Organ Distribution of Rat Histidine–Pyruvate Aminotransferase Isoenzymes

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The organ distribution of rat histidine–pyruvate aminotransferase isoenzymes 1 and 2 was examined by using an isoelectric-focusing technique. Isoenzyme 1 (pI 8.0) is present only in the liver and its activity is increased by the injection of glucagon, whereas isoenzyme 2 (pI 5.2) is distributed in all tissues (liver, kidney, brain and heart) tested, and is not affected by glucagon injection. Isoenzyme 2 of the liver, kidney, brain and heart was purified by the same procedure and characterized. Isoenzyme 2 preparations from these four tissues were nearly identical in physical and enzymic properties. These properties differed from those previously found for the highly purified isoenzyme 1 preparation of rat liver. Isoenzyme 2 was active with pyruvate but not with 2-oxoglutarate as amino acceptor. Amino donors were effective in the following order of activity: tyrosine > histidine > phenylalanine > kynurenine > tryptophan. Very little activity was found with 5-hydroxytryptophan. The apparent K_m for histidine was about 0.45 mM. The K_m for pyruvate was about 4.5 mM with histidine as amino donor. The aminotransferase activities of isoenzyme 2 towards phenylalanine and tyrosine were inhibited by histidine. The ratio of aminotransferase activities towards these three amino acids was constant through gel filtration, electrophoresis, isoelectric focusing and sucrose-density-gradient centrifugation of the purified isoenzyme 2 preparations. These results suggest that these three activities are properties of the same enzyme protein. Sephadex G-150 gel filtration and sucrose-density-gradient centrifugation yielded mol.wts. of approx. 95000 and 92000 respectively. The pH optimum was between 9.0 and 9.3.

The presence of two distinct isoenzymes of histidine–pyruvate aminotransferase (EC 2.6.1.–) in rat liver has been documented. One is localized in the mitochondria; the other is restricted to the cytosol. Spolter & Baldridge (1964) reported that the two isoenzymes differed in K_m value for histidine and pyruvate, in pH profile and in heat lability. Morris et al. (1973) suggested that the mitochondrial and cytosol histidine–pyruvate aminotransferase are different proteins on the basis of the different responses of the two activities to hormones, and noting the findings of Spolter & Baldridge (1964). We reported that both the mitochondrial and supernatant fractions of rat liver contained two forms of histidine–pyruvate aminotransferase (Noguchi et al., 1976). One, designated isoenzyme 1, has a pI of 8.0 and is induced by the injection of glucagon; the other, designated isoenzyme 2, has a pI of 5.2 and is not affected by glucagon. The present report describes the organ distribution of rat isoenzymes 1 and 2, and their response to glucagon. Purification, characterization and identification of isoenzyme 2 from various tissues of rats are also described.

Experimental

Materials

L-Kynurenine was prepared by ozonolysis of L-tryptophan by the method of Warnell & Berg (1954). The following compounds were obtained from commercial sources: L-phenylalanine, L-tyrosine, L-histidine and L-tryptophan, from Tanabe Amino Acid Foundation, Osaka, Japan; sodium pyruvate and pyridoxal 5'-phosphate, from Nakarai Chemicals, Kyoto, Japan; monosodium 2-oxoglutarate and crystalline glucagon, from Sigma Chemical Co., St. Louis, MO, U.S.A.; 5-hydroxy-L-tryptophan, from Calbiochem, San Diego, CA, U.S.A.; DEAE-cellulose and hydroxyapatite, from Seikagaku Co., Tokyo, Japan; Sephadex G-150, from Pharmacia Fine Chemicals, Uppsala, Sweden; Ampholine carrier ampholytes for electrofocusing, from LKB Produkter AB, Stockholm, Sweden.

Methods

Aminotransferase assays with histidine, phenylalanine, tyrosine, tryptophan and 5-hydroxytrypto-
phan were based on the arsenate-catalysed formation of aromatic 2-oxo acid-enol borate complexes, which show characteristic absorption spectra in the 300 nm region (Lin et al., 1958). Details of these assays have been described (Noguchi et al., 1976). The assay mixtures (0.8 ml) contained, unless specified otherwise, l- amino acid (3 mm), 20 mm-pyruvate, 40 mm-pyridoxal 5'-phosphate, enzyme preparation and 0.2 M-Tris/ HCl, pH 9.2. Incubation was at 37°C. In the blank, pyruvate was added after incubation and inactivation.

Phenylalanine aminotransferase and tyrosine aminotransferase activities in the presence of histidine were determined as previously described (Noguchi et al., 1976).

Kynurenine aminotransferase was assayed by a minor modification of the method of Mason (1954). The assay mixture (0.8 ml) contained 3 mm-L-kynurenine, 20 mm-pyruvate, 40 mm-pyridoxal 5'-phosphate, enzyme preparation and 0.2 M-potassium phosphate buffer, pH 8.0. In the blank, pyruvate was added after the reaction was terminated. After incubation, the reaction was stopped by the addition of 0.2 ml of 25% (w/v) trichloroacetic acid. The mixture was centrifuged, and 0.5 ml of clear supernatant fluid was mixed with 2.5 ml of 0.5 M-potassium phosphate buffer, pH 7.5. The E_333 and E_565 of the solution were read against 0.5 M-potassium phosphate buffer, pH 7.5. These values were used to calculate the amount of kynureninic acid formed (Knox, 1953).

Enzyme activity was calculated from the linear part of the progress curve. A unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 mmol of product/h under the above conditions. Specific activity is defined as enzyme units per mg of protein. The apparent K_m values were determined from double-reciprocal plots of initial velocity and substrate concentration.

Catalase (H_2O_2→H_2O_2, oxidoreductase, EC 1.1.1.6) (Chance & Maehly, 1955), alcohol dehydrogenase (alcohol-NAD^+ oxidoreductase, EC 1.1.1.1) (Büttner, 1965) and monoamine oxidase [monoamine-oxygen oxidoreductase (deaminating), EC 1.4.3.4] (Tottmar et al., 1973) were assayed as described in the cited references.

The protein content was determined by the biuret method (Gornall et al., 1949) in crude extracts, or by the method of Lowry et al. (1951) in purified preparations. Bovine serum albumin was used in preparing a standard curve.

Polyacrylamide-disc-gel electrophoresis, isoelectric focusing and approximate molecular-weight determination by sucrose-density-gradient centrifugation and Sephadex G-150 gel filtration were carried out under the conditions previously described (Noguchi et al., 1976).

Results

Organ distribution of histidine-pyruvate aminotransferase isoenzymes in the rat

We have reported that the isoelectric focusing of rat liver extract resulted in the detection of two peaks of histidine-pyruvate aminotransferase activity with pI values 8.0 and 5.2 respectively (Noguchi et al., 1976). In the present work, the distribution of histidine-pyruvate aminotransferase isoenzymes in various rat tissues was examined by using an isoelectric-focusing technique. Two separated activity peaks with pI values 8.0 and 5.2 respectively were obtained with liver extract. However, only a single activity peak with a pI of 5.2 was obtained with any extract of the kidney, brain and heart. Histidine-pyruvate aminotransferase with a pI of 8.0 is referred to hereafter as 'isoenzyme 1' and that with a pI of 5.2 as 'isoenzyme 2'.

Response of histidine-pyruvate aminotransferase isoenzymes to the injection of rats with glucagon

We have previously reported that isoenzyme 1 activity, but not isoenzyme 2 activity, of rat liver was increased by the injection of glucagon (Noguchi et al., 1976). In the present study, the response in vivo to glucagon of isoenzymes 1 and 2 in various rat tissues was examined by using an isoelectric-focusing technique. Fig. 1 shows the focusing profiles of liver and kidney extracts of both control and glucagon-injected rats. In comparison with control rat liver, isoenzyme 1 of glucagon-treated rat liver showed an approx. 20-fold increase in total activity. However, isoenzyme 2 activities of the liver, kidney, brain and heart were not affected by glucagon injection. The profiles for brain and heart for both control and glucagon-injected rats did not differ significantly from those obtained for kidney (Fig. 1).

Purification of isoenzyme 2 from various rat tissues

Isoenzyme 1 has been highly purified from rat liver and characterized (Noguchi et al., 1976). In the present investigation, rat liver, kidney, brain and heart isoenzyme 2 were purified. With each tissue the same procedure was used. All operations were carried out at 0-4°C. Potassium phosphate buffer, pH 7.5, containing 200 mm-pyridoxal 5'-phosphate was used throughout.

Preparation of crude extracts. For this, 45 male rats (100-150 g body wt.) of the Donryu strain were killed by decapitation, and livers (225 g), brains (68 g), kidneys (56 g) and hearts (32 g) were removed. Each tissue was homogenized in a Waring blender for 2 min with 5 vol. of 5 mm buffer. After sonication for 4 min at 20 kHz with a Kubota sonicator (Tokyo, Japan), each homogenate was centrifuged at 105000 g for 30 min and the precipitate discarded.
Rats were housed in wire-bottomed cages and maintained at about 20°C in a room with a 12h light/12h dark cycle. Food and water were available ad libitum. Some were injected subcutaneously with glucagon suspended in 0.15% KCl (0.35mg, every 8h for 2 days). Control rats received 0.15% KCI every 12h for 2 days. Rats were decapitated with a guillotine and the livers, kidneys, brains and hearts were removed. Each tissue was homogenized in 5vol. of ice-cold 5mM-potassium phosphate buffer, pH7.5, in a Potter-Elvehjem tissue grinder with a Teflon pestle. After sonication at 20kHz for 4min, each homogenate was centrifuged at 105000g for 30min. Each resulting supernatant was separately subjected to isoelectric focusing on a pH3.5-10 Ampholine gradient in amounts corresponding to 1g of liver, 0.5g of kidney, 2.0g of brain and 2.0g of heart respectively. Fractions (2ml) were collected. pH values (○) and histidine-pyruvate aminotransferase activities (●) were determined as described in the text. The Figure shows focusing profiles of (a) liver and (b) kidney extracts from a control rat and those of (c) liver and (d) kidney extracts from a glucagon-injected rat. Profiles for brain and heart extracts resembled those shown for kidney.

**(NH₄)₂SO₄ fractionation.** To the supernatant solid (NH₄)₂SO₄ was added, with gentle stirring, to 30% saturation. After 30min, the precipitate was removed by centrifugation at 5000g for 20min and discarded. (NH₄)₂SO₄ was added to the supernatant to 60% saturation, and the precipitate was collected by centrifugation at 5000g for 20min and dissolved in 5mM buffer.

**Heat treatment.** The enzyme solution was diluted to a protein concentration of about 10mg/ml. Pyridoxal 5'-phosphate and pyruvate were added to final concentrations of 0.2 and 2mM respectively. The solution was warmed rapidly to 70°C and maintained at this temperature, with constant stirring, for 15s, after which it was quickly chilled in a 0.9% NaCl/ice bath at −7°C. The precipitate was removed by centri-
fugation at 5000g for 20 min and solid (NH₄)₂SO₄
was added to the supernatant to 65% saturation.
After centrifugation at 5000g for 20 min, the precipi-
tate was dissolved in 5 mM buffer and desalted by dia-
ysis against the same buffer overnight. The inactive precipitate formed during dialysis was removed by
centrifugation at 10000g for 1 h.

**DEAE-cellulose chromatography.** The non-diffus-
ible solution was applied to a column (3.5 cm x 7 cm; or 6.5 cm x 7 cm for the liver enzyme preparation)
equilibrated with 5 mM buffer. The column was
washed sequentially with 200 ml (500 ml for liver enzyme preparation) each of 5 mM and then 15 mM
buffer, and then the enzyme was eluted with 50 mM
buffer. The active fractions were pooled and concen-
trated by ultracentrifugation.

**Isoelectric focusing.** The concentrated enzyme solution was subjected to isoelectric focusing on a
pH 4.6 Ampholine gradient. The focusing resulted in
the detection of a single peak of activity with a pI of
5.2 with enzyme preparation from each of the four sources. The active fractions were pooled and concen-
trated by ultracentrifugation.

**Sephadex G-150 gel filtration.** The concentrated enzyme solution was added to a column (2.5 cm x
100 cm) of Sephadex G-150, which had been
equilibrated with 50 mM buffer. The column was
equilibrated with the same buffer at a flow rate of 25 ml/h.
The effluent was collected in 3 ml fractions. The active fractions were pooled, and concentrated by ultra-
centrifugation.

Results of typical purifications of isoenzyme 2
preparations from the liver, kidney, brain and heart of
rats are shown in Table 1. Isolezyme 2 activity was
purified 44.7-fold over the liver extract, 142-fold over the
kidney extract, 437-fold over the brain extract and
131-fold over the heart extract. Liver isoenzyme 2
activity especially was obtained with a poor apparent
percentage recovery, owing to the removal of an addi-
tional histidine–pyruvate aminotransferase iso-
zyme 1 (with a pI of 8.0) during purification. Purified isoenzyme 2 preparations from the kidney, brain and heart were of nearly identical specific activity. The liver preparation had a lower specific activity.

The purified enzyme preparations (in 50 mM-
potassium phosphate buffer, pH 7.5, containing
200 μM-pyridoxal 5'-phosphate) from the liver,
kidney, brain and heart may be stored frozen at –20°C.

<p>| Table 1. Purification of histidine–pyruvate aminotransferase (isoenzyme 2) from rat liver, kidney, brain and heart |
|------------------|------------------|------------------|------------------|
| Details of purification and assay methods are given in the text. L, Liver; K, kidney; B, brain; H, heart. |</p>
<table>
<thead>
<tr>
<th>Total activity (units)</th>
<th>Specific activity (units/mg of protein)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>L</td>
<td>K</td>
<td>B</td>
</tr>
<tr>
<td>18977</td>
<td>3234</td>
<td>2223</td>
<td>3195</td>
</tr>
<tr>
<td>11888</td>
<td>1670</td>
<td>569</td>
<td>285</td>
</tr>
<tr>
<td>104</td>
<td>23</td>
<td>3.4</td>
<td>3.2</td>
</tr>
<tr>
<td>8.6</td>
<td>22</td>
<td>0.27</td>
<td>0.42</td>
</tr>
</tbody>
</table>

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for at least 6 weeks without loss of activity towards histidine, phenylalanine and tyrosine.

Criteria of purity

On polyacrylamide-disc-gel electrophoresis at pH 8.9 in 7% gel, a major protein component which represented about 70–80% of total protein, and two or three other protein bands, were obtained with isoenzyme 2 preparations from the kidney, brain or heart. Unstained gels run in parallel were sliced into 2mm sections and added to the assay system for determination of activities; the major protein band and all the measured aminotransferase activities, i.e. histidine, phenylalanine or tyrosine with pyruvate, were coincident. Electrophoresis of isoenzyme 2 preparation from the liver revealed multiple protein bands, one of which coincided with all three aminotransferase activities. Isoenzyme 2 preparations from these four tissues showed the same mobility of 0.50 (relative to that of a Bromophenol Blue marker, Fig. 2), which differs from that (0.19) showed by the purified rat liver isoenzyme 1 previously described (Noguchi et al., 1976). With all isoenzyme 2 preparations, the ratio of aminotransferase activities towards histidine, phenylalanine and tyrosine was unchanged after electrophoresis.

With all the isoenzyme 2 preparations, Sephadex G-150 gel filtration (for molecular-weight determinations) gave a single symmetrical peak, with protein and all three aminotransferase activities coincident. Similarly sedimentations in a sucrose density gradient (for molecular-weight determination) showed a single peak for protein and the three aminotransferase activities.

Properties of isoenzyme 2 from various rat tissues

Isoenzyme 2 preparations from the liver, kidney, brain and heart of rats had nearly identical pH optima, isoelectric points, molecular weights and substrate specificities. Isoenzyme 2 differed in these properties from isoenzyme 1 previously purified in this laboratory from rat liver (Noguchi et al., 1976).

pH optimum. Aminotransferase activities of purified isoenzyme 2 towards histidine, phenylalanine and tyrosine were investigated as a function of

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Fig. 2. Polyacrylamide-disc-gel electrophoresis of isoenzymes 1 and 2

Each purified isoenzyme 2 preparation was applied to three gels, which were used for assays of histidine-pyruvate aminotransferase (○), tyrosine-pyruvate aminotransaminase (△) and phenylalanine-pyruvate aminotransaminase (△) respectively. Only histidine-pyruvate aminotransferase was assayed with liver isoenzyme 1 preparation. After electrophoresis, the gels were cut into 2mm slices and these were added separately to the assay mixture. The Figure shows electrophoretic profiles of (a) liver isoenzyme 1 (7μg), (b) liver isoenzyme 2 (70μg), (c) kidney isoenzyme 2 (27μg), (d) brain isoenzyme 2 (28μg) and (e) heart isoenzyme 2 (30μg).
Table 2. Relative activities of isoenzyme 2 for various aromatic amino acids

Assay conditions were as described in the text. The final concentration of each L-amino acid and pyruvate were 3.0 and 20 mM respectively. Relative activity values are given, signifying transamination rates compared with that for L-histidine. N.D., Not detected.

<table>
<thead>
<tr>
<th>L-Amino acid</th>
<th>Organ</th>
<th>Liver</th>
<th>Kidney</th>
<th>Brain</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine</td>
<td>1.2</td>
<td>1.3</td>
<td>1.1</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.65</td>
<td>0.70</td>
<td>0.72</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>1</td>
<td>1</td>
<td>0.17</td>
<td>0.17</td>
<td>0.19</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.17</td>
<td>0.16</td>
<td>0.41</td>
<td>0.55</td>
<td>0.55</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>0.42</td>
<td>N.D.</td>
<td>0.55</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5-Hydroxytryptophan</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pH over the range 7.0–9.6. Potassium phosphate buffer was used between pH 7.0 and 8.0, and Tris/HCl between pH 8.0 and 9.6. All three aminotransferase activities with pyruvate showed nearly identical pH profiles, showing pH optima between 9.0 and 9.3.

Isoelectric point. Purified isoenzyme 2 was subjected to isoelectric focusing on a pH 4–6 Ampholine gradient. The focusing resulted in the detection of a single activity peak with a pI of 5.2, possessing aminotransferase activity towards histidine, phenylalanine and tyrosine with pyruvate. The activity ratio of isoenzyme 2 towards these three amino acids was unchanged after the focusing.

Determination of approximate molecular weight. Sucrose-density-gradient centrifugation gave a mol wt. of about 92000 with bovine catalase ($s_{20, w} = 11.3\text{S}$) as internal standard. The mol wt. was also estimated as about 95000 by Sephadex G-150 gel filtration.

Substrate specificity. With L-histidine, L-phenylalanine and L-tyrosine, isoenzyme 2 from each of the four sources was specific for pyruvate, exhibiting no measurable activity with 2-oxoglutarate. The relative initial velocities of isoenzyme 2 with various L-amino acids (3 mM) were determined by using pyruvate (20 mM) as amino acceptor. Results are summarized in Table 2. Isoenzyme 2 utilized a wide range of amino acids. These amino acids were effective as amino donors in the following order of activity: tyrosine > histidine > phenylalanine > kynurenine > tryptophan. Very little activity was detected with 5-hydroxytryptophan. The apparent $K_m$ values for histidine with pyruvate at 20 mM were 0.45, 0.40, 0.42 and 0.44 mM for isoenzyme 2 preparations from the liver, kidney, brain and heart respectively. Corresponding $K_m$ values for pyruvate with L-histidine at 3 mM were 4.0, 4.7, 4.2 and 4.5 mM respectively. Aminotransferase activities towards phenylalanine (3 mM) and tyrosine (3 mM) with 20 mM-pyruvate were inhibited by about 30 and 45% respectively by the addition of 3 mM-L-histidine with all four isoenzyme 2 preparations. These results suggest that transamination of histidine, phenylalanine and tyrosine with pyruvate is catalysed by a single enzyme protein.
Elution patterns of the enzyme from hydroxyapatite and DEAE-cellulose columns are identical with those of isoenzyme 2. Moreover we have applied an isoelectric-focusing technique to extracts of rat liver, kidney, brain and heart and detected a single kynurenine-pyruvate aminotransferase activity peak with a pI of 5.2 that has also histidine- and phenylalanine-pyruvate aminotransferase activities; no kynurenine-pyruvate activity was observed from the liver extract in the isoenzyme 1 (pI = 8.0) position (not detailed in the Results section). On the basis of these observations, it is suggested that kynurenine-pyruvate aminotransferase is also identical with isoenzyme 2.

References