Partial Purification and Properties of Frog Liver Tyrosine Aminotransferase

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Hepatic tyrosine aminotransferase of the frog *Rana temporaria* was partially purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation and successive chromatography on DEAE-cellulose DE-52, Ultrogel AcA-34, DEAE-cellulose DE-52 again and, finally, hydroxyapatite. During the last step, the enzyme activity separated into two fractions; traces of a third fraction were also found. The major form was purified 6000-fold to a specific activity of 200 units/mg of protein; it was about 50% pure by electrophoretic criteria. It had mol.wt. about 85000 as determined by gel filtration on a Sephadex G-100 column. It was not activated by added pyridoxal 5'-phosphate. The enzyme was, however, inactivated by the pyridoxal phosphate reactants canaline and amino-oxyacetate. The enzyme was specific for 2-oxoglutarate as the amino group acceptor. Homogentisate inhibited the enzyme and adrenaline was an activator; both effects were seen at low concentrations of the effectors. The relationship between initial rate and tyrosine or 2-oxoglutarate concentration was abnormal and complex. Form-2 enzyme had similar or identical molecular weight, cofactor requirements, oxo acid specificity and kinetics.

The complex regulation of hepatic L-tyrosine-2-oxoglutarate aminotransferase (EC 2.6.1.5) of the rat has interested many biochemists during the past decade. The enzyme is induced by many hormones (Greengard & Dewey, 1967; Hager & Kenney, 1968; Wicks, 1968; Tomkins *et al.*, 1972) and tyrosine (Mendelson *et al.*, 1971), whereas glucose lowers its activity (Mendelson *et al.*, 1971; J. J. Ohisalo, unpublished work).

In frog liver, only tyrosine, dibutyl cyclic AMP and glucose have been shown to affect the enzyme activity (Chan & Cohen, 1964; Ohisalo & Pispä, 1975). The enzymes from both sources have been reported to have several subforms, the significance of which remains unknown (Ohisalo & Pispä, 1976). The role of pyridoxal 5'-phosphate in the regulation of the enzyme has been discussed in the literature (Black & Axelrod, 1969; Badawy, 1972; Ohisalo *et al.*, 1974). The enzyme is known to have a very short half-life in rat liver (about 1.5h), which has been suggested to be connected with the dissociability of the cofactor (Litwack & Rosenfield, 1973). The rat enzyme has been purified and reported to have a mol.wt. of 115000 and to bind four molecules of pyridoxal phosphate per molecule of enzyme (Valeriote *et al.*, 1969). The enzyme binds some of its cofactor molecules tightly, but the others can be removed by dialysis (Valeriote *et al.*, 1969; Iwasaki *et al.*, 1973). It has been proposed that the dissociation of the cofactor from the apoenzyme results

in denaturation and degradation of the enzyme (Reynolds & Thompson, 1974).

To clarify the regulation of the non-inducible enzyme further, we have partially purified tyrosine aminotransferase from the liver of the frog *Rana temporaria*. The molecular weights of two subforms have been determined, and their oxo acid specificities and cofactor requirements have been investigated. We have shown that, in addition to hormonal regulation, there are direct mechanisms of regulation of the enzyme activity by homogentisate, one of the products of the catabolic pathway of tyrosine. The regulation of the transamination of tyrosine will be discussed in the light of the present results.

Experimental

Animals

The frogs were provided by the Porla Fisheries, Lohja, Finland. They were stored at 4°C and equilibrated at room temperature (20°C) for 5 days before being killed. The average weight of the animals was about 30g. Both sexes were used unsegregated; females outnumbered males.

Purification of the enzyme

A total of 282 frogs were killed by decapitation at noon. The livers (total 182g) were removed immediately, rinsed in ice-cold 0.9% NaCl and homogenized in 3 vol. of 100mM-potassium phos-

phate buffer, pH 6.8 at 4°C. The homogenate was centrifuged (in two portions) at 100000g for 60 min. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to a relative saturation of 35% (at 4°C) and the resulting precipitate was removed by centrifugation at 10000g for 10 min. More $(\text{NH}_4)_2\text{SO}_4$ was added to a final saturation of 70%, and the precipitate was collected by centrifugation and dissolved in a small volume of buffer A (50 mM-potassium phosphate buffer, pH 7.6, containing 0.1 mM-dithiothreitol and 1 mM-EDTA), and then dialysed twice against 4 litres of the same buffer. The dialysed sample was then applied to a column (6.5 cm $\times$ 12.5 cm) of DEAE-cellulose DE-52 equilibrated with buffer A. The column was washed with the same buffer, and the bound proteins were then eluted with a continuous linear KCl gradient (0–400 mM) in buffer A; the total volume of the gradient was 4.4 litres. The fractions containing tyrosine aminotransferase activity (see the Results section) were combined and concentrated by an Amicon ultrafiltrator under a pressure of 300 kPa (3 atm) of N_2 by using a PM-10 membrane. The concentrated preparation was dialysed for 4 h against 100 vol. of buffer A to which 5 mM-2-oxoglutarate and 50 μM -pyridoxal 5'-phosphate had been added (buffer B). The sample was then applied to a column (5 cm $\times$ 92 cm) of Ultrogel AcA-34 equilibrated with buffer B. The column was run with the same buffer and the active fractions were combined, concentrated and dialysed against buffer A. The dialysate was rechromatographed on a column (2.5 cm $\times$ 12 cm) of DEAE-cellulose (total volume of the gradient was 1 litre; gradient as in the previous run). The combined and concentrated active fractions were dialysed against 100 mM-potassium phosphate buffer, pH 6.8, after which the dialysed enzyme preparation was applied to a column (2.5 cm $\times$ 7 cm) of hydroxyapatite equilibrated with the same buffer. After washing, the column was eluted with a continuous linear gradient of potassium phosphate (100–500 mM; total volume 1 litre). The peaks obtained were concentrated and dialysed against 50 mM-Tris/HCl, pH 7.0, containing 5 mM-2-oxoglutarate and 50 μM -pyridoxal 5'-phosphate, in which buffer they were preserved at 4°C. These rather stable preparations were used in subsequent experiments. As the oxo acid specificities and cofactor requirements were studied, the enzyme forms were first exhaustively dialysed against buffer A.

Determination of molecular weights

This was performed on a column (2.5 cm $\times$ 65 cm) of Sephadex G-100 by conventional methods (Andrews, 1965), with catalase (mol.wt. 240000), yeast alcohol dehydrogenase (mol.wt. 150000), bovine serum albumin (mol.wt. 68000), chymotrypsinogen (mol.wt. 25000) and cytochrome *c* (mol.wt. 12400) as the standards.

Assays

Tyrosine aminotransferase activity was determined by a modification of the method of Diamondstone (1966) as described previously (Ohisalo & Pispá, 1976). The units of tyrosine aminotransferase activity are μmol of *p*-hydroxyphenylpyruvate formed/min at 37°C. Bovine serum albumin was added to the assay mixture up to a final concentration of 0.3–1 mg/ml to stabilize the enzyme. Protein was measured, with bovine serum albumin as the standard, by the method of Lowry *et al.* (1951). In column-chromatography experiments, the protein-elution profile was monitored by recording the A_{280} .

Materials

Ultrogel AcA-34 was obtained from LKB, Bromma, Sweden, and Sephadex G-100 from Pharmacia AB, Uppsala, Sweden. DEAE-cellulose DE-52 was purchased from Whatman, Maidstone, Kent, U.K. Hydroxyapatite was prepared by the method of Levin (1962). The following reagents were used: canaline (2-amino-4-amino-oxybutyrate) (Sigma, St. Louis, MO, U.S.A.), amino-oxyacetate (Aldrich, Beersse, Belgium), adrenaline, dopamine (3,4-dihydroxyphenethylamine), metanephrine [1-(4-hydroxy-3-methoxyphenyl)-2-methylaminoethanol], normetanephrine [1-(4-hydroxy-3-methoxyphenyl)-2-aminoethanol], tyramine, homogentisate (Sigma), phenethylamine and phenylethanolamine (Aldrich). The other reagents used were of reagent grade.

In the experiments described in Table 2 and Fig. 3 the bovine serum albumin was defatted before use (Michael, 1962).

Results

Comments on the purification

In the first DEAE-cellulose DE-52 column chromatography, a small fraction of the total tyrosine aminotransferase activity was eluted in the buffer front. This fraction is identical with the cytoplasmic isoenzyme of L-aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1), which is known to be capable of transaminating tyrosine (Ohisalo & Pispá, 1976). This fraction was discarded and ignored when calculating final purification. In the subsequent step, gel filtration on an Ultrogel AcA-34 column, the enzyme activity was eluted as a single sharp peak. After rechromatography on DEAE-cellulose DE-52, the active fractions were chromatographed on a column of hydroxyapatite. Two peaks were obtained. The first of these contained most of the activity. Traces of a third peak were obtained after the other two. Peaks (forms) 1 and 2 were concentrated and studied separately; the amount of the third fraction obtained was insufficient to permit any characterization whatsoever. After the hydroxyapatite step, the

enzyme in the first peak had a specific activity of 203 units per mg of protein and had thus been purified 6000-fold. In polyacrylamide-gel electrophoresis, the enzyme preparation was about 50%

Fig. 1. Electrophoresis of form-1 enzyme in 5% (w/v) polyacrylamide

About 20 μ g of form-1 enzyme was used. The buffer was 50 mM-Tris/glycine, pH 8.5, the current 5 mA/cm² and the temperature 12°C. Coomassie Blue was used for staining. The cathode is at the top. All tyrosine aminotransferase activity was found at a place corresponding to the upper band seen in the gel.

pure. The contamination consisted mainly of one single inactive protein band. All the tyrosine aminotransferase activity migrated as a single fraction (Fig. 1). A summary of the purification is presented in Table 1.

Characterization of form-1 enzyme

The mol.wt. of this enzyme was estimated to be about 85000 by gel filtration on a column of Sephadex G-100.

The enzyme was not activated at all by added pyridoxal 5'-phosphate, and high concentrations were even found to be slightly inhibitory. Canaline and amino-oxyacetate, compounds known to react with the cofactor (Rahiala *et al.*, 1971; Huovinen, 1968), could, however, inactivate the enzyme almost totally. The effect of preincubation with different concentrations of amino-oxyacetate on the enzyme activity is presented in Fig. 2. The enzyme could not be reactivated thereafter by exhaustive dialysis against buffer A, but addition of 50 μ M-pyridoxal 5'-phosphate or pyridoxamine 5'-phosphate restored approx. 10–20% of the activity. Pyridoxine and pyridoxamine had no effect.

The enzyme was specific for 2-oxoglutarate as the amino group acceptor; oxaloacetate, pyruvate and acetoacetate, each tested at 1–10 mM, could not serve as substrates.

Several structural analogues of tyrosine and 2-oxoglutarate and some metabolically relevant compounds were screened for potential effects on the enzyme activity *in vitro*. Homogentisate, one of the intermediates in the catabolic pathway of tyrosine, inhibited the enzyme. Half-maximal inhibition was obtained with 15 μ M-homogentisate. This inhibition could be partly overcome by increasing concentrations of tyrosine. However, owing to the low solubility of tyrosine and to the complex reaction kinetics (see below), no linear reciprocal plots for calculation of the inhibition factors could be obtained. Preincubation with 150 μ M-homogentisate at 37°C for 15 min did not inactivate the enzyme.

Adrenaline activated the enzyme. Maximal activation was about 50%, half-maximal activation being obtained with 3.5 μ M-adrenaline. Maximal activation

Table 1. Summary of the purification of form 1 of tyrosine aminotransferase from frog liver

Step	Protein (mg)	Specific activity (units/mg of protein)	Yield (%)	Purification (fold)
Homogenate	20 500	0.034	100	1
10 ⁵ g supernatant	10 100	0.052	80	1.5
(NH ₄) ₂ SO ₄ fractionation	6610	0.057	55	1.7
DEAE-cellulose	581	0.503	42	15
Ultrogel	201	1.22	36	36
DEAE-cellulose	86.8	4.09	51	123
Hydroxyapatite	0.76	203	22	6000

Fig. 2. Effect of preincubation of the enzyme forms with amino-oxyacetate

The enzyme was preincubated for 15 min at 37°C in the presence of different concentrations of amino-oxyacetate in 125 mM-potassium phosphate, pH 7.6, as shown. The assay mixture was then added so that the enzyme solution was diluted tenfold. ●, Pyridoxal 5'-phosphate present, 50 μM; ○, no pyridoxal phosphate added. (a) Form 1; (b) form 2. About 1 munit of each form was used per assay.

Fig. 3. Effect of adrenaline on the enzyme activity

Adrenaline was added to the usual assay-mixture in the final concentration shown before addition of the enzyme. The activation is expressed as the percentage of activity in the absence of adrenaline. About 0.8 munit of enzyme was used per assay.

was accomplished with 7–10 μM-adrenaline; activation by higher concentrations was somewhat less. The effect of increasing concentration of adrenaline on the enzyme activity is displayed in Fig. 3. The effect was independent of the concentration of tyrosine.

Table 2. Effects of some analogues of tyrosine on the activity of form-1

The experiments were carried out as described in the legend to Fig. 2. The activities are expressed relative to the control.

	Concentration (μM)	Activity (range)	No. of independent experiments
No addition	—	1.00	—
Adrenaline	50	1.31 (1.18–1.47)	4
Noradrenaline	12.5	1.17 (1.15–1.19)	3
Metanephrine	500	1.17 (1.14–1.21)	3
Dopa	25	1.17 (1.12–1.23)	3
Dopamine	50	1.08 (1.04–1.12)	3
Phenethylamine	500	1.03 (1.02–1.04)	2
Phenylethanolamine	500	1.03 (1.01–1.04)	2
Tyramine	500	1.01 (0.98–1.03)	3
Homogentisate	50	0.65 (0.53–0.80)	6

The effects of some other derivatives of tyrosine are shown in Table 2. Noradrenaline, metanephrine and dopamine also activated the enzyme, whereas phenethylamine, phenylethanolamine and tyramine had no effect. Oxaloacetate, fumarate, citrate, succinate and phenylalanine (at 0.5 mM concentration) had no effect.

The enzyme did not cross-react with antibodies against rat liver tyrosine aminotransferase in Ouchterlony double-diffusion experiments (Ouchterlony, 1949).

Fig. 4. *Lineweaver-Burk plot of form-2 enzyme for 2-oxoglutarate*

(a) and (b) represent the same experiment; the only difference is the scale. About 3 munits of enzyme were used per assay.

Fig. 5. *Lineweaver-Burk plot of form-2 enzyme for tyrosine*
About 3 munits of enzyme were used per assay.

Characteristics of form-2 enzyme

This form was obtained in much smaller amounts. It also had a mol.wt. of about 85000 and was specific for 2-oxoglutarate. Its cofactor requirements, the effects of canaline and amino-oxyacetate (Fig. 2) and kinetic properties (see below) were also as observed with form 1. Owing to the small amount recovered, we were unable to study this form any further.

Kinetic features

The reaction kinetics was found to be extremely complex. In Fig. 4, the double-reciprocal plot of form-2 enzyme for 2-oxoglutarate is displayed. There is a distinct plateau at about 0.2 mM-substrate concentration. Another plateau occurs at 0.6 mM-2-oxoglutarate. This plateau is not evident in the

same scale (Fig. 4a), but can be seen in the scale of Fig. 4b (both curves are plotted from the same experimental points). The plateaux or turning points have been seen in many separate and independent experiments with both the forms isolated in this work and also with less-purified preparations.

The double-reciprocal plot of form-2 enzyme for tyrosine is shown in Fig. 5. Here again, a constantly non-linear curve was obtained. A closer study of the form of the curve is hindered by the low solubility of the substrate. In the assay buffer, tyrosine is soluble up to 6–7 mM. On standing, however, a precipitate is formed even at this concentration. This tends to cause an upward inflexion at the left-side of the double-reciprocal plot, so that the form of the curve cannot be caused by this. The possibility that the serum albumin added to the assay might interfere with the kinetics was excluded by three independent lines of evidence. Firstly, the kinetics of less purified preparations that needed no albumin for stability was identical. Secondly, both forms separated in the present work did exhibit similar kinetics in the absence of albumin; these results, however, are not quite reliable, as the preparations were not quite stable without this addition and the substrates might have a stabilizing effect. Thirdly, albumin did not cause any aberrations from linearity in the $1/v$ versus $1/[S]$ plots of purified rat liver tyrosine aminotransferase.

Discussion

In the present report, a procedure is described for the isolation of hepatic tyrosine aminotransferase from frog liver. There are two additional procedures that could have been used in the purification. First, injection of tyrosine methyl ester or dibutyl cyclic AMP before killing can induce the enzyme in the frog (Ohisalo & Pispas, 1975). This was not done because

we do not know for certain that the induced enzyme is, in all respects, identical with the uninduced enzyme. Secondly, advantage could have been taken of the fact that the enzyme is highly resistant to heating in the presence of pyridoxal phosphate and 2-oxoglutarate (Ohisalo & Pispa, 1976). We do not think, however, that such methods should be used in the purification of enzymes, as their catalytic and structural properties may be strongly affected.

No differences were found in the molecular weights of the two enzyme forms isolated in the present work. This indicates that the heterogeneity of the enzyme is not due to its polymerization. As the forms had been eluted through a column of Ultrogel AcA-34 as a single peak before the chromatography on hydroxyapatite, it is also improbable that the different forms were polymers but lost their structural difference during or after hydroxyapatite column chromatography.

The activity of both forms was unaffected by added pyridoxal phosphate. They were identically inactivated by canaline and amino-oxyacetate. This is in agreement with the suggestion that the heterogeneity is not due to different amounts of cofactor bound to the enzyme. As all the properties of the two forms studied in this report proved to be identical, the molecular basis and physiological significance of the heterogeneity still remains obscure.

Both forms were different from the rat enzyme in their cofactor saturation (see Valeriote *et al.*, 1969). In this respect, they resemble the aspartate aminotransferases more closely. This finding is noteworthy, as it has been proposed that the short half-life of the rat enzyme *in vivo* is due to the dissociability of the cofactor (Litwack & Rosenfield, 1973). Reynolds & Thompson (1974) have reported a cysteine-dependent system that inactivates hepatic tyrosine aminotransferase in rat liver; according to their view, cysteine (or a related compound) is bound to the cofactor of the enzyme, which leads to its dissociation from the apoenzyme. The apoenzyme is then irreversibly inactivated and subsequently loses its antigenic properties. The frog enzyme was almost irreversibly inactivated by the pyridoxal phosphate reactants, which is in agreement with the theory described above.

The effect of homogentisate on the frog enzyme was quite different from the reported effect of the compound on rat liver tyrosine aminotransferase. The latter is irreversibly inactivated in the presence of homogentisate; this inactivation can be prevented by pyridoxal phosphate (Civen *et al.*, 1970). The frog enzyme was inhibited but not inactivated by homogentisate. As it is known that homogentisate forms a complex with pyridoxal phosphate (Civen *et al.*, 1970), it is possible that the difference is due to different dissociability of the cofactor. The physiological significance of the inhibition is obvious. If homo-

gentisate begins to accumulate, the transamination of tyrosine is slowed down unless there is an excess of tyrosine. The substrate is also known to increase the enzyme activity in frog liver when administered *in vivo* (Ohisalo & Pispa, 1975).

Adrenaline elevates hepatic tyrosine aminotransferase activity in rat liver (Wicks, 1968). This catecholamine activated the frog enzyme *in vitro*. Half-maximal enhancement was obtained by $3.5 \mu\text{M}$ -adrenaline. The physiological intracellular concentration of adrenaline, owing to its compartmentation and nature as an extracellularly functioning hormone, is not known exactly, but it is highly improbable that the concentration in the liver cell would ever be high enough to activate tyrosine aminotransferase to any marked extent. The effect is possibly related to the peculiar activation of the enzyme by its substrate tyrosine, which is the parent compound of the catecholamines.

The kinetic behaviour of the enzyme is of extreme complexity as revealed by Figs. 4 and 5. If we express the Fig. 4 curve as

$$y = f(x)$$

where $y = 1/v$ and $x = 1/[2\text{-oxoglutarate}]$, then, since there are two inflexions, the equation of the second differential

$$y'' = f''(x) = 0$$

has two positive real roots, which would mean that the original function is of at least fourth degree. Further discussion of the kinetics is beyond the scope of the present work. For physical interpretation of analogous kinetic anomalies the reader is referred to the article by Levitzki & Koshland (1969) on negative co-operativity in regulatory enzymes.

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