Purification and properties of kynurenine aminotransferase from rat kidney

Madhumalti R. MAWAL, Arindam MUKHOPADHYAY and Devendra R. DESHMUKH*

Department of Pediatrics, Children's Hospital of Michigan, Wayne State University, Detroit, MI 48201, U.S.A.

Previous reports indicated that a single protein exhibits kynurenine aminotransferase (KAT) and α -aminoadipate aminotransferase (AadAT) activities. However, recently we discovered that KAT and AadAT activities are associated with two different proteins. KAT from rat kidney supernatant fraction was purified to electrophoretic homogeneity by $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-Sephacel and hydroxyapatite chromatography. This procedure separated KAT from AadAT and improved the overall yield and the degree of purification over previously published methods. Some of the properties of purified KAT, such as M_r , subunit structure and the inhibition by dicarboxylic acids, were identical with those reported previously. However, the substrate specificity and pI of purified KAT were different from earlier reports. The same procedure can also be used to purify KAT from rat kidney mitochondria. These results support our earlier observation that KAT and AadAT activities are associated with two proteins and suggest that cytosolic KAT may be structurally similar to the mitochondrial enzyme.

INTRODUCTION

Kynurenine aminotransferase (KAT; EC 2.6.1.7), an enzyme of the tryptophan catabolic pathway, catalyses the irreversible transamination reaction between L-kynurenine and α -oxoglutarate to form kynurenic acid and L-glutamate. In mammals, KAT activity is present in various tissues such as liver, kidney, small intestine and brain and in different subcellular fractions such as mitochondria and cytosol [1–4]. The mitochondrial KAT also catalyses the transamination of 3-hydroxykynurenine into xanthurenate [5]. α -Aminoacidase aminotransferase (AadAT; EC 2.6.1.39), an enzyme of the lysine catabolic pathway, catalyses the reversible transamination reaction between α -aminoacidase and α -oxoglutarate to produce α -oxoadipate plus L-glutamate [3,4].

Earlier methods of KAT purification were time-consuming, provided low yield and could not separate KAT from AadAT [1–3]. In all these studies, both activities co-purified with a constant ratio of specific activities, showed similar chromatographic profiles and exhibited identical patterns of inactivation by heat and by dicarboxylic acids. Based on these results, it was concluded that a single protein catalyses both activities [1–3]. However, recently we discovered that the two activities can be separated by affinity chromatography [6]. The results described in the present paper demonstrate that the two activities can be separated by DEAE-Sephacel column chromatography, confirming that AadAT and KAT are associated with two distinct proteins. An improved procedure for purifying KAT from rat kidney supernatant fraction is described, and the properties of the purified enzyme are reported.

MATERIALS AND METHODS

L-Kynurenine sulphate, kynurenic acid, α -aminoadipic acid, α -oxoglutaric acid, pyridoxal 5'-phosphate, standard protein molecular-mass markers, standard pI markers and other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Adult male albino rats (Sprague–Dawley) weighing

250–300 g were purchased from Charles River Laboratories, Wilmington, DE, U.S.A.

Enzyme assays

KAT activity was assayed as described by Knox [7]. The reaction mixture contained 100 mM-imidazole/HCl, pH 6.5, 3.3 mM-kynurenine sulphate, 5 mM- α -oxoglutarate and 0.2 mM-pyridoxal 5'-phosphate in a total volume of 0.6 ml. The assay mixture was incubated for 30 min at 37 °C, and the reaction was terminated by adding 10 ml of 1% boric acid in 95% (v/v) ethanol. α -Oxoglutarate was added to control tubes after termination of the reaction. Denatured protein was removed by centrifugation at 5000 g for 10 min, and the A_{333} corresponding to kynurenic acid was measured against boric acid in ethanol. AadAT was assayed as described by Nakatani *et al.* [8]. Protein concentration was determined by the protein dye-binding assay of Bradford [9], with BSA as a standard.

Purification of KAT

Rats were killed by decapitation and their kidneys were immediately removed. All operations were carried out at 0–4 °C, unless stated otherwise. The kidneys were homogenized (10%, w/v) in 0.25 M-sucrose containing 0.1 mM-EDTA and 10 mM-Tris, pH 7.4. The homogenate was centrifuged at 600 g for 10 min and the pellet was discarded. To separate mitochondria, the supernatant was centrifuged at 12000 g for 10 min. The purity of mitochondrial and supernatant fractions was checked by assaying glutamate dehydrogenase (EC 1.4.1.3) and lactate dehydrogenase (EC 1.1.1.27) activities respectively [10,11].

The procedure for purifying KAT from rat kidney supernatant fraction is summarized in Table 1. Initial steps of purification, including acid precipitation and $(\text{NH}_4)_2\text{SO}_4$ fractionation, were carried out as described by Tobes & Mason [2]. The pellet obtained after $(\text{NH}_4)_2\text{SO}_4$ fractionation was suspended in 8 mM-potassium phosphate buffer, pH 6.2, containing 10 mM-2-mercaptoethanol and 0.05 mM-pyridoxal 5'-phosphate (buffer A). The sample was dialysed for 20 h against the same buffer and loaded on the DEAE-Sephacel column (1 cm $\times$ 14 cm) pre-equilibrated with buffer A. The column was eluted with buffer A,

Abbreviations used: KAT, kynurenine aminotransferase; AadAT, α -aminoadipate aminotransferase.

* To whom correspondence should be addressed.

Table 1. Purification of KAT from rat kidney supernatant fraction

Details of the purification procedure are described in the text. One unit of activity is defined as the amount of enzyme that catalyses the formation of 1 μ mol of product/min at 37 °C.

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Supernatant	5.79	640	0.009	100	1
Acid precipitation	4.90	331	0.015	85	1.7
(NH ₄) ₂ SO ₄	4.13	72.7	0.057	71	6.3
DEAE-Sephacel	3.14	3.36	0.94	54	104
Hydroxyapatite	2.28	0.54	4.22	39	469

and 1.0 ml fractions were collected (Fig. 1). The fractions containing KAT activity were pooled, dialysed for 20 h against 8 mM-potassium phosphate buffer, pH 7.0, containing 10 mM-2-mercaptoethanol and 0.05 mM-pyridoxal 5'-phosphate (buffer B) and subjected to hydroxyapatite column chromatography. The hydroxyapatite column (1 cm $\times$ 15 cm) was equilibrated with buffer B and washed with 2 column vol. of the same buffer. The column was eluted with a linear gradient of 8–210 mM-potassium phosphate buffer, pH 7.0, and 1.2 ml fractions were collected and assayed for KAT activity. The active fractions were pooled, dialysed against buffer B, concentrated by Centricon-10 (Amicon) filtration and used for further studies. A_{280} was used to identify protein peaks during DEAE-Sephacel and hydroxyapatite column chromatography.

Polyacrylamide-gel electrophoresis

To check the purity of KAT, non-gradient polyacrylamide-gel (7.5%) electrophoresis was carried out at pH 8.3 [12]. The M_r of the subunits was estimated by gel electrophoresis in SDS [13]. The enzyme was denatured by treatment with 1% SDS solution containing 1% 2-mercaptoethanol at 100 °C for 5 min. BSA (M_r 66000), ovalbumin (M_r 45000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000) and trypsinogen (M_r 24000), used as M_r markers, were treated in the same way.

M_r of the native enzyme

A Sephadex G-200 column (1.5 cm $\times$ 46 cm) was equilibrated with 50 mM-potassium phosphate, pH 7.0, containing 10 mM-2-mercaptoethanol [14]. The column was calibrated with the following standard M_r markers: thyroglobulin (M_r 670000), β -globulin (M_r 158000), ovalbumin (M_r 44000), myoglobin (M_r 17000) and vitamin B-12 (M_r 1350). Proteins were eluted with the same buffer, and the elution was monitored by measuring the A_{280} . A plot of fraction number against log M_r was used to determine the M_r of purified KAT.

Isoelectric focusing

AadAT activity was purified as reported previously [6]. Isoelectric focusing of purified KAT and AadAT was carried out on 7.5%-acrylamide gels containing 2% ampholyte and 10% sucrose, carrier ampholytes in the pH range 5–8 being used. The gels were focused at 4800 V-h, fixed in 10% (w/v) trichloroacetic acid containing 5% (w/v) sulphosalicylic acid and stained for 10–12 h with 0.2% Coomassie Blue R-250. The gels were destained with ethanol/acetic acid/water (4:1:5, by vol.).

Substrate specificity

The substrate specificity of purified KAT was determined by using a number of α -amino acids as amino donors and with either α -oxoglutarate or pyruvate as amino-group acceptor. The

Fig. 1. Chromatographic separation of KAT and AadAT

A partially purified enzyme obtained after (NH₄)₂SO₄ fractionation was dialysed and loaded on a DEAE-Sephacel column (1 cm $\times$ 14 cm), and 1.0 ml fractions were collected. Details of chromatography are described in the text. Enzyme activities are expressed as units/ml.

transamination reaction with aromatic amino acids was carried out by the method of Takada & Noguchi [15].

Amino acid analysis

Amino acid analysis was carried out at the protein-sequencing facility of Wayne State University, Detroit, MI, U.S.A. The purified (unoxidized) sample was freeze-dried and subjected to vapour-phase hydrolysis at 150 °C for 90 min. The sample was then treated with phenyl isothiocyanate, and the derivative was used for amino acid analysis by the Waters Pico-tag system [16]. The loss of serine (17%) and threonine (13%) during hydrolysis was corrected for accordingly.

Absorption spectra

The absorption spectrum for purified KAT (200 μ g/ml) was obtained at pH 5.0 and 8.2 in the presence of 0.05 mM-mercaptoethanol [17].

RESULTS

Purification of KAT

The mitochondrial fraction from rat kidney did not contain any detectable lactate dehydrogenase activity, and the supernatant fraction was devoid of glutamate dehydrogenase activity. These results confirmed that the mitochondrial and supernatant fractions were not contaminated by other subcellular components. The purification procedure for KAT is summarized in Table 1. On DEAE-Sephacel column chromatography, KAT activity separated from AadAT activity (Fig. 1). During this

Fig. 2. SDS/PAGE of KAT

Purified enzyme (30 μ g) was electrophoresed on 10%-polyacrylamide gels in presence of 1% SDS, as described in the Materials and methods section. The direction of migration was top (cathode) to bottom (anode). Lane 1, standard M_r markers: BSA (M_r 66000), ovalbumin (M_r 44000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36000), carbonic anhydrase (M_r 29000) and trypsinogen (M_r 24000). Lane 2, KAT.

Fig. 3. Isoelectric focusing of KAT

Purified enzyme (30 μ g) was electrophoresed on 7.5%-polyacrylamide gels as described in the Materials and methods section. Lane 1, purified KAT. Lane 2, standard pI markers: myoglobin from horse heart (pI 7.2), myoglobin from horse heart (pI 6.8), carbonic anhydrase I from human erythrocytes (pI 6.6), carbonic anhydrase II from bovine erythrocytes (pI 5.9), carbonic anhydrase II from bovine erythrocytes (pI 5.4). Lane 3, AaAT purified from rat kidney supernatant fraction.

step, aspartate aminotransferase, which is usually associated with AaAT and KAT activities, was completely removed. The KAT activity was purified 470-fold, with an overall yield of 40%. The same procedure could also be used to purify KAT from rat kidney mitochondria (results not shown).

Table 2. Substrate specificity of KAT purified from rat kidney

In all assays 10 μ g of purified KAT and 50 mM of either α -oxoglutarate or pyruvate was used. The final concentrations were: kynurenine, 3.3 mM; aromatic amino acids, 2.0 mM; DL-2-aminopimelic acid, 80 mM; and other amino acids, 40 mM. Enzyme activities were assayed as described in the Materials and methods section and are expressed as the percentage of activity obtained with kynurenine and α -oxoglutarate as substrates. The data shown are averages of duplicate analyses.

Amino-group donor	α -Oxoglutarate	Pyruvate
Kynurenine	100	4
5-Hydroxykynurenine	60	8
Histidine	15	11
Aminoadipate	0	0
DL-2-Aminopimelic acid	1	5
L-Norleucine	1	4
Phenylalanine, tyrosine, tryptophan	< 8	< 4

Table 3. Effect of dicarboxylic acids on KAT activity purified from rat kidney

Purified KAT (10 μ g) was preincubated with dicarboxylic acid (final concn. 6 mM; neutralized to pH 7.0) at 37 $^{\circ}$ C for 30 min in imidazole buffer, pH 6.5. Enzyme activity was then determined as described in the Materials and methods section. The data shown are averages of duplicate analyses.

Dicarboxylic acid added	Inhibition (%)
None	0
Decanoic acid	41
Adipic acid	40
Diethylglutaric acid	30
Pimelic acid	23
Azelaic acid	21
Dimethylglutaric acid	12
3-Methylglutaric acid, glutaric acid, oxaloacetic acid	< 3

Purity, M_r and subunit structure

The purified KAT migrated as a single band on native PAGE, indicating that the preparation was homogeneous. On Sephadex G-200 column chromatography, the purified enzyme was eluted between β -globulin (M_r 158000) and ovalbumin (M_r 44000), indicating the M_r of 91000 (mean of two observations). The M_r of the subunit structure, estimated by PAGE in the presence of 1% SDS, was found to be about 44000 ± 1200 ($n = 3$; Fig. 2). These results indicate that KAT is composed of two apparently identical subunits.

Isoelectric focusing and kinetic parameters

The pI of KAT was found to be 5.9 ± 0.07 (Fig. 3). The K_m values for kynurenine and α -oxoglutarate were 4.0 mM and 0.02 mM respectively.

Substrate specificity

The ability of KAT to catalyse transamination between various amino acids and keto acids is shown in Table 2. Maximum activity was obtained with kynurenine as an amino-group donor and α -oxoglutarate as the amino-group acceptor. A significant activity was also observed with 5-hydroxykynurenine as an

Table 4. Amino acid composition of KAT purified from rat kidney

Purified KAT was hydrolysed *in vacuo* at 150 °C for 90 min and the amino acids were analysed as described in the text.

Amino acid	Content (residues/molecule)
Glutamic acid	124.0
Aspartic acid	82.0
Serine	86.0
Glycine	75.0
Histidine	11.8
Arginine	30.5
Threonine	39.4
Alanine	88.0
Proline	34.6
Tyrosine	29.3
Valine	59.0
Methionine	10.0
Isoleucine	30.8
Leucine	46.8
Phenylalanine	38.0
Lysine	39.8

Fig. 4. Absorption spectra of KAT

Curve I represents the absorption spectrum of KAT in 0.1 M-acetate, pH 5.2, and curve II is that in 0.05 M-Tris, pH 8.2.

amino-group donor and α -oxoglutarate as amino-group acceptor. No measurable activity was observed when either α -amino adipate or aromatic amino acids were used as amino-group donor and α -oxoglutarate or pyruvate as the amino group acceptor. The purified KAT exhibited some (< 15%) activity with histidine as an amino-group donor and either pyruvate or α -oxoglutarate as amino-group acceptor (Table 2).

Effect of dicarboxylic acids and metabolites

Several dicarboxylic acids were tested as inhibitors of KAT from kidney supernatant (Table 3). Adipic acid and decanoic acid caused the maximum inhibition. Dimethylglutaric acid caused about 30% inhibition. However, oxaloacetic acid and 3-methylglutaric acid had no significant effect on the KAT activity. The effect of various metabolites of the lysine and tryptophan pathway on the KAT activity was studied. Amino adipate and other metabolites did not cause any change in KAT activity (results not shown).

Amino acid analysis

The amino acid composition of purified KAT is shown in Table 4. The purified enzyme had a high content of aspartic acid, glutamic acid, serine and alanine as compared with other amino

acids, whereas histidine and methionine were present in low amounts. The high content of glutamic acid and aspartic acid presumably reflects a substantial contribution from the hydrolysis of glutamine and asparagine respectively, since the pI value indicates only a moderate excess of acidic over basic amino acid side chains.

Absorption spectra

At pH 5.2 KAT exhibited two absorption maxima at 330 nm and 400 nm, whereas at pH 8.2 only one absorption maximum at 330 nm was observed (Fig. 4).

DISCUSSION

Earlier studies indicate that KAT and AadAT, purified from rat liver mitochondria as well as from rat kidney supernatant fractions, have a pI of 6.6 [2,3]. However, we found that the pI values of KAT and AadAT purified from rat kidney supernatant fraction were 5.9 and 6.6 respectively (Fig. 3). In previous studies, a broader range of ampholytes (pH range 3–10) was used, whereas we used a narrow range of ampholytes (pH 5–8). The isoelectric-focusing data are consistent with our observation that two activities are resolved on DEAE-Sephacel chromatography at pH 6.2 (Fig. 1). KAT and AadAT activities from rat kidney mitochondria were also separated on DEAE-Sephacel column chromatography at pH 6.2 (results not shown). These results indicate that KAT and AadAT activities from mitochondrial fractions represents two distinct proteins and that the pI of the mitochondrial KAT may be the same as that of supernatant enzyme.

In the previous studies, aspartate aminotransferase activity was associated with both AadAT and KAT activities until the last stage of purification, and even the purified KAT/AadAT exhibited a small amount of aspartate aminotransferase activity [2]. In our studies, aspartate aminotransferase activity was completely removed during DEAE-Sephacel column chromatography. Therefore additional steps of purification after hydroxyapatite column chromatography used in other studies were not necessary.

The M_r and subunit structure of the KAT purified from rat kidney supernatant fraction were identical with those of enzymes purified from other sources [1–4,18]. KAT from rat kidney supernatant fraction also resembled that from other sources in the pattern and the degree of inhibition by dicarboxylic acids (Table 3).

Although there are several reports on the purification of KAT from different sources, the amino acid composition of the enzyme has not yet been determined. The amino acid composition of rat kidney KAT (Table 4) was similar to that of AadAT from bovine kidney [18], except that the numbers of lysine and proline residues in rat kidney enzyme were less than those in bovine kidney AadAT. High levels of glutamic acid and aspartic acid observed in our study may be due to the hydrolysis of glutamine and asparagine, because the Pico-tag method used in our study does not distinguish between aspartic acid and asparagine and between glutamic acid and glutamine.

The absorption spectrum of KAT (Fig. 4) resembles that of AadAT and of most other aminotransferases in that it absorbs light in the wavelength range of 300–500 nm. At low pH (5.2) one absorption maximum appeared at 415 nm, which can be ascribed to a phosphopyridoxal–aldimine enzyme complex, and the second maximum was observed at 335 nm. At high pH (8.2) only one absorption maximum, at 335 nm, was observed. These spectra resemble those of aspartate aminotransferase from pig heart [17] and are characteristic of pyridoxal phosphate-requiring enzymes.

The K_m values for kynurenine and α -oxoglutarate for the purified enzyme in our studies were comparable with those reported [1–3]. However, the substrate specificity of KAT from rat kidney supernatant (Table 2) was different from that reported previously. For example, previous studies indicated that, in addition to kynurenine, other amino acids such as DL-3-hydroxykynurenine, DL- α -aminoadipate, DL-aminopimelate, L-norleucine and L-tryptophan also serve as amino donors, and α -oxoglutarate serves as amino-group acceptor [2,18,19] for KAT activity. However, we did not observe any activity with aminoadipate, norleucine or DL-aminopimelate as amino group donors and with α -oxoglutarate as amino-group acceptor (Table 2). Aminoadipate was reported to be a competitive inhibitor of KAT activity partially purified from rat kidney supernatant fraction and also from rat liver mitochondria [1,3]. However, we did not observe any inhibition of KAT activity on addition of aminoadipate. This difference could be due to removal of AadAT activity from our preparations.

Recently we separated KAT from AadAT activity from rat kidney supernatant fraction [6]. However, the method was time-consuming, provided a low yield and required antibodies against AadAT. The present method is rapid, separates KAT from AadAT, and provides improved yield and degree of purification over previously published methods.

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