Crystallization and characterization of human liver kynurenine–glyoxylate aminotransferase

IDENTITY WITH ALANINE–GLYOXYLATE AMINOTRANSFERASE AND SERINE–PYRUVATE AMINOTRANSFERASE

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Kynurenine–glyoxylate aminotransferase, alanine–glyoxylate aminotransferase and serine–pyruvate aminotransferase were co-purified and crystallized as yellow cubes from human liver particulate fraction. The crystalline enzyme was homogeneous by the criteria of electrophoresis, isoelectric focusing, gel filtration, sucrose-density-gradient centrifugation and analytical ultracentrifugation. The molecular weight of the enzyme was calculated as approx. 90 000, 89 000 and 99 000 by the use of gel filtration, analytical ultracentrifugation and sucrose-density-gradient centrifugation respectively, with two identical subunits. The enzyme has a $s_{20,w}$ value of 5.23 S, an isoelectric point of 8.3 and a pH optimum between 9.0 and 9.5. The enzyme solution showed absorption maxima at 280 and 420 nm. The enzyme catalysed transamination between several L-amino acids and pyruvate or glyoxylate. The order of effectiveness of amino acids was alanine > serine > glutamine > glutamate > methionine > kynurenine = phenylalanine = asparagine > valine > histidine > lysine > leucine > isoleucine > arginine > tyrosine = threonine > aspartate, with glyoxylate as amino acceptor. The enzyme was active with glyoxylate, oxaloacetate, hydroxypyruvate, pyruvate, 4-methylthio-2-oxobutyrates and 2-oxobutyrates, but showed little activity with phenylpyruvate, 2-oxoglutarate and 2-oxoadipate, with kynurenine as amino donor. Kynurenine–glyoxylate aminotransferase activity was competitively inhibited by the addition of L-alanine or L-serine. From these results we conclude that kynurenine–glyoxylate aminotransferase, alanine–glyoxylate aminotransferase and serine–pyruvate aminotransferase activities of human liver are catalysed by a single protein. Kinetic parameters for the kynurenine–glyoxylate aminotransferase, alanine–glyoxylate aminotransferase, serine–pyruvate aminotransferase and alanine–hydroxypyruvate aminotransferase reactions of the enzyme are presented.

Kynurenine aminotransferase (EC 2.6.1.7, L-kynurenine–2-oxoglutarate aminotransferase) catalyses the formation of kynurenic acid from kynurenine. Several enzyme species that catalyse the transamination of kynurenine have been reported in mammalian species (Noguchi *et al.*, 1975; Noguchi & Kido, 1976; Tobes & Mason, 1977, 1978). We have previously reported the purification and characterization of aromatic amino acid–glyoxylate aminotransferase from monkey and rat liver, which also catalyses the transamination between kynurenine and glyoxylate (Harada *et al.*, 1978). To

our knowledge there has been no report of kynurenine aminotransferase in humans.

Alanine–glyoxylate aminotransferase was partially purified from human liver, and the possible involvement of this enzyme in primary hyperoxaluria of oxalosis was discussed (Thompson & Richardson, 1967). The enzyme was highly purified and characterized from rabbit liver (Feld & Sallach, 1973) and rat, mouse, cat and dog liver (Noguchi *et al.*, 1978a). The enzyme is distributed in a wide range of animal species, and relatively high activity is associated with carnivorous species (cat, dog,

common frog, common toad, salamander and roach) (RowSELL *et al.*, 1969), suggesting the association of the enzyme with gluconeogenesis from amino acids (RowSELL *et al.*, 1969, 1972a; Snell, 1971; Snell & Walker, 1972). We have suggested that alanine-glyoxylate aminotransferase isoenzyme 1 of rat, mouse, cat and dog liver is identical with serine-pyruvate aminotransferase (Noguchi *et al.*, 1978a). In the present paper, it is reported that kynurenine aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activities are co-purified as a single protein from human liver particulate fraction.

Experimental

Materials

Human liver was obtained from autopsy 4–5 h post mortem of a 65-year-old man who died of lung cancer, which metastasized to the brain but not to the liver, and was stored at -20°C until used. L-Kynurenine was prepared by ozonolysis of L-tryptophan by the method of Warnell & Berg (1954). 4-Methylthio-2-oxobutylate, 2-oxoadipate, sodium hydroxypyruvate, sodium phenylpyruvate, lactate dehydrogenase (L-lactate- NAD^{+} oxidoreductase, EC 1.1.1.27) and D-glycerate dehydrogenase (D-glycerate- NAD^{+} oxidoreductase, EC 1.1.1.29) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and DEAE-cellulose and hydroxyapatite were from Seikagaku Kogyo Co., Tokyo, Japan. Sephadex G-150 was from Pharmacia Fine Chemicals, Uppsala, Sweden, and Ampholine carrier ampholytes were from LKB-Product AB, Stockholm, Sweden. All other chemicals used were commercial products of the highest grade available.

Enzyme assays

Enzyme assays were done under conditions giving linear progress curves and where the initial rate was proportional to enzyme concentration. Incubation of the assay of alanine-glyoxylate aminotransferase activity was for 10 min at 37°C after 5 min preincubation, and those of other aminotransferase activities were for 60 min at 37°C .

Kynurenine aminotransferase activity was in principle assayed by the method of Mason (1954). The reaction mixture contained, unless specified otherwise, 3 mM-L-kynurenine, 5 mM-2-oxo acid, 40 μM -pyridoxal 5'-phosphate, enzyme preparation and 0.15 M-potassium phosphate buffer, pH 8.0, in a total volume of 100 μl . The reaction was started by the addition of 2-oxo acid. After incubation, 7 μl of 50% (w/w) trichloroacetic acid was added. In the blank, 2-oxo acid was added just before the addition of trichloroacetic acid after incubation. Kynurenic acid formed was determined spectrophotometrically

as previously described (Knox, 1953; Harada *et al.*, 1978).

Other aminotransferase assays. The assay mixture contained, unless specified otherwise, 40 mM-L-amino acid, 5 mM-2-oxo acid, 40 μM -pyridoxal 5'-phosphate, 0.15 M-potassium phosphate buffer, pH 8.0, and enzyme preparation in a total volume of 0.4 ml.

Transamination between alanine or serine and glyoxylate was in principle assayed by the method of RowSELL *et al.* (1972b). Pyruvate or hydroxypyruvate formed was determined in the presence of Tris by using lactate dehydrogenase or spinach D-glycerate dehydrogenase and NADH respectively. In the presence of Tris, these two 2-oxo acids formed are substrates for lactate dehydrogenase and D-glycerate dehydrogenase, but glyoxylate is not. Details of the assay have been described (Noguchi *et al.*, 1978a).

Transamination between serine and pyruvate was assayed as described by Snell & Walker (1974). Hydroxypyruvate formed was determined with NADH and spinach D-glycerate dehydrogenase as described previously (Noguchi *et al.*, 1978a).

Transamination between aromatic amino acids (except kynurenine) and 2-oxo acid (glyoxylate or pyruvate) was in principle determined by the methods of Lin *et al.* (1958) and Nakano (1967). This assay is based on the arsenate-catalysed formation of aromatic 2-oxo acid enol-borate complexes. Details of this assay have been described (Noguchi *et al.*, 1976a).

Transamination between aspartate and glyoxylate was determined by the formation of oxaloacetate. Oxaloacetate formed was determined in the presence of Tris by using 0.25 unit of malate dehydrogenase, 0.5 unit of lactate dehydrogenase and NADH. Because of the spontaneous conversion of oxaloacetate into pyruvate, lactate dehydrogenase was used together with malate dehydrogenase for the determination of oxaloacetate as in the assay of citrate synthase by Moellering & Gruber (1966). In the presence of Tris, pyruvate formed is a substrate for lactate dehydrogenase, but glyoxylate is not.

Transamination between other amino acids, including branched-chain amino acids and glyoxylate or pyruvate, was determined by measuring the amount of unchanged glyoxylate or pyruvate with lactate dehydrogenase and NADH as described in principle by Noguchi *et al.* (1978a) for the assay of the transamination between glycine and pyruvate.

A unit of enzyme activity is defined as the amount of enzyme that catalyses a formation of product or a decrease in substrate of 1 $\mu\text{mol/h}$ at 37°C . Specific activity is expressed as enzyme units per mg of protein.

Protein was determined by the method of Lowry

et al. (1951). Bovine serum albumin was used in preparing a standard curve.

Polyacrylamide-disc-gel electrophoresis

Electrophoresis was performed in a continuous 7% polyacrylamide-gel column (0.5 cm × 8 cm) containing 0.184% bisacrylamide, by the original procedure of Davis (1964). A Tris/glycine buffer, pH 8.9 (0.6 g of Tris and 2.8 g of glycine/litre) was used as the running buffer, and Bromophenol Blue was used as tracking dye. Electrophoresis was carried out at 4°C for 180 min at a current of 2.0 mA/column; the sample was applied in 5% (w/v) sucrose solution (dissolved in the running buffer). For enzyme assay immediately after electrophoresis, the gel was cut into 2 mm slices, which were rinsed in 400 µl of 5 mM-potassium phosphate buffer, pH 7.5, containing 40 µM-pyridoxal 5'-phosphate overnight at 4°C.

Polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969). The following marker proteins were used for the estimation of the subunit molecular weight of the crystallized enzyme: bovine serum albumin (mol.wt. 67 000), ovalbumin (43 000), bovine liver alcohol dehydrogenase (41 000), carboxypeptidase A (34 600), trypsin (23 000) and cytochrome *c* (12 380).

Approximate molecular-weight determination by sucrose-density-gradient centrifugation

The method of Martin & Ames (1961) was used, with a sucrose gradient (5–20%, w/v) in 50 mM-potassium phosphate buffer, pH 7.5. Centrifugation was carried out at 100 000 *g* with a RPS-50 swinging-bucket rotor in a Hitachi 65P centrifuge (Hitachi, Tokyo, Japan) at 4°C for 16 h. Fractions (three drops) were collected by using a Mitsumi gradienter SJ-1300UD (Mitsumi Scientific Industry Co., Tokyo, Japan) and analysed for enzyme activity. Bovine catalase was used as internal standard, with an assumed mol.wt. of 250 000 (Martin & Ames, 1961).

Analytical ultracentrifugation

Analytical ultracentrifugation was performed with a Hitachi Analytical Ultracentrifuge UCA-1A instrument. Sedimentation coefficient was determined by standard procedures (Schachman, 1957) at 51 200 rev./min at 20°C. Photographs were taken at 6-min intervals. According to Martin & Ames (1961), a crude estimation of the molecular weight (MW) can be obtained from the sedimentation coefficient alone:

$$\frac{s_1}{s_2} = \left(\frac{MW_1}{MW_2} \right)^{2/3}$$

with yeast alcohol dehydrogenase, with $s_{20,w} = 7.4$ S and mol.wt. 150 000, as standard (Martin & Ames, 1961).

Analytical gel filtration

Gel filtration on a Sephadex G-150 column (2.5 cm × 100 cm) was calibrated with the following proteins of known molecular weight: ovalbumin (43 000), bovine serum albumin (67 000), bovine liver alcohol dehydrogenase (83 000), yeast alcohol dehydrogenase (150 000) and catalase (250 000). Elution profiles of these marker proteins from the column were detected by measurement of either A_{280} or enzyme activity. Elution volumes were plotted against the logarithm of the respective molecular weights (Andrews, 1964).

Preparation of apoenzyme

Apoenzyme was prepared by acid dialysis treatment and (NH₄)₂SO₄ precipitation. The crystallized enzyme (about 0.5 mg of protein) was dissolved in 0.05 M-potassium phosphate buffer, pH 7.5. L-Alanine (40 mM final concn.) was added to the enzyme solution, and then the enzyme solution was dialysed against 1000 ml of 0.05 M-potassium phosphate buffer, pH 6.2, for 10 h at 4°C in a collodion bag. After dialysis the preparation was concentrated to its original volume *in vacuo* in the collodion bag. This procedure was repeated twice. After the acid-dialysis treatment, (NH₄)₂SO₄ powder was added to the enzyme solution to produce the enzyme precipitate. After centrifugation at 10 000 *g* for 30 min, the supernatant was discarded and the precipitate was dissolved in 0.05 M-potassium phosphate buffer, pH 7.5. The enzyme activity was almost completely (98.5%) lost in the absence of pyridoxal 5'-phosphate.

Results

Co-purification and crystallization of human liver kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase

All manipulations were carried out at 0–4°C unless stated otherwise. Glass-distilled water and potassium phosphate buffer, pH 7.5, containing 10 mM-mercaptoethanol and 200 µM-pyridoxal 5'-phosphate were used throughout. Kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activities were always recovered in the same fraction through the purification steps.

Frozen human liver (150 g) was thawed and cut into small pieces and homogenized in 5 vol. of 0.25 M-sucrose (dissolved in water) in a Potter-Elvehjem homogenizer by two strokes of a Teflon

pestle. The homogenate was centrifuged at 10000g for 20 min and the resulting pellet was washed once with 0.25 M-sucrose and then suspended in 5 mM buffer. After sonication for 4 min with Branson Sonifier B-12 (Branson Sonic Power Co., Danbury, CT, U.S.A.), the suspension was centrifuged at 100000g for 30 min and the precipitate was discarded.

Heat treatment. The resulting supernatant was warmed rapidly to 65°C and maintained at this temperature with constant stirring for 1 min. Then it was quickly chilled in an ice/water bath, and the precipitate was removed by centrifugation at 10000g for 30 min.

(NH₄)₂SO₄ fractionation. To the supernatant, solid (NH₄)₂SO₄ was added with gentle stirring to 35% saturation. After 30 min, the precipitate was removed by centrifugation at 10000g for 30 min and discarded. (NH₄)₂SO₄ was added to the supernatant to 75% saturation. After 30 min the precipitate was collected by centrifugation (10000g for 30 min) and dissolved in 5 mM buffer. The enzyme solution was desalted by dialysis against the same buffer solution overnight.

DEAE-cellulose chromatography. The dialysed enzyme solution was applied to a column (5 cm × 8 cm) equilibrated with 5 mM buffer. Pyridoxal 5'-phosphate was omitted from the elution buffer. The enzyme was eluted with 5 mM buffer.

Hydroxyapatite chromatography. The enzyme solution was directly applied to a hydroxyapatite column (2.5 cm × 7 cm) equilibrated with 5 mM buffer. After washing with 100 ml of 5 mM buffer, the enzyme was eluted with a linear gradient formed from 500 ml of 5 mM buffer and 500 ml of 250 mM buffer. The active fractions were pooled and concentrated by ultrafiltration with a Diaflo apparatus (Amicon Far East Ltd., Tokyo, Japan) with a YM10 filter.

Sephadex G-150 gel filtration and crystallization. The concentrated enzyme solution was added to a column (2.5 cm × 100 cm) of Sephadex G-150, equilibrated with 50 mM buffer. The column was eluted with the same buffer at a flow rate of 40 ml/h. The effluent was collected in 3.0 ml fractions and monitored for enzyme activity. The active fractions (fractions 79–89) were pooled and concentrated by using a collodion bag *in vacuo*, by dialysis against 1000 ml of 50 mM-potassium phosphate buffer, pH 7.5 (pyridoxal 5'-phosphate and mercaptoethanol were omitted from the dialysis buffer). On concentration, some enzyme crystals appeared in the collodion bag. The yellow crystals and the supernatant were separated by centrifugation at 10000g for 30 min. The crystallized enzyme showed very low solubility without 10 mM-mercaptoethanol. From the supernatant solution, further yellow crystals were formed by the addition of powdered

(NH₄)₂SO₄. The crystals were easily soluble in 50 mM buffer without addition of mercaptoethanol. Specific activities of both crystallized enzymes were very similar. Both crystallized enzymes were recrystallized with (NH₄)₂SO₄, and had the same specific activity as the separated crystallized enzymes.

Results of a typical purification are shown in Table 1. The enzyme was purified about 700-fold over the human liver particulate fraction, giving 8% yield. The crystallized enzyme possessed the activities of kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase. During purification the proportions kynurenine-glyoxylate aminotransferase activity: alanine-glyoxylate aminotransferase activity: serine-pyruvate aminotransferase activity remained almost constant. On polyacrylamide-disc-gel electrophoresis at pH 8.9 in 7% gel, the purified enzyme migrated toward the anode with mobility of 0.06 relative to Bromophenol Blue (the tracking dye) as a single protein band, which coincided with kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activities (Fig. 1).

Kynurenine-glyoxylate aminotransferase activity was competitively inhibited by the addition of 20 mM-L-alanine or 20 mM-L-serine (Fig. 2).

Kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activities of the crystallized enzyme were not separated by Sephadex G-150 gel filtration, sucrose-density-gradient centrifugation, analytical polyacrylamide-disc-gel electrophoresis and isoelectric focusing, and the activity ratios were not changed before and after these treatments.

Enzymic properties

pH optimum. Aminotransferase activities were investigated as a function of pH over the range pH 6.0–9.7. Kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activities showed nearly identical optimal pH/activity profiles between pH 9.0 and 9.5 (Fig. 3).

Kinetic properties of the enzyme. The Michaelis constants for the aminotransferase activities of the crystallized enzyme were measured. The reciprocal values of $V_{\max}$ for amino acids and 2-oxo acids at several concentrations of the co-substrate were estimated. A secondary plot of these values against the reciprocal of the co-substrate concentrations permits the estimation of $V_{\max}$ at infinite co-substrate concentration and calculation of the absolute K_m values for alanine-glyoxylate aminotransferase as shown in Figs. 4(a) and 4(b).

Table 1. Co-purification of kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase from human liver particulate fraction

Assay methods are described in the text. Abbreviations: KGT; kynurenine-glyoxylate aminotransferase; AGT, alanine-glyoxylate aminotransferase; SPT, serine-pyruvate aminotransferase.

Purification step	KGT				AGT				SPT				Specific activity ratio		
	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	AGT KGT	SPT KGT	
10 000 g Pellet	7628	390	0.051	100	1	46 500	6.09	100	1	3830	0.502	100	119	9.84	
Extract	4928	451	0.092	116	1.80	44 800	9.09	93.3	1.49	3820	0.775	99.7	98.8	8.42	
Heat	4795	481	0.100	123	1.96	46 900	9.78	101	1.61	4090	0.853	107	97.8	8.53	
Ammonium sulphate	188	323	1.72	82.8	33.7	29 400	156	63.2	25.6	2430	12.9	63.4	90.7	7.50	
DEAE-cellulose	108	290	2.69	74.3	52.7	28 800	267	61.9	43.8	2350	21.8	61.4	99.3	8.10	
Hydroxyapatite	12.7	217	17.1	55.6	335	21 400	1690	46.0	278	1760	139	46.0	98.8	8.13	
Sephadex G-150	4.24	127	30.0	32.6	588	12 200	2880	26.2	473	1030	243	27.0	96.0	8.10	
Crystals	0.85	31.4	36.9	8.05	723	3020	3550	6.49	583	254	299	6.63	96.2	8.10

Fig. 1. Disc-gel electrophoresis of the crystalline enzyme

Approx. 20 μ g of the enzyme preparation was applied to a 7% polyacrylamide gel as described in the Experimental section. Of a pair of polyacrylamide gels, one was stained for protein with Amido Black and scanned at 625nm with a Digital Densitrol DMU-33C (Toyo Kagaku Sangyo Co., Tokyo, Japan), and the other was cut into 2mm slices for enzyme assay as described in the text. —, A_{625} on densitometry of protein stained; Δ , kynurenine-glyoxylate aminotransferase; O , alanine-glyoxylate aminotransferase; $\bullet$, serine-pyruvate aminotransferase.

Fig. 2. Double-reciprocal plots of initial velocity of kynurenine-glyoxylate aminotransferase activity of the crystalline enzyme against L-kynurenine concentration in the presence of 20 mM-L-alanine (O) and 20 mM-L-serine ($\bullet$), and in the absence of both amino acids (Δ)

Assay conditions were as described in the Experimental section except the concentration of kynurenine.

K_m values at infinite co-substrate concentration for serine-pyruvate aminotransferase and alanine-hydroxypyruvate aminotransferase were obtained as for alanine-glyoxylate aminotransferase. The

Fig. 3. Effect of pH on kynurenine-glyoxylate aminotransferase, alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase activity of the crystalline enzyme

Assay conditions were as described in the Experimental section, except that potassium phosphate buffer was used at pH 6.0–8.0 and pyrophosphate/KOH buffer at pH 7.6–9.7. Kynurenine-glyoxylate aminotransferase (Δ), alanine-glyoxylate aminotransferase ($\circ$) and serine-pyruvate aminotransferase ($\bullet$) activities are shown.

absolute K_m values of the alanine-glyoxylate aminotransferase reaction were 13.3 mM for alanine and 0.6 mM for glyoxylate, those of the serine-pyruvate aminotransferase reaction were 10.0 mM for serine and 1.5 mM for pyruvate, and those of the alanine-hydroxypyruvate aminotransferase reaction (a reverse reaction of serine-pyruvate aminotransferase) were 5.0 mM for alanine and 16.7 mM for hydroxypyruvate. K_m values for kynurenine and glyoxylate were obtained from straight-line double-reciprocal plots. The apparent K_m values of the kynurenine-glyoxylate aminotransferase reaction were 5.0 mM for kynurenine with 5 mM-glyoxylate and 0.075 mM for glyoxylate with 3 mM-kynurenine. The apparent Michaelis constant for pyridoxal 5'-phosphate of the apoenzyme prepared as described in the Experimental section was measured by use of the reaction of alanine-glyoxylate aminotransferase. The K_m value was estimated as 6.5 μ M when 100 mM-alanine and 5 mM-glyoxylate were used.

Substrate specificity. The purified enzyme showed broad specificity for L-amino acids (40 mM) with glyoxylate (5 mM) or pyruvate (5 mM). Alanine was the most active of the 21 amino acids investigated (Table 2) with glyoxylate as amino acceptor, and serine was the most active with pyruvate, but little or no activity was observed for cysteine, tryptophan and 3-iodotyrosine with either 2-oxo acid. Glyoxylate, oxaloacetate, hydroxypyruvate, pyruvate, 2-oxobutyrate and 4-methylthio-2-oxobutyrate were active amino-group acceptors, and

Fig. 4. Kinetic properties of the crystalline enzyme

Double-reciprocal plots of V_{max} of the alanine-glyoxylate aminotransferase activity of the enzyme against the concentration of L-alanine (a) and glyoxylate (b) are shown. Assay conditions were as described in the Experimental section, except L-amino acid concentration and 2-oxo acid concentration.

Table 2. *Substrate specificity of the crystalline enzyme for various L-amino acids with glyoxylate and pyruvate as amino acceptor*

Enzyme assays were carried out as described in the Experimental section. The final concentration of L-amino acid was 40 mM, except for L-tyrosine and L-3-iodotyrosine (6.5 mM) and L-kynurenine (3 mM), and that of glyoxylate and pyruvate was 5 mM. Abbreviation: N.D., not detected.

L-Amino acid	Specific activity (units/mg)	
	Glyoxylate	Pyruvate
Alanine	3547	—
Serine	799	257
Kynurenine	37.4	15.9
Aspartate	8.2	—
Asparagine	37.4	34.9
Glutamate	42.5	15.9
Glutamine	50.7	10.8
Lysine	21.6	2.5
Cysteine	N.D.	N.D.
Ornithine	N.D.	8.2
Arginine	10.8	N.D.
Threonine	9.5	9.5
Methionine	39.3	N.D.
Valine	29.1	8.2
Isoleucine	10.8	10.8
Leucine	15.9	3.2
Tyrosine	9.5	9.5
Histidine	24.1	17.1
Tryptophan	N.D.	N.D.
Phenylalanine	37.4	21.6
3-Iodotyrosine	N.D.	N.D.

Table 3. *Relative activities of transamination between L-kynurenine and various 2-oxo acids of the crystalline enzyme*

Assay conditions are described in the Experimental section. The final concentration of L-kynurenine was 3 mM and that of each 2-oxo acid was 5 mM. Relative activity values are given, signifying transamination rates compared with that for glyoxylate.

2-Oxo acid	Relative activity
Glyoxylate	100
Oxaloacetate	78
Hydroxypyruvate	70
Pyruvate	50
4-Methylthio-2-oxobutyrates	46
2-Oxobutyrates	46
Phenylpyruvate	20
2-Oxoglutarate	10
2-Oxoadipate	8

phenylpyruvate, 2-oxoglutarate and 2-oxoadipate showed little activity, for kynurenine (Table 3).

Activation and inhibition. Alanine-glyoxylate

Fig. 5. *Double-reciprocal plots of initial velocity of alanine-glyoxylate aminotransferase activity of the crystalline enzyme against L-alanine concentration in the presence of 30 μg of bovine serum albumin (□) or 10 mM-mercaptoethanol (●) or in the absence of both reagents (○)*

Assay conditions were as described in the Experimental section except the concentration of L-alanine.

aminotransferase activity of the purified enzyme was activated by 32% by the addition of 30 μg of bovine albumin to the reaction mixture, but the activity was inhibited by about 50% in the presence of 10 mM-mercaptoethanol. There was no change in the K_m value for alanine in the presence or absence of albumin or mercaptoethanol (Fig. 5).

Physical properties

Absorption spectrum of the enzyme. The absorption spectrum of the crystallized enzyme dissolved in 0.1 M-potassium phosphate buffer, pH 7.5, is shown in Fig. 6. The absorption maximum at 280 nm is caused by aromatic amino acid residues in the polypeptide chains. The peak at 420 nm is probably due to pyridoxal 5'-phosphate bound to the enzyme, and that at 330 nm is caused by pyridoxamine 5'-phosphate bound to the enzyme, as is known for other pyridoxal phosphate enzymes (Jenkins & Sizer, 1957, 1960; Fischer *et al.*, 1958). The ratio A_{280}/A_{420} of the pyridoxal form of the enzyme is about 9:1. Addition of 20 mM-alanine resulted in decrease of the A_{420} and increase of the A_{330} . Apoenzyme prepared as described in the Experimental section did not show the absorption maxima at 420 nm and 330 nm, showing the elimination of the coenzyme from the enzyme, and the apoenzyme almost completely lost (98.5%) activity

Fig. 6. U.v.-absorption spectra of (a) the crystalline enzyme in the presence (-----) and absence (—) of 20 mM-L-alanine and (b) the apoenzyme

Spectra were taken in 50 mM-potassium phosphate buffer pH 7.5. Preparation of apoenzyme was described in the Experimental section.

in the absence of pyridoxal 5'-phosphate from the reaction mixture.

Determination of approximate molecular weight. In the analytical ultracentrifuge the enzyme moved as a single component (Fig. 7). The sedimentation coefficient at 3 mg of protein/ml was calculated as 5.23S. By using this value, a mol.wt. of approx. 89 000 was calculated as described in the Experimental section. The mol.wt. was estimated as $90\,000 \pm 5000$ by Sephadex G-150 gel filtration and as $99\,000 \pm 4000$ by sucrose-density-gradient centrifugation. The crystallized enzyme was subjected to electrophoresis on a polyacrylamide disc gel containing sodium dodecyl sulphate, giving a single protein band that had an estimated mol.wt. of $47\,000 \pm 2000$, showing that this enzyme probably consists of two identical subunits.

Determination of isoelectric pH. The isoelectric pH of the crystallized enzyme was determined to be 8.3 by the use of a 110 ml isoelectric-focusing column with a pH 3.5–10 Ampholine gradient. Kynurenine-glyoxylate aminotransferase, alanine-

Fig. 7. Analytical-ultracentrifugal pattern of the crystalline enzyme

The protein concentration was 3.0 mg/ml of 50 mM-potassium phosphate buffer, pH 7.5. Photographs were taken 6 min (right) and 30 min (left) after the rotor reached 51 200 rev./min at 20°C.

glyoxylate aminotransferase and serine-pyruvate aminotransferase activities were coincidentally focused, showing a single activity peak.

Discussion

In the present investigation, kynurenine-glyoxylate aminotransferase was co-purified with alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase from human liver particulate fraction. The specific-activity ratios alanine-glyoxylate aminotransferase/kynurenine-glyoxylate aminotransferase or serine-pyruvate aminotransferase/kynurenine-glyoxylate aminotransferase maintained constant values during all purification steps. These ratios were unchanged after a variety of treatments of the purified enzyme, namely gel filtration, sucrose-density-gradient centrifugation, polyacrylamide-disc-gel electrophoresis and isoelectric focusing. These ratios were also unchanged after repeated crystallization. The results show that the three aminotransferase activities are properties of the same protein.

It has been reported that there are at least four enzymes possessing kynurenine aminotransferase activity in rat tissues (Noguchi *et al.*, 1975; Noguchi & Kido, 1976; Tobes & Mason, 1977, 1978; Harada *et al.*, 1978). Aromatic amino acid-glyoxylate aminotransferase of the rat, an enzyme showing kynurenine aminotransferase activity, is induced by the injection of glucagon. This enzyme is presumably identical with glucagon-inducible phenylalanine-pyruvate aminotransferase (Harada

et al., 1978). We have also reported that glucagon-inducible aminotransferase activities such as phenylalanine-pyruvate, histidine-pyruvate, serine-pyruvate and alanine-glyoxylate aminotransferase activities are properties of a single protein (Noguchi *et al.*, 1976a,b, 1978a; Minatogawa *et al.*, 1977). Therefore we infer that rat liver aromatic amino acid-glyoxylate aminotransferase is identical with glucagon-inducible alanine-glyoxylate aminotransferase and also serine-pyruvate aminotransferase. The enzyme purified from human liver in the present investigation showed some similar properties to those of aromatic amino acid-glyoxylate aminotransferase of monkey and rat liver, such as mol.wts. (approx. 90 000–99 000 for the present enzyme and approx. 80 000 for the monkey and rat enzymes) and isoelectric points (pH 8.0–8.3 for the three enzymes), and showed similar enzymic properties to those of the monkey enzyme. However, a difference in substrate specificity was observed between the present enzyme and the rat aromatic amino acid-glyoxylate aminotransferase. Both these enzymes utilized glyoxylate, pyruvate and other 2-oxo acids as amino acceptor for kynurenine. The rat enzyme was active with 2-oxoglutarate, but the human enzyme was not. In addition, the human enzyme showed the same or lower activity for phenylalanine, histidine, tyrosine and tryptophan even in high concentration compared with that for kynurenine (Table 2), though the rat enzyme showed remarkably high activity with these aromatic amino acids (Harada *et al.*, 1978).

The molecular weight estimated by sucrose-density-gradient centrifugation ($99\,000 \pm 4000$) was larger than that by analytical ultracentrifugation (approx. 89 000) and by Sephadex G-150 gel filtration ($90\,000 \pm 5000$). The disagreement with sucrose-density-gradient centrifugation may be due to the large differences between the molecular weight of the present enzyme and that of the bovine catalase (mol.wt. 250 000) used as internal standard.

The present enzyme contained pyridoxal 5'-phosphate as a coenzyme, and the pyridoxal form of the enzyme was converted into the pyridoxamine form by the addition of alanine (Fig. 6).

Figs. 5(a) and 5(b) suggest a Ping Pong Bi Bi mechanism for alanine-glyoxylate aminotransferase, and similarly for serine-pyruvate aminotransferase and alanine-hydroxypyruvate aminotransferase. The absolute K_m values for alanine-glyoxylate aminotransferase of the present enzyme are somewhat higher than the apparent K_m values of previously reported human, rat, mouse, cat and dog liver enzymes (Thompson & Richardson, 1967; Noguchi *et al.*, 1978a; Kamoda *et al.*, 1980).

Several findings, such as the difference in solubility of the enzyme crystallized in the presence or absence of mercaptoethanol, the inhibition of the

enzyme activity by the addition of high concentration of mercaptoethanol and the activation by bovine serum albumin, suggest that the enzyme is modified by mercaptoethanol and albumin. However, after treated with mercaptoethanol and bovine serum albumin, the V_{max} of the enzyme was changed, but the K_m value for alanine was not (Fig. 5).

The subcellular distribution of serine-pyruvate aminotransferase in neonatal human liver is reported by Noguchi & Takada (1978). They claimed that the enzyme was present only in the peroxisomal fraction, but not in mitochondria. However, in the present investigation, the enzyme was obtained from 10 000 g precipitate from frozen and thawed liver of an adult man, and without sonication it is difficult to solubilize the enzyme. The peroxisomes are so easily broken by homogenization (Noguchi & Takada, 1978) that we cannot deduce that only peroxisomes in the adult contain the present enzyme. We have found that dog liver alanine-glyoxylate aminotransferase and serine-pyruvate aminotransferase are mainly localized in the mitochondrial fraction, but not in the peroxisomal fraction (Okuno *et al.*, 1979). In rat liver, alanine-glyoxylate aminotransferase is mainly localized in the mitochondria and to some extent in the peroxisomal fraction, and the serine-pyruvate aminotransferase is evenly distributed in both fractions. Glucagon injection resulted in an increase of both enzyme activities in the mitochondria, but not in the peroxisomes (Noguchi *et al.*, 1978b).

Alanine-glyoxylate aminotransferase of rat liver suggested an association with gluconeogenesis from amino acids, and relatively high activity is associated with carnivorous species and humans (Rowell *et al.*, 1969, 1972a; Snell, 1971; Snell & Walker, 1972; Kamoda *et al.*, 1980); also, gluconeogenesis from serine via hydroxypyruvate (Dickens & Williamson, 1959; Rowell *et al.*, 1969, 1972c; Lardy *et al.*, 1969; Sallach *et al.*, 1972; Snell & Walker, 1974; Snell, 1974; Williamson & Ellington, 1975) may be associated with the enzyme. To our knowledge there has been no report about human kynurenine aminotransferase, although it has been reported that kynurenic acid, a product of the kynurenine aminotransferase reaction, is excreted in human urine (Price & Dodge, 1956) and is increased by a tryptophan load in vitamin B-6-deficient women (Yeh & Brown, 1977). We have found that the activity of kynurenine-glyoxylate aminotransferase was much higher than that of kynurenine-2-oxoglutarate aminotransferase in homogenates of human liver (Y. Minatogawa & R. Kodo, unpublished work) and monkey liver (Y. Minatogawa, T. Noguchi & R. Kodo, unpublished work). Therefore the enzyme may contribute not only in gluconeogenesis from alanine and serine, but also in the

metabolism of tryptophan, a precursor of kynurenine.

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