	TAT
Rat, HA 2 ^e	1.1	0.3	5.0	±	0	None	TAT
Rat, HA 3 ^e	1.3	0.3	4.8	-	0	None	TAT
Rat, IF α ^f	1.1	0.3	4.72	-	0	None	TAT
Rat, IF β ^f	1.4	0.3	4.98	-	0	None	TAT
Rat, IF γ ^f	1.3	0.4	5.30	-	0	None	TAT
Rat, IF η ^f	8.0	0.04	9.8	+	15	25	mASAT
Frog, HA A ^{b,g}	4.2	0.05	6.35, 6.50 and 6.90	+	0	75	sASAT
Frog, HA B ^{b,g}	15	0.05	9.0 and 9.4	+	80	30	mASAT
Frog, HA C 1	1.1	^a	N.D. ^c	-	0	^a	TAT
Frog, HA C 2	1.0	^a	N.D.	-	0	^a	TAT
Frog, HA C 3	1.1	^a	N.D.	-	0	^a	TAT

^a = Substrate activation (see text). ^b = Mixtures of at least two forms (see Fig. 5). ^c = Not determined. ^d $K_m(\text{Tyr}) = K_m$ for tyrosine; $K_m(\alpha\text{-KG}) = K_m$ for α -ketoglutarate. ^e See Fig. 3. ^f See Figs. 1 and 2. ^g See Fig. 4.

C 1, C 2 and C 3). The fraction of TAT activity from rat liver that had a pI above 9 had a K_m for tyrosine of 8 mM, which further suggests that this form is, in fact, identical to mASAT.¹⁸ Group A of multiple forms of TAT activity from hydroxylapatite column chromatography fractionation of frog liver cytosol (studied as a combination of all three subforms in roughly equal amounts) had a K_m for tyrosine of 4 mM. It is possible that this form can transaminate tyrosine under physiological conditions. The multiple forms of group B, when combined, had somewhat higher K_m -values for tyrosine. The K_m 's for α -ketoglutarate will be discussed under a separate heading.

All the multiple forms except those of group B of the frog were found to be heat stable. The labile multiple forms were denaturated to 20 % by the heating (Table 1).

Mitochondrial ASAT separated from frog liver (unpublished) was found to share the following properties with group B: the ratio of ASAT and TAT activities, isoelectric point(s), K_m for tyrosine, heat lability, K_m for α -ketoglutarate, inhibition by α -ketoglutarate and ability to use pyruvate as the keto acid. Thus, group B is probably identical to mASAT.

Effects of α -ketoglutarate on the activity of the multiple forms of TAT. The K_m -values for α -ketoglutarate for the multiple forms of TAT of the rat are shown in Table 1. It can be seen that there are no gross differences between the three major forms. The form that has a high pI has a significantly lower K_m for α -ketoglutarate than the three main fractions. This further lends support to the suggestion that this form is identical to mitochondrial ASAT.¹⁸ The K_m -values of the major forms are quite near to those reported by Iwasaki *et al.*⁶

The combined fractions of group A from frog liver (suggested to be subforms of sASAT) were strongly inhibited by α -ketoglutarate under the normal assay conditions (α -ketoglutarate, 10 mM). The inhibition was stronger at lower pH values. The K_m -value of the combination was very low, about 0.05 mM, which indicates that it is always saturated with this substrate.¹⁹

The combined fractions of group B (that showed similarities to mASAT) had also a low K_m for α -ketoglutarate but were inhibited to a much lesser extent by this substrate at pH 7.6

(25 % *vs.* 75 % inhibition at 10 mM α -ketoglutarate). This property is shared with mASAT from frog liver. The peaks of group B were combined in these studies because so little enzyme protein was obtained.

The three forms proposed to be subforms of TAT (C 1, C 2 and C 3) seemingly did not follow Michaelis-Menten kinetics. Instead, they exhibited substrate activation by α -ketoglutarate. All the forms showed this same tendency. Studies are in progress to further characterize this phenomenon.

DISCUSSION

Three major fractions that have tyrosine aminotransferase activity have been resolved from rat liver by isoelectric focussing, hydroxylapatite column chromatography, electrophoresis in agar gel⁶ and ion exchange chromatography by CM-Sephadex.⁹ Sepharose fails to separate these forms⁹ as does Sephadex G-200 gel filtration.⁶ This suggests that these subforms differ in their isoelectric points and net charges but probably not in molecular weights. These multiple forms are interconverted in homogenates⁹ which, together with the above-mentioned facts, suggests that the different forms are posttranslational modifications that arise from a single molecular species. The physiological significance of the heterogeneity is not known at present. The present results show, however, that this phenomenon is independent of the complex hormonal regulation of the enzyme seen in rat liver, because hepatic TAT of the frog also has subforms but is not induced by corticosteroids.⁵

It was interesting to note that, in addition to tyrosine aminotransferase, both the mitochondrial and soluble isoenzymes of aspartate aminotransferase obtained from frog liver have subforms that differ in their isoelectric points. This indicates that the heterogeneity is a property common to at least three aminotransferases (TAT, sASAT and mASAT). Earlier reports on ASAT reveal that the two isoenzymes of ASAT obtained from different mammalian tissues also have subforms. The only difference so far known between them is in their isoelectric points.²⁰⁻²² The heterogeneity of the cytoplasmic isoenzyme of aspartate aminotransferase has been noted in preparations from

sheep liver,²⁰ beef heart²¹ and rat brain.²² Aspartate aminotransferase is far better characterized enzymologically than TAT.²⁰⁻²⁸ The amino acid sequence for sASAT is completely known and that for mASAT almost completely known.^{27,28} The subforms of sASAT from pig heart have identical amino acid compositions.²² The subforms from sheep liver are immunologically identical.²⁰

Summing up, we conclude that the heterogeneity is common to many aminotransferases. It is possible that in each case it is based on similar, minor posttranslational modification. The heterogeneity may be important in the regulation of gluconeogenesis from amino acids in higher animals.

While this manuscript was in preparation, Smith *et al.*²⁹ reported on a particle bound factor that interconverts the subforms of TAT. It will be very interesting to see if this factor can interconvert the subforms of sASAT and mASAT as well.

The cytoplasmic isoenzyme of ASAT from frog liver could also transaminate tyrosine while that of the rat is known to have little TAT activity.³⁰ It is possible that the aminotransferases have evolved from a common ancestral gene during evolution. This would explain their common properties such as their heterogeneity and overlapping substrate spectra.

Another fraction of tyrosine aminotransferase activity that has a high K_m for tyrosine and is eluted from hydroxylapatite columns before the three "major" subforms has been reported and designated "form 1" by Iwasaki *et al.*⁶ This form was denaturated by heating to 60 °C for 5 min and had a high K_m for α -ketoglutarate, all properties that clearly separate it from the aspartate aminotransferases. Other authors have failed to demonstrate this form. In our studies we have sometimes seen a small notch in the beginning of the phosphate gradient in hydroxylapatite column chromatography. One such notch is demonstrated in Fig. 3. We also sometimes noticed a minor form of activity at about pH 8 (see Results) in isoelectric focussing experiments of rat cytosol. It is not known if these forms are identical; because of the small amount of the enzyme characterization was not feasible. It is possible that the cathodal "isoenzymes" reported by

Mertvetsov *et al.*^{31,32} are related to these forms; most probably, however, they represent mitochondrial ASAT activity (also known as mitochondrial TAT).

From the results presented in this paper and from a study of the literature we conclude that several aminotransferases have subforms. The subforms probably arise from single molecular species through minor chemical modifications; this can be seen *in vitro* but the modification may be catalyzed by specific factors *in vivo*. The peculiar kinetic behaviour of the subforms of TAT from frog liver suggests a possible allosteric mechanism of regulation; this is presently being studied in our laboratory.

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