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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 300nm region (Lin *et al.*, 1958). Details of these assays have been described (Noguchi *et al.*, 1976). The assay mixtures (0.8ml) contained, unless specified otherwise, L amino acid (3mM), 20mM-pyruvate, 40μM-pyridoxal 5'-phosphate, enzyme preparation and 0.2M-Tris/HCl, pH9.2. 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 (0.8ml) contained 3mM-L-kynurenine, 20mM-pyruvate, 40μM-pyridoxal 5'-phosphate, enzyme preparation and 0.2M-potassium phosphate buffer, pH8.0. In the blank, pyruvate was added after the reaction was terminated. After incubation, the reaction was stopped by the addition of 0.2ml of 25% (w/v) trichloroacetic acid. The mixture was centrifuged, and 0.5ml of clear supernatant fluid was mixed with 2.5ml of 0.5M-potassium phosphate buffer, pH7.5. The E_{333} and E_{365} of the solution were read against 0.5M-potassium phosphate buffer, pH7.5. These values were used to calculate the amount of kynurenic 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 1μmol 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, EC1.11.1.6) (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, pH7.5, containing 200μM-pyridoxal 5'-phosphate was used throughout.

Preparation of crude extracts. For this, 45 male rats (100–150g body wt.) of the Donryu strain were killed by decapitation, and livers (225g), brains (68g), kidneys (56g) and hearts (32g) were removed. Each tissue was homogenized in a Waring blender for 2min with 5vol. of 5mM buffer. After sonication for 4min at 20kHz with a Kubota sonicator (Tokyo, Japan), each homogenate was centrifuged at 105000*g* for 30min and the precipitate discarded.

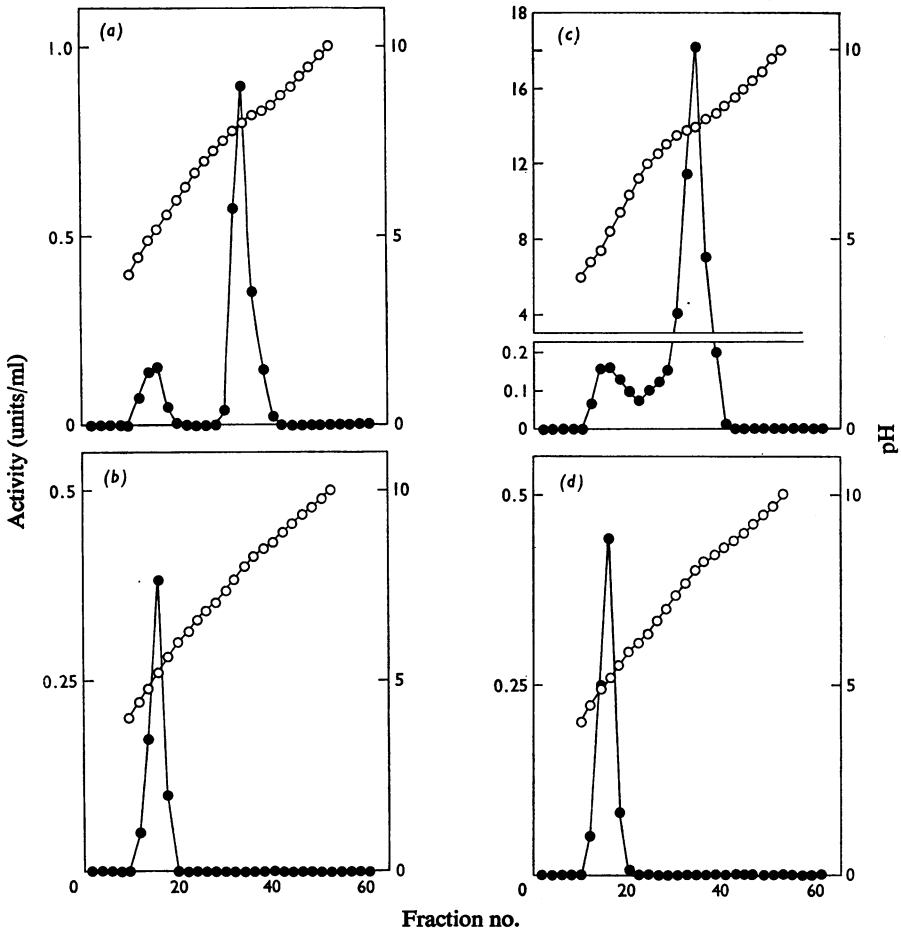

Fig. 1. *Isoelectric-focusing profiles of tissue extracts of both control and glucagon-injected rats*

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% KCl 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 105000*g* 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.

$(NH_4)_2SO_4$ fractionation. To the supernatant solid $(NH_4)_2SO_4$ was added, with gentle stirring, to 30% saturation. After 30min, the precipitate was removed by centrifugation at 5000*g* for 20min and discarded. $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation, and the precipitate was collected by centrifugation at 5000*g* 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 5000*g* for 20min and solid $(NH_4)_2SO_4$ was added to the supernatant to 65% saturation. After centrifugation at 5000*g* for 20min, the precipitate was dissolved in 5mM buffer and desalted by dialysis against the same buffer overnight. The inactive precipitate formed during dialysis was removed by centrifugation at 10000*g* for 1h.

DEAE-cellulose chromatography. The non-diffusible solution was applied to a column (3.5cm×7cm; or 6.5cm×7cm for the liver enzyme preparation) equilibrated with 5mM buffer. The column was washed sequentially with 200ml (500ml for liver enzyme preparation) each of 5mM and then 15mM buffer, and then the enzyme was eluted with 50mM buffer. The active fractions were pooled and concentrated by Diaflo ultrafiltration (Bio-engineering Co., Tokyo, Japan) by using a G10T filter, and diluted with water to adjust the buffer concentration to 5mM.

Hydroxyapatite chromatography. The concentrated enzyme solution was applied to a hydroxyapatite column (3cm×5cm) previously equilibrated with 5mM buffer and the enzyme was eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration.

Isoelectric focusing. The concentrated enzyme solution was subjected to isoelectric focusing on a pH4–6 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 concentrated by ultrafiltration.

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

Results of typical purifications of isoenzyme 2 preparations from the liver, kidney, brain and heart of rats are shown in Table 1. Isoenzyme 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 additional histidine–pyruvate aminotransferase isoenzyme 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 50mM-potassium phosphate buffer, pH7.5, containing 200μM-pyridoxal 5′-phosphate) from the liver, kidney, brain and heart may be stored frozen at −20°C

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.

	Total protein (mg)				Total activity (units)				Specific activity (units/mg of protein)				Recovery (%)				Purification (fold)			
	L	K	B	H	L	K	B	H	L	K	B	H	L	K	B	H	L	K	B	H
Extract	18797	3234	2223	336	3195	420	89	44	0.17	0.13	0.04	0.13	100	100	100	100	1	1	1	1
$(NH_4)_2SO_4$ fractionation	11888	1630	569	285	1902	424	46	37	0.16	0.26	0.08	0.13	60	101	51	85	0.9	2	2	1
Heat treatment	1302	336	101	36	1953	403	40	23	1.5	1.2	0.40	0.64	61	96	45	53	8.8	9.2	10.0	4.9
DEAE-cellulose	353	76	11	9.0	812	175	16	12	2.3	2.3	1.4	1.3	25	42	18	27	13.5	17.7	35.0	10.0
Hydroxyapatite	104	23	3.4	3.2	135	97	9.9	12	1.3	4.2	2.9	3.6	4.2	23	11	26	7.6	32.3	72.5	27.7
Isoelectric focusing and Sephadex G-150 gel filtration	8.6	2.2	0.27	0.42	65.4	41	4.7	7.1	7.6	18.5	17.5	17.0	2.0	9.7	5.3	16	44.7	142.0	437	131

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

Criteria of purity

On polyacrylamide-disc-gel electrophoresis at pH8.9 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

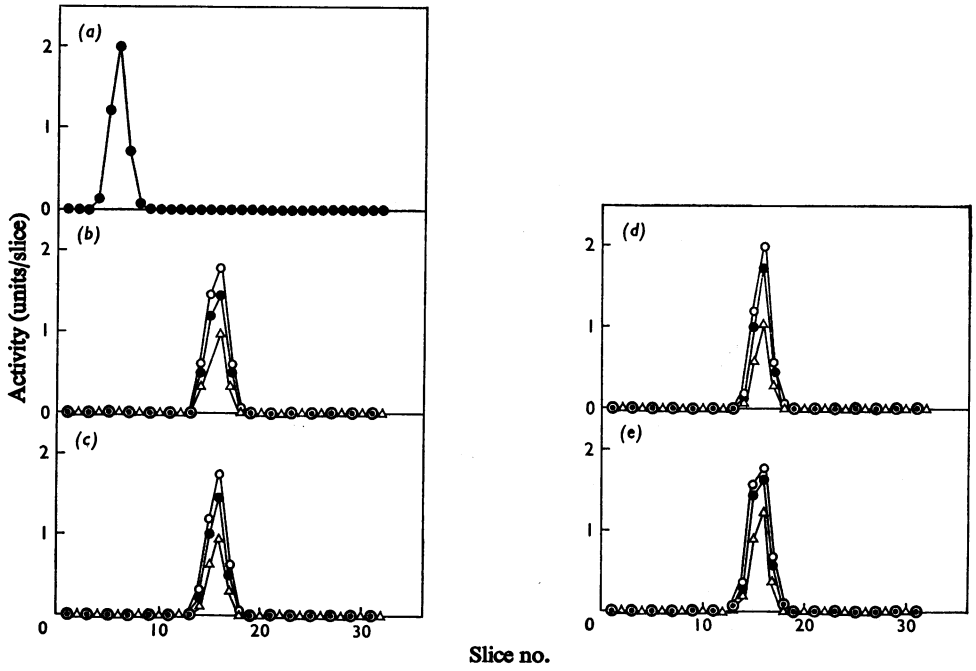

Fig. 2. *Polyacrylamide-disc-gel electrophoresis of isoenzymes* 1 *and* 2

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 (7 μg), (*b*) liver isoenzyme 2 (70 μg), (*c*) kidney isoenzyme 2 (27 μg), (*d*) brain isoenzyme 2 (28 μg) and (*e*) heart isoenzyme 2 (30 μg).

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 20mM respectively. Relative activity values are given, signifying transamination rates compared with that for L-histidine. N.D., Not detected.

	Relative isoenzyme 2 activity			
L-Amino acid Organ ...	Liver	Kidney	Brain	Heart
Tyrosine	1.2	1.3	1.1	1.3
Phenylalanine	0.65	0.70	0.72	0.72
Histidine	1	1	1	1
Tryptophan	0.17	0.16	0.17	0.19
Kynurenine	0.42	0.41	0.55	0.55
5-Hydroxytryptophan	N.D.	N.D.	N.D.	N.D.

pH over the range 7.0–9.6. Potassium phosphate buffer was used between pH7.0 and 8.0, and Tris/HCl between pH8.0 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 pH4–6 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 ($s_{20,w}$ = 11.3S) 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 (3mM) were determined by using pyruvate (20mM) 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 20mM were 0.45, 0.40, 0.42 and 0.44mM for isoenzyme 2 preparations from the liver, kidney, brain and heart respectively. Corresponding K_m values for pyruvate with L-histidine at 3mM were 4.0, 4.7, 4.2 and 4.5mM respectively. Aminotransferase activities towards phenylalanine (3mM) and tyrosine (3mM) with 20mM-pyruvate were inhibited by about 30 and 45% respectively by the addition of 3mM-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.

Discussion

The present paper describes the organ distribution and response to glucagon of rat histidine–pyruvate aminotransferase isoenzymes 1 and 2, with pI values of 8.0 and 5.2 respectively. Isoenzyme 1 is present only in the liver but not in the kidney, brain and heart, whereas isoenzyme 2 is present in all these tissues. Isoenzyme 1 is responsive to glucagon, but not isoenzyme 2. Physical and enzymic properties of rat liver isoenzyme 1 have been described for the highly purified enzyme preparation, but for rat liver isoenzyme 2 only with the crude enzyme preparation (Noguchi *et al.*, 1976). In the present study, isoenzyme 2 from the liver, kidney, brain and heart of rat was highly purified and characterized by identical procedures. Isoenzyme 2 preparations from these four tissues were remarkably similar in isoelectric point, pH optimum, molecular weight, electrophoretic migration, activity ratio for various aromatic amino acids, K_m values for histidine and pyruvate, and degree of inhibition of phenylalanine and tyrosine aminotransferase activities by histidine. These results suggest the identity of isoenzyme 2 preparations from these four tissues. In each case the activity ratio of isoenzyme 2 towards histidine, phenylalanine and tyrosine was constant after electrophoresis, gel filtration, isoelectric focusing and sucrose-density-gradient centrifugation, showing that these activities are properties of the same enzyme protein.

Isoenzyme 2 was found to catalyse transamination between kynurenine and pyruvate. Kynurenine–pyruvate aminotransferase is found in the liver (Nakatani *et al.*, 1974), kidney (Noguchi *et al.*, 1975), brain (Minatogawa *et al.*, 1973) and small intestine (Noguchi *et al.*, 1973; Nakamura *et al.*, 1973). This enzyme is also specific for pyruvate as amino acceptor, exhibiting no measurable activity with 2-oxoglutarate.

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.

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