Neurochemistry International 133 (2020) 104653

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Purification, characterization and identification of rat brain cytosolic tyrosine transaminase as glutamine Transaminase-K

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ARTICLE INFO

Keywords:
Tyrosine
Transamination
Glutamine transaminase-K
Kynurenine transaminase-I
Aminotransferase
Aromatic amino acids

ABSTRACT

The current study was undertaken to investigate the spectrum of tyrosine transaminases enzymes in a cytosolic fraction of rat brain and to specifically purify and characterize a previously identified cytosolic brain enzyme possessing tyrosine/glyoxylate transaminase activity. Based upon extensive biochemical and immunochemical characterization of purified brain tyrosine/glyoxylate transaminase, we concluded the purified enzyme is glutamine transaminase-K (EC 2.6.1.64). This conclusion was based on: 1.) a concurrent enrichment in the tyrosine/glyoxylate and glutamine/phenylpyruvate transaminase activities during purification, 2.) demonstration of a co-substrate specificity for amino acids and α-keto acids that was highly consistent with published information for glutamine transaminase-K, 3.) results from detailed kinetic analysis, 4.) glutamine was a potent inhibitor of *in vitro* tyrosine/glyoxylate transamination, 5.) biochemical characterization, including pH optimum of 8.5 and spectrophotometric analysis and 6.) immunoanalytical analysis using a specific antiserum to rat renal glutamine transaminase-k. In addition, immunochemical characterization of a crude soluble extract of whole brain suggests that the *in vitro* tyrosine transaminase activity for several different α-keto acid co-substrates likely reflect the activity of glutamine transaminase-K. In conclusion, this investigation confirmed the presence of multiple tyrosine transaminase enzymes in a cytosolic extract of rat brain. Moreover, we concluded glutamine transaminase-K represents a predominant cytosolic enzyme in rat brain that's capable of catalyzing *in vitro* transamination of p-tyrosine and other aromatic amino acids, including the neurotransmitter precursors L-dopa and 5-hydroxytryptophan. The purified transaminase possesses a broad co-substrate specificity with preferential reactivity with α-keto acids derived from neutral aliphatic and aromatic amino acids. Lastly, we identified a heterogeneous regional distribution of tyrosine/glyoxylate transaminase (glutamine transaminase-K) in rat brain with a significantly higher level of *in vitro* activity in cerebellum.

1. Introduction

The intraneuronal concentration of free tyrosine is one factor that can influence the synthesis of catecholamine neurotransmitters and trace amines (Wurtman et al., 1974; Gibson and Wurtman, 1978; Edwards, 1982). Consequently, the flux of tyrosine into various metabolic pathways has the capability of influencing the synthesis of neurotransmitters and trace amines within brain by modulating the level of free precursor tyrosine. This notion is supported by the inverse relationship between tyrosine hydroxylation and decarboxylation (Jurio, 1979; Jurio and Jones, 1981) and by the accumulation of tyrosine in rat brain after inhibition of AADC (Carlsson et al., 1972).

Transamination is one of several biochemical pathways that has the potential to influence the intraneuronal concentration of free tyrosine by transferring the α-amino group to an acceptor co-substrate. Transamination is a reversible reaction that can either synthesize or degrade tyrosine depending on the concentration of the respective co-substrates. To date a large body of data has been amassed concerning the properties and functional aspects of enzymes that can catalyze the reversible transamination of tyrosine. Accordingly, tyrosine transamination has been hypothesized to be a pathway for potential regulation of neurotransmitter synthesis (Oja, 1968; Gibb and Webb, 1969; Gabay and Clarke, 1983). The presence of multiple tyrosine transaminase activities in brain has been suggested by a number of studies (Benuck et al., 1971; Minatogawa et al., 1973) and tyrosine has been demonstrated to undergo *in vitro* transamination with a wide range of α-keto acids (Oja, 1968; Minatogawa et al., 1973).

Tyrosine aminotransferase (EC 2.6.1.5) which utilizes α-

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https://doi.org/10.1016/j.neuint.2019.104653
Received 6 August 2019; Received in revised form 17 December 2019; Accepted 19 December 2019
Available online 23 December 2019
0197-0186/

ketoglutarate as the principal amino acceptor is a well-characterized inducible hepatic enzyme that degrades tyrosine (Kenney, 1959; Jacoby and La Du, 1964; Groenewald et al., 1984). Even though low levels of tyrosine/α-ketoglutarate transaminase activity is detected in most organs, including brain, authentic tyrosine aminotransferase is viewed largely as being a hepatic specific inducible enzyme (Belarbi et al., 1979; Hargrove, 1984). The rat liver enzyme is reported to have a molecule weight of about 100,000 and an isoelectric point of 4.3 (Belarbi et al., 1977; Groenewald et al., 1984; Beck et al., 1978). The isoenzymes of aspartate aminotransferase (EC 2.6.1.1) have been reported to catalyze the *in vitro* transamination of tyrosine (\l "bib57" \Ohisalo and Pispa, 1976; Belarbi et al., 1979; Hargrove and Mackin, 1984). Yet, the *in vitro* reactivity of these α-ketoglutarate-dependent isoenzymes towards tyrosine is low (Miller and Litwack, 1971); consequently, a physiological role in the modulation of intraneuronal tyrosine levels is unlikely (Ohisalo et al., 1982; Hargrove and Mackin, 1984). Glutamine transaminase-K (EC 2.6.1.64) is another enzyme that has the potential to catalyze tyrosine transamination. This enzyme possesses a broad substrate specificity that includes the aliphatic α-keto acids, glyoxylate and pyruvate (Van Leuven, 1975, 1976; Noguchi et al., 1977; Cooper and Meister, 1974, 1981), but demonstrates preferential reactivity with large neutral amino acids, including tyrosine (Cooper and Meister, 1974; Van Leuven, 1976; Cooper, 1978). In addition, glutamine transaminase-K has been reported to be present in brain (Bowsher and Henry, 1985; Cooper and Anders, 1990) where it has been suggested to play diverse roles in metabolic regulation of ammonia and glutamine levels, conservation of carbon chains for essential amino acids, metabolic mechanism transport and homeostasis of amino acid and as a potential detoxification pathway (Cooper, 2004, 2016; Cooper and Kuhara, 2014). More recently, this same enzyme was identified and extensively characterized in brain as kynurenine aminotransferase-I (Mosca et al., 1994; Guidetti et al., 1997; Han et al., 2004, 2010; Han et al., 2010a,b). Branched-chain aminotransferase (EC 2.6.1.42) is also present in brain (Garcia-Espinosa et al., 2007; Hull et al., 2012, 2015; Sperringer et al., 2017), but its co-substrate specificity does not extend to tyrosine.

During earlier work involving investigation of tyrosine decarboxylation in rat tissues (Bowsher and Henry, 1983), we identified a tyrosine/glyoxylate transaminase enzyme that tended to co-purify with the renal tyrosine decarboxylase enzyme. Accordingly, we subsequently reported purification and extensive characterization of this aminotransferase and concluded it was glutamine transaminase-K (Bowsher and Henry, 1985). The current study extends previous work to investigate the spectrum of enzymes in a cytosolic fraction of rat brain that have the potential to catalyze *in vitro* transamination of p-tyrosine and other aromatic amino acid. While this study confirms the presence of multiple enzymes in crude soluble extract of rat brain that can catalyze *in vitro* tyrosine transamination, we concluded that glutamine transaminase-K comprises the predominant cytosolic tyrosine transaminase across a wide range of α-keto acid co-substrates.

2. Materials

All chemicals were high-quality, reagent grade obtained from EMD Sigma chemical company. The following were obtained from Amersham (Arlington Heights, IL): L-[ring-3,5-^{3}H]tyrosine (50 Ci/mmol) and L-[ring-4-^{3}H]phenylalanine (23.5 Ci/mmol). Econofluor was purchased from New England nuclear Corp. (Boston, MA). Chromatographic materials, including phenyl-Sepharose, Sephadex-G 25 (fine), and DEAE-Sephacel were obtained from GE Healthcare (formerly Pharmacia Fine Chem.) Bio-Gel HTP and electrophoretic reagents were purchased from Bio-Rad (Richmond, CA). IgGsorb, formalin-fixed and heat-treated *Staphylococcus aureus*, was purchased from The Enzyme Center, Inc. (Malden, MA).

3. Methods

3.1. Enzyme assays

3.1.1. Tyrosine transaminase

Tyrosine transaminase activity was quantified by measuring the rate of conversion of tritiated tyrosine to labeled p-hydroxyphenylpyruvic acid. Prior to use in transaminase assays, radiolabeled tyrosine was acidified with formic acid to a final concentration of 0.2 M, extracted with 10 vol of toluene-isopentyl alcohol (3:1) and 25 μL aliquots were dried under vacuum in 12 × 75 mm borosilicate culture tubes. This manipulation resulted in substantial assay blank reduction by removal of an acid-extractable nonpolar contaminant.

Each 100-μL reaction was performed in a 12 × 75 mm borosilicate culture tube and contained the following components: 0.1 μmol of L-[ring-3,5-^{3}H]tyrosine, 15 μmol of K borate, pH 8.5, 0.1 μmol EDTA, 0.1 μmol of DTT and 25 μL of enzyme. The transaminase reaction was initiated by a 25 μL addition containing the α-keto, usually 1 μmol of glyoxylate, and then incubated for 20 min at 37 °C. Under these conditions, the enzymatic formation of p-hydroxyphenylpyruvate proceeded at a linear rate for at least 30 min when incubated with a cytosolic extract derived from 5 mg of whole rat brain tissue or greater than 60 min when incubated with 1 μg of 670-fold purified transaminase. All samples were quantified in duplicate and blank tubes containing water instead of α-keto acid were included in all assays. Under the assay conditions, substrate utilization was routinely < 2%.

The transaminase reaction was terminated by a 50 μL addition of 2 M phosphoric acid and the reaction product, p-hydroxyphenylpyruvate, was extracted using 1.25 mL of toluene-isopentyl alcohol (9:1). After vigorous mixing using a mechanical vortexer and phase separation by brief centrifugation at ambient temperature, a 1-mL aliquot was transferred to a glass scintillation vial that contained 10 mL of Econofluor. Vials were capped and the radioactivity was measured by liquid scintillation spectrometry with a counting efficiency of 42%. Under these assay conditions the recovery of tritiated p-hydroxyphenylpyruvic acid was > 75%, while labeled tyrosine as < 0.1%.

Assay sensitivity, defined as the amount of synthesized p-hydroxyphenylpyruvate necessary to yield twice the background cpm, was 0.15 nmol. The reaction product was confirmed as being isographic with authentic p-hydroxyphenylpyruvic acid by thin-layer chromatography using a Whatman LK5DF silica gel plate as the stationary phase and a mobile phase consisting of ethyl acetate/methanol/glacial acetic acid/water (86:12:1:1).

3.1.2. Glutamine Transaminase-K

Two different assay methods were used for quantitative determination of glutamine transaminase-K activity. The first one employed the specific type-K co-substrates, phenylalanine and α-ketomethiolbutyrate (Cooper, 1978; Cooper and Meister, 1984). Each 100 μL reaction was performed using 0.2 μmol of L-[ring-4-^{3}H]phenylalanine (25 mCi/mmol) and 0.5 μmol α-ketomethiolbutyrate. Blank tubes received water instead of α-keto acid. The assay conditions and product isolation procedure were the same, as those used for the tyrosine transaminase assay.

The second assay procedure was a modification of the spectrophotometric method of Cooper (1978) using the co-substrates glutamine and phenylpyruvate (Cooper and Meister, 1981, 1984, 1985). Each 100 μL-reaction was conducted in a 12 × 75 mm borosilicate tube and contained 2.0 μmol of glutamine, 0.04 μmol phenylpyruvate, 0.1 μmol of Bis Tris-propane pH 8.5 (37 °C) and 25 μL of enzyme. Blank tubes received water instead of glutamine. After incubation for 20 min at 37 °C, the transaminase reaction was terminated by a 1-mL addition of 3.3 M NaOH. The rate of disappearance of phenylpyruvate was determined using a disposable acrylic cuvette by measuring the decrease in absorbance at 322 nm. Under these conditions, the assay was linear for at least 30 min. Even though the sensitivity of this assay was less than the radiometric based method, it was regarded as being a

specific marker for glutaminase transaminase-K (Cooper, 1978) and, therefore, better suited for application with brain extracts.

3.1.3. Glutamine Transaminase-L

Glutaminase transaminase-L transaminase was measured by a spectrophotometric assay using the co-substrates L-albizziin and glyoxylate (Cooper and Meister, 1981, 1984, 1985).

3.1.4. Aspartate aminotransferase

Aspartate aminotransferase was determined by the spectrophotometric method of Reitman and Frankel (1957) using a commercially available from Sigma Chemical company. Each 300-μL reaction contained the following components: 50 μmol of D,L-aspartate, 0.45 μmol of α-ketoglutarate, 30 μmol Na phosphate, pH 7.5 and 0.4–5.0 mU of enzyme, as a 50 mL addition. Blank tubes received water instead of enzyme. After incubation for 60 min at about 37 C, the transaminase reaction was terminated by a 0.25 mL addition of 1 M HCl which contained 0.05 mg of 2,4-dinitrophenylhydrazine. Upon addition the tubes were incubated for 20 min at ambient temperature at which time 2.5 mL of 0.4 M NaOH. Following addition of base, the tubes were incubated for approximately 5 min and the absorbance was measured at 505 nm. Asp transaminase was estimated by dose interpolation from a calibration curve.

3.1.5. Aromatic amino acid aminotransferase

Aromatic amino acid transaminase was quantified in experiments involving purified brain enzyme by forming the arsenate catalyzed enol-borate complex of each respective aromatic α-keto acid product (George et al., 1967). Molar extinction coefficient values for the aromatic α-keto acid-enol-borate complex were determined empirically or taken from literature values (Noguchi et al., 1976).

Each 150-μL reaction contained the following components: 600 nmol of aromatic L-amino acid, 600 nmol of α-ketomethiolbutyrate, 15 μmol of Bis Tris-propane, pH 8.5 (37 °C), 15 nmol of EDTA, 15 nmol of dithiothreitol and 80 μU of purified brain transaminase. Blank tubes were generated by adding α-ketomethiolbutyrate at the end of the incubation period immediately prior to quenching the reaction by adding 50 μL of 1 M HCl followed by a 1 mL addition of 1 M of potassium arsenate/borate, pH 6.5. Each mixture was incubated for 20 min at ambient temperature at which time the absorbance was measured at the appropriate wavelength. The absorption maxima were determined empirically to be Phe (295 nm), m-Tyr (300 nm), p-Tyr (310 nm), His (292 nm), DOPA (330 nm), Try (330 nm), and 5-HTP (334 nm). The molar extinction coefficient values were determined empirically or taken from published values.

3.2. Immunotitration of transaminase activities

Immunotitration experiments were performed to characterize the highly purified rat brain tyrosine/glyoxylate transaminase enzyme and a fresh crude soluble tissue brain extract from Wistar rats. Aliquots of samples were combined with an equal volume of 25 mM PBS/0.2 mM EDTA/0.1% BSA that contained various proportions of rabbit polyclonal antiserum to highly purified rat renal glutamine transaminase-K. Mixtures were incubated at 4 °C for about 16 h and then centrifuged at 40,000 × *g* for 20 min. The supernatants were subsequently treated with Protein-A (IgGsorb) for about 30 min, centrifuged at 3000 × *g* for 15 min. The supernatants were assayed directly for transaminase activity.

4. Results and discussion

4.1. Distribution in Wistar rat tissues

The distribution of cytosolic tyrosine transaminase was investigated in rat tissues using both glyoxylate and α-ketoglutarate as the amino acceptor co-substrates. As reported in Table 1, kidney contained the highest level of tyrosine/glyoxylate transaminase activity that was about six-times higher than the level in liver. In contrast, when α-ketoglutarate, a marker for tyrosine aminotransferase (EC 2.6.1.5), was used as the co-substrate, the rate of tyrosine transamination by kidney was < 2% of liver. Not unexpectedly, the patterns for distribution for glyoxylate and α-ketoglutarate dependent transaminase activities were different across rat tissues. For most tissues, the *in vitro* rate of tyrosine transamination was greater with glyoxylate with the exceptions being liver and stomach. Extracts of heart and duodenum demonstrated similar rates of transamination for both α-keto acids.

Table 1
Distribution of cytosolic tyrosine/glyoxylate transaminase in rat tissues.

	[a]Transamination Rate (mUnits/g tissue)	
Tissue	Glyoxylate	α-Ketoglutarate
Kidney	445.0 (79.7)	3.88 (0.90)
Liver	74.2 (7.1)	210.0 (17.5)
Stomach	17.6 (1.7)	26.8 (1.3)
Heart	13.5 (1.1)	14.7 (2.8)
Colon	13.3 (2.6)	4.52 (1.29)
Ileum	12.8 (2.3)	2.48 (0.32)
Mid-Jejunum	10.1 (2.6)	1.32 (0.33)
Brain	8.75 (1.48)	1.73 (0.54)
Lung	7.65 (1.31)	3.27 (1.00)
Duodenum	5.15 (1.67)	5.30 (1.72)

[a] Each reaction was incubated with crude tissue soluble extract after centrifugation at 40,000 × *g* using the tyrosine transaminase conditions described in the methods section. Each value represents the mean of duplicate determinations and results are expressed as the mean (S.E.M.) for 4 determinations. 1 Unit = formation of 1 μmol p-hydroxyphenylpyruvate per min.

The heterogeneous tissue distribution of tyrosine transaminase activities confirmed that multiple transaminase enzymes are present in rat tissues. Undoubtedly, the high level of tyrosine/α-ketoglutarate transaminase activity present in liver extracts was due to tyrosine aminotransferase (Lin and Knox, 1958; Groenwald et al., 1984). The isoenzymes of aspartate aminotransferase have also been reported to catalyze tyrosine transamination (Miller and Litwack, 1971; Shrawder and Martinex-Carrion, 1972; Spencer and Gelehrter, 1974; Hargrove and Mackin, 1984), which provides an explanation for the presence of tyrosine/α-ketoglutarate in nonhepatic tissues. The pattern for the distribution of tyrosine/glyoxylate transaminase activity in rat tissues highly consistent with published data for glutamine transaminase-K (Cooper and Meister, 1974, 1981).

4.2. Purification of tyrosine/glyoxylate transaminase from rat brain

We previously reported a 507-fold purification of an enzyme possessing tyrosine/glyoxylate aminotransferase activity to electrophoretic homogeneity from rat kidney (Bowsher and Henry, 1985). This work served as the foundation for development of an optimized strategy for purifying tyrosine/glyoxylate aminotransferase from a cytosolic fraction of whole rat brain. The resulting purification scheme for brain tyrosine/glyoxylate transaminase is summarized in Table 2. For the rat brain enzyme, the overall purification achieved was 670-fold with a 68% recovery.

Since our research interest focused on a potential role of Tyr transamination in modulating biosynthesis of catecholamines and trace amines, this investigation was aimed specifically at isolation of transaminases from the cytosolic fraction of brain. Procedural steps in the purification scheme were performed at 0-4 °C. Upon attaining fresh tissue from Wistar rats (101 g), brains were minced and homogenized with 4 vol of ice cold 0.25 M sucrose per g of tissue using a Brinkmann Polytron at one-half maximal setting. The resulting homogenate was centrifuged at 40,000 × *g* for 20 min and the supernatant underwent another highspeed centrifugation in an ultracentrifuge at 220,000 × *g* at about 4 °C for 1 h. This procedure permitted isolation of cytosolic transaminases, while limiting enzyme liberation from mitochondria and other subcellular organelles. This conclusion was verified by an

Table 2
Purification of tyrosine/glyoxylate transaminase from rat brain.

Procedure	Activity[a]	Yield	Protein	Specific Activity	Purification
	(mUnits)	(%)	(mg)	(mUnits/mg)	(-fold)
220,000 × *g* supernatant	317	100	4300	0.073	1.0
31–57% ammonium sulfate	367	116	900	0.41	5.6
Phenyl-Sepharose	300	95	100	3.0	41.0
DEAE-Sephacel	250	79	15.0	17.0	230
Hydroxyapatite	217	68	4.4	49.0	670

[a] Tyrosine transaminase was quantified using the optimized assay conditions described in the Methods section. For this assessment the tyrosine concentration was 1 mM, the glyoxylate concentration was 10 mM and the incubation buffer consisted of potassium borate, pH 8.5. An enzyme unit catalyzed the formation of 1 μmol of p-hydroxyphenylpyruvate/min.

investigation of subcellular tissue compartmentation involving known marker enzymes (data not shown).

4.2.1. Ammonium sulfate fractionation

The crude preparation was subsequently treated with solid ammonium sulfate between 31 and 57% saturation (1.2–2.2 M) (Wood, 1976). The protein precipitate was collected by centrifugation at 40,000 × *g* for 10 min and resuspended with 25 mL of 0.1 M K phosphate/0.1 mM EDTA/0.1 mM PLP, pH 7.5. Batch ammonium sulfate protein precipitation proved to be highly efficient at removing aspartate aminotransferase (Table 3). As reported in several investigations, we noted that aspartate aminotransferase is precipitated only at a high level of ammonium sulfate saturation (Aunis et al., 1971; Magee and Phillips, 1971; Miller and Litwach, 1971). Accordingly, we determined that efficient precipitation of aspartate aminotransferase by ammonium sulfate required > 60% saturation, whereas tyrosine/glyoxylate transaminase activity only necessitated 40% saturation for complete precipitation. Thus, for routine purification of the brain enzyme, we employed solid ammonium sulfate fractionation using 31–57% saturation which yielded > 100% recovery of tyrosine/glyoxylate transaminase activity, but simultaneously eliminated aspartate aminotransferase activity by more than 90% (Table 3). The low % saturation needed for precipitation was consistent with the reported data for purification of rat kidney glutamine transaminase-K (Cooper and Meister, 1974).

4.2.2. Phenyl-sepharose chromatography

Phenyl-sepharose chromatography served as the first chromatographic procedure in the purification scheme. In brief, a 30 × 1.6 cm phenyl-sepharose column was equilibrated with > 500 mL of 0.1 M potassium phosphate/0.1 mM EDTA, pH 7.5. Upon loading the crude protein, the column was eluted with the same phosphate buffer until the 280 nm absorbance returned to baseline. The column was then step-eluted by reducing the ionic strength of the mobile phase to 1 mM K phosphate/0.1 mM EDTA, pH 7.5. Fractions containing the peak activity were combined, concentrated in an Amicon ultrafiltration unit using a PM-10 membrane and equilibrated with 25 mM Bis-Tris/10% glycerol/0.1 mM EDTA, pH 6.5. Phenyl-sepharose chromatography yielded nearly a 10-times improvement in fold purification with high recovery, while simultaneously reducing recovery of aspartate aminotransferase to only 0.02%.

4.2.3. DEAE-Sephacel chromatography

Tyrosine/glyoxylate transaminase activity bound efficiently to this anion-exchange media in Bis-Tris buffer at pH 6.5 and eluted at around 0.1 M NaCl using a liner gradient of NaCl. These anion-exchange conditions were deemed to be useful for removing contamination from other known tyrosine transaminases. Hepatic tyrosine aminotransferase is reported to bind to a cation-exchange resin at pH 6.5 (Johnson et al., 1973; Donner et al., 1978; Belarbi et al., 1979; Hargrove et al., 1980). Likewise, the mitochondrial form for aspartate aminotransferase also binds to CM-cellulose at pH values of 6.4–7.2 (Magee and Phillips, 1971; Miller and Litwack, 1971).

4.2.4. Hydroxyapatite chromatography

Hydroxyapatite chromatography, the final step in the purification scheme, was conducted using 5 mM K phosphate/10% glycerol/0.1 mM DTT, pH 7.5 as the adsorption buffer. Tyrosine glyoxyate activity was retained only slightly and eluted shortly after the non-adsorbed peak. In contrast, tyrosine aminotransferase and both isoenzymes of aspartate aminotransferase adsorb to hydroxyapatite column at a phosphate concentration > 0.1 M (Iwaski and Pitot, 1971; Shrawder and Martinez-Carrion, 1972; Ohisalo and Pispa, 1976; Hargrove et al., 1980; Hargrove and Mackin, 1984).

Collectively, the chromatographic procedures employed in this study were engineered to efficiently eliminate contamination from other known tyrosine transaminase enzymes. As reported in Table 3, the ratio of the recoveries of tyrosine/glyoxylate and glutamine/phenylpyruvate transaminase activities remained constant throughout the entire purification scheme. In contrast, the purified enzyme preparation was devoid of aspartate aminotransferase activity. Based on the criteria of co-purification of aminotransferase activities, our data indicated that rat brain tyrosine/glyoxylate transaminase and glutamine/phenylpyruvate transaminase, a marker of glutamine transaminase-K (Cooper, 1978), are indeed the same enzyme. Purity of the brain enzyme was confirmed by SDS-PAGE which demonstrated a single major peak with only 2 minor bands. Thus, we concluded that a single purified enzyme was responsible for catalyzing both the tyrosine/glyoxylate and glutamine/phenylpyruvate transaminase reactions. In agreement with Cooper and Meister (1984, 1985), the purified enzyme was stable for an extended period in dilute phosphate buffer containing 10% glycerol/1 mM DTT/10 μM pyridoxal-5′-phosphate.

4.3. Immunochemical analysis of brain tyrosine transaminase

A rabbit polyclonal antiserum to highly purified rat renal tyrosine/glyoxylate transaminase (glutamine transaminase-K) was used to

Table 3
Recovery of different aminotransferase activities through the rat brain enzyme purification scheme.

Procedure	% Recovery		
	Tyrosine/glyoxylate	Glutamine/phenylpyruvate	Aspartate/α-ketoglutarate
220,000 × *g* supernatant	100	100	100
31–57% ammonium sulfate	116	121	9.3
57–82% ammonium sulfate	9.2	6.3	95.0
Phenyl-Sepharose	95	104	0.02
DEAE-Sephacel	79	80	0.004
Hydroxyapatite	68	65	0.003

[a]The different transaminase activities were measured using the assay conditions described in the Methods section. For this assessment the tyrosine concentration was 1 mM, the glyoxylate concentration was 10 mM and the incubation buffer consisted of potassium borate, pH 8.5.

conduct immunochemical evaluations of brain tyrosine/glyoxylate transaminase. In brief, the antiserum was created by immunizing 2 New Zealand white rabbits subcutaneously with approximately 250 μg of purified rat kidney enzyme in complete Freund's adjuvant. The rabbits then were injected intradermally at multiple sites with an additional 100 μg of purified enzyme in incomplete Freund's adjuvant at 21-day intervals. Fourteen days after the second and third boosts, production bleeds were taken from the ear veins. Whole blood was allowed to clot at ambient temperature. After about 30 min, the serum was collected by centrifugation for about 15 min at 2500 × *g* at 4 °C. Sera from both rabbits were determined to be positive for tyrosine/glyoxylate transaminase (glutamine transaminase-k) reactive antibodies by double immunodiffusion agar gels and immunoprecipitation experiments. The final antiserum was prepared by pooling the crude antisera from both rabbits and storing it in aliquots frozen at −80 °C.

This antiserum completely neutralized both the tyrosine/glyoxylate and phenylalanine/α-keto-methiolbutyrate transaminase reactions in extracts of rat kidney (Bowsher and Henry, 1985). Because the co-substrates, phenylalanine/α-keto-methiolbutyrate, are a specific marker for glutamine transaminase-K (Cooper, 1978; Cooper and Meister, 1981, 1984, 1985), we concluded purified renal tyrosine transaminase was glutamine transaminase-K. In addition, the antiserum did not demonstrate any neutralization of rat liver tyrosine/α-ketoglutarate transaminase which indicated that antiserum did not cross-react with hepatic tyrosine aminotransferase (EC 2.6.1.5).

For the current study we determined that the specific rabbit antiserum produced an equivalent degree of neutralization of both the tyrosine/glyoxylate transaminase and glutamine/phenylpyruvate transaminase activities present in the purified brain enzyme (r^2 = 0.976) (Fig. 1). Similarly, the antiserum efficiently neutralized the transaminases present in a crude cytosolic extract of whole rat brain (r^2 = 0.990) (Fig. 2). These immunochemical findings strongly support the idea that the same enzyme is catalyzing both transaminase reactions and documented that glutamine transaminase-K represents a major *in vitro* tyrosine transaminase in a cytosolic extract of rat brain. Verification of the presence of glutamine transaminase-K in rat brain supports numerous earlier reports regarding its potential role in CNS metabolism of glutamine and kynurenine (Cooper et al., 2008; Han et al., 2010a,b; Cooper and Kuhara, 2014) and increases awareness of this enzyme's *in vitro* reactivity towards other aromatic acids, including tyrosine.

4.4. Immunochemical analysis of tyrosine transaminase in a soluble extract of rat brain with different α-keto acid co-substrates

An immunoanalytical experiment was also performed to specifically investigate the spectrum of different tyrosine transaminase enzymes present in a soluble extract of Wistar rat brain. For this experiment 20 g of fresh whole rat brain tissue, including cerebellum, was homogenized with 4 vol of 0.25 M sucrose using a Polytron homogenizer and centrifuged at 40,000 × *g* for 20 min. The supernatant was adjusted to 82% saturation with solid ammonium. After centrifugation, the pelleted protein was resuspended with 15 mL of buffer and dialyzed for about 18 h at 4 °C against 5 L of PBS/0.1 mM EDTA/0.01 mM pyridoxal-5′-phosphate, pH 7.5.

The extent tyrosine transamination by the cytosolic brain extract was investigated using eight different α-keto acids before and after treatment with the specific rabbit anti-renal tyrosine/glyoxylate transaminase (glutamine transaminase-K) antiserum. As reported in Table 4, the tyrosine transaminase activity associated with glyoxylate, α-keto-caproate, α-keto-methiolbutyrate, phenylpyruvate, and α-keto-butyrate were neutralized efficiently (> 70%) upon antibody treatment. In contrast, the antiserum was only partially effective at neutralizing tyrosine transamination when pyruvate and oxaloacetate served as the amino acceptors. When α-ketoglutarate was used as the co-substrate the degree of neutralization of tyrosine transamination was only 13% relative to control. Antiserum treatment also neutralized > 90% of the glutamine/phenylpyruvate transaminase activity, the marker for

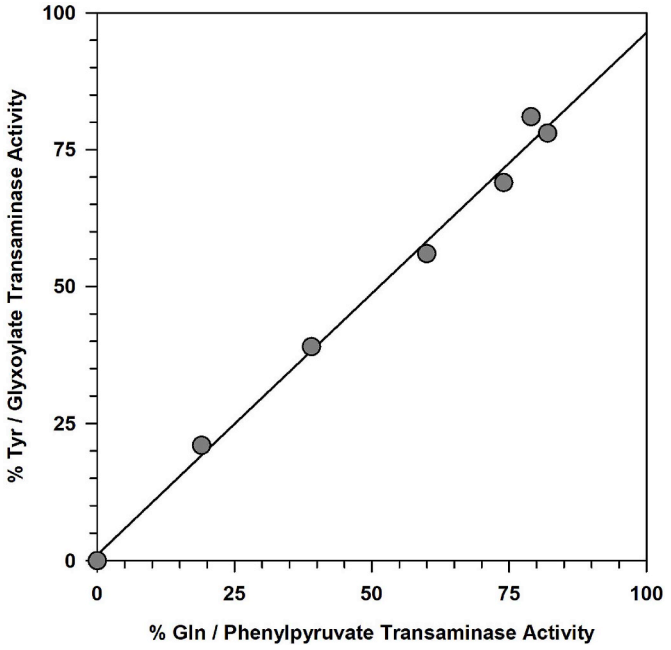

Fig. 1. Immunological evaluation of the tyrosine/glyoxylate and glutamine/phenylpyruvate transaminase enzyme activities of purified rat brain cytosolic tyrosine transaminase. As described in the Methods section, 670-fold purified enzyme was incubated with varying concentrations of the rabbit polyclonal antiserum to rat renal tyrosine transaminase (glutamine transaminase-K). Antibody treatment produced an equivalent degree [y = 0.95(x) + 1.09; r^2 = 0.976] in the reduction in both transaminase activities. In addition, when added to the incubation at a high level, the antiserum completely neutralized both transaminases. This finding strongly supports the identification of the purified brain enzyme as glutamine transaminase-K.

glutamine transaminase-K, but only produced a small degree of neutralization of aspartate aminotransferase. In this experiment the α-keto acids that demonstrated the highest rates of *in vitro* tyrosine transamination were the same ones that were most efficiently neutralized by the specific antiserum. Thus, while differences in the effectiveness of the antiserum to neutralize the different α-keto acid dependent tyrosine transamination reactions supports the presence of multiple enzymes in rat brain, it also strongly supports the view that glutamine transaminase-K should be regarded as a major *in vitro* tyrosine transaminase across a range of α-keto acid co-substrates.

4.5. Characterization of purified rat brain tyrosine/glyoxylate transaminase

4.5.1. α-Keto acid specificity

The first property of the purified brain tyrosine transaminase examined was the relative rates of enzymic activity with respect to different α-keto acids. The relative rates were evaluated at a final co-substrate concentration of 0.5 mM and 10 mM. As reported in Table 5, tyrosine underwent detectable transamination with all α-keto acids tested, except α-ketoglutarate. This finding verified that the purified enzyme was neither tyrosine aminotransferase nor aspartate aminotransferase.

As shown in Fig. 3, some generalizations are apparent regarding the enzyme's α-keto acid co-substrate specificity. The highest rates of tyrosine transamination were obtained with the α-keto acids derived from large neutral or aromatic amino acids (*i.e.,* tyrosine, methionine, and phenylalanine) (black diamonds) when evaluated at a concentration of 0.5 mM. Each of these co-substrates demonstrated appreciable substrate inhibition when analyzed at 10 mM. In contrast, the observed rate of tyrosine transamination for all linear aliphatic α-keto acids (black circles) was greater at 10 mM with no evidence of substrate inhibition and increased in response to increased length of the sidechain.

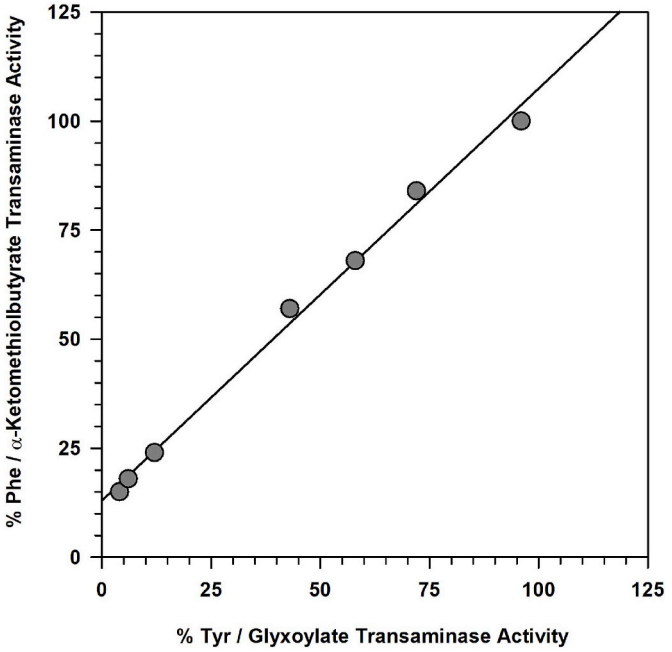

Fig. 2. Immunological evaluation of the tyrosine/glyoxylate and phenylalanine/α-keto-methiolbutyrate transaminase activities in a soluble extract of whole rat brain. As described in the Methods section, a crude soluble extract of whole rat brain was treated with varying amounts of the rabbit polyclonal antiserum to rat renal tyrosine transaminase (glutamine transaminase-K). Antibody treatment produced a proportional reduction in both transaminase activities [y = 0.945x + 13.0; r^2 = 0.990]. At a high level the antiserum completely removed the tyrosine/glyoxylate aminotransferase activity and about 90% of the phenylalanine/α-ketomethiolbutyrate transaminase activity. This finding strongly supports identification of rat brain tyrosine/glyoxylate transaminase as glutamine transaminase-K. Of note, about 10% the phenylalanine/α-ketomethiolbutyrate transaminase persisted in the supernatant after antibody neutralization. Incomplete removal by the antiserum suggests the presence of other tyrosine transaminases that differ in their antigenicity.

Table 4
Immunoprecipitation of transaminase activities in a cytosolic extract of whole rat brain.

	[a]Activity (mUnits/mg protein)		
Co-substrates	Before PAb Treatment (A)	After PAb Treatment (B)	[b]% Activity Removed
Tyr/α-keto-methiolbutyrate	0.83	0.12	86
Tyr/phenylpyruvate	0.54	0.08	85
Tyr/α-keto-caproate	0.46	0.05	89
Tyr/α-keto-butyrate	0.41	0.11	73
Tyr/glyoxylate	0.36	0.01	97
Tyr/pyruvate	0.34	0.11	68
Tyr/oxaloacetate	0.09	0.05	44
Tyr/α-ketoglutarate	0.08	0.07	13
Asp/α-ketoglutarate	7380.	7020.	5
Gln/phenylpyruvate	6.37	0.54	92

[a] The different transaminase reactions were conducted using the assay conditions described in the Methods section. For this assessment the tyrosine concentration was 1 mM, the concentration of the various α-keto acids was 10 mM and the incubation buffer consisted of potassium borate, pH 8.5.

[b] % Activity removed = [(A – B)/B] x 100.

Transamination rates for the iso-analogs of the aliphatic α-keto acids (red triangles) was less than for their corresponding linear forms. Negligible rates of tyrosine transamination were obtained when α-keto-isovalerate, the α-keto acid of valine, and the dicarboxylic acids, oxaloacetate and α-ketoglutarate (blue squares), were used as the amino acceptors. Their reaction rates did not show a marked enhancement upon increasing the concentration from 0.5 to 10 mM. Our reported α-keto acid co-substrate specificity profile agrees well with the published

Table 5
Relative rates of tyrosine transamination by purified rat brain enzyme with different α-keto acid co-substrates.

	[a]Tyrosine Transamination Rate (mUnits/mg protein)	
α-Keto acid	0.5 mM	10 mM
p-hydroxyphenylpyruvate	173.0	57.0
α-keto-methiolbutyrate	151.0	60.3
phenylpyruvate	125.0	21.5
α-keto-caproate	59.5	101.0
α-keto-isocaproate	22.3	65.3
α-ketovalerate	18.5	90.0
glyoxylate	13.0	68.5
α-keto-butyrate	7.17	77.2
pyruvate	5.83	60.5
oxaloacetate	1.50	5.67
α-keto-isovalerate	1.00	0
α-ketoglutarate	0	0

[a] Transaminase activities were measured using the assay conditions described in the Methods section. For this assessment the incubation included 1 mM tyrosine and included 0.65 μg of purified enzyme.

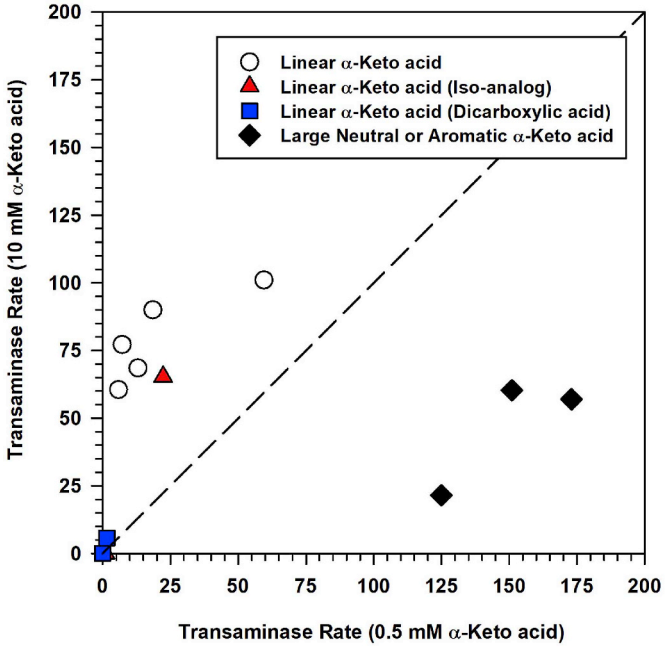

Fig. 3. Evaluation of the impact of α-keto acid co-substrate concentration on tyrosine transaminase reaction rates. The relative rates of *in vitro* tyrosine transamination by the purified rat brain enzyme were investigated using 12 different α-keto acids at the final concentrations of 0.5 mM and 10 mM. Upon inspection of the plot some generalizations are apparent. The line of identity is depicted as the dashed line. The rates of transamination were greatest with the α-keto-acids derived from tyrosine, methionine and phenylalanine (black diamonds) at a concentration of 0.5 mM. All demonstrated marked substrate inhibition when added to the incubation at 10 mM. Reaction rates for linear α-keto acids (open circles) generally increased with respect to the length of their aliphatic side chain. Moreover, these α-keto-acids did not show substrate inhibition at a concentration of 10 mM. Both of the iso-α-keto-acid analogs tested (red triangles) demonstrated a reduced rate of transamination relative to their linear aliphatic forms. The dicarboxylic acid containing α-keto acids (blue squares) showed much reduced rates of transamination. While a low level of tyrosine transamination was measurable with oxaloacetate, none was detectable when α-ketoglutarate was used as the co-substrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

specificity of glutamine transaminase-K (Cooper and Meister, 1974, 1981, 1984 and 1985) and kynurenine aminotransferase-I (Han et al., 2004, 2010). It's likely that the pronounced inhibition seen for some α-keto acids is one reason for potential differences reported in the reactivity of some amino acceptor co-substrates.

4.5.2. Kinetic analysis

The kinetics of tyrosine transamination were investigated using several different α-keto acids. Of these, the highest affinity and maximal velocity were obtained with α-ketomethiolbutyrate (Table 6). The Michaelis constant for p-tyrosine (1.41 ± 0.46 mM) was calculated by averaging the estimated K_M values from secondary report data for each α-keto acid.

Detailed kinetic evaluation of selected α-keto acids supports the findings reported in Table 5 and depicted in Fig. 3. The largest V_M/K_M ratio was obtained with α-ketomethiolbutyrate. Substantially lower values were obtained for α-keto-caproate and glyoxylate. The kinetic results reported in Table 6 agree well with data published previously by Cooper and Meister (1974) and kinetic constants reported by Van Leuven (1976). Pyruvate was determined to function as a co-substrate for purified tyrosine transaminase (Table 5). Yet, its catalytic efficiency was only 0.3% of that for α-ketomethiolbutyrate. Thus, this finding suggests that pyruvate should only be regarded as a potential co-substrate for *in vivo* tyrosine transamination by glutamine transaminase-K in tissues that have a low concentration of other α-keto acids.

4.5.3. Specificity for aromatic amino acids

Because aromatic amino acids serve as precursors for the synthesis of monoamine neurotransmitters and trace amine neuromodulators, the substrate specificity of the purified rat brain tyrosine transaminase was evaluated with respect to these amino acids. For this series of experiments, α-ketomethiolbutyrate was used as the amino acceptor, as it was one of the best α-keto acid co-substrates for the purified enzyme (Table 7) and it did not absorb in the near uv. As described in our earlier preliminary report, all aromatic acids tested were determined to be substrates for the purified brain transaminase, including L-dopa and L-5-hydroxytryptophan (Bowsher and Henry, 1985). More recently other investigators have also reported that L-dopa is a substrate for glutamine transaminase-K (Cooper et al., 2008). Combined with the more recent information regarding reactivity with kynurenine, our data confirm that glutamine transaminase-K possesses broad substrate specificity which includes large neutral and aromatic amino acids in addition to glutamine.

4.6. Regional distribution of tyrosine/glyoxylate transaminase in rat brain

We examined the regional distribution of tyrosine/glyoxylate transaminase activity in rat brain to investigate its potential functional significance. Whole brains were collected from 4 male Wistar rats (250 g) and immediately immersed in chilled isotonic saline. After a few min, the brains were placed on a chilled metal surface and then dissected anatomically according to the procedure of Glowinski and Iverson (1966). Each tissue was homogenized with 4 vol of 0.25 M sucrose per g tissue using a Polytron homogenizer and centrifuged for about 15 min at 30,000 × *g* to obtain the cytosolic fraction. The supernatants were assayed directly by the validated tyrosine/glyoxylate transaminase assay as described in the Methods.

Table 6
Kinetic characterization of selected α-keto co-substrate with purified rat brain tyrosine transaminase.

α-Keto co-substrate	K_M (mM)	V_M (mUnits/mg)	V_M/K_M
glyoxylate	1.40	100	71.4
α-keto-caproate	3.36	455	135.
pyruvate	7.29	51.2	7.0
α-keto-methiolbutyrate	0.34	783	2300.

Table 7
Transamination of aromatic amino acids by purified rat brain tyrosine transaminase.

[a]Amino acid	Activity (mUnits/mg protein)
L-phenylalanine	1030
D,L-m-tyrosine	750
L-p-tyrosine	717
L-histidine	717
L-3,4-dihydroxyphenylalanine (DOPA)	317
L-tryptophan	163
L-5-hydroxytryptophan (5-HTP)	28.3

[a] Each transaminase reaction was incubated with 4 mM amino acid and 4 mM α-keto-methiolbutyrate using the assay conditions described in the Methods section.

The regional distribution of tyrosine transaminase activity was mostly consistent across all brain areas (547 ± 38 nmol/h/g tissue, mean ± S.E.M.), except for cerebellum (Fig. 4). The *in vitro* rate of tyrosine transamination was significantly higher in this brain region (1320 ± 51 nmol/h/g tissue) by a factor of about 2½. This finding indicates that either the concentration of enzyme is higher in cerebellar tissue or other undetermined factors were present in the tissue extract milieu that resulted in a higher rate of *in vitro* tyrosine transamination.

Several studies have reported a uniform distribution for various aromatic amino acid transaminases in mammalian brain (Benuck and Lajtha, 1975; Gabay and Clarke, 1983). Most investigations directed specifically at tyrosine/α-ketoglutarate transaminase have also found a relatively homogeneous distribution of this activity in rat brain (Miller and Litwack, 1969; Mark et al., 1970; Benuck et al., 1972). In contrast, a study by Oja (1968) reported a significantly higher level of aromatic amino acid transaminase activity in rat cerebellum. Thus, our findings agree with Oja and support a heterogeneous regional distribution in rat brain with a higher level of *in vitro* transaminase activity in cerebellum. This finding is one major difference that distinguishes the *in vitro* transamination of tyrosine when glyoxylate and α-ketoglutarate are used as the respective α-keto acid co-substrates. More recently, Malherbe et al. (1995) reported that unlike cortical neurons and astroglial cells, the cerebellum of rat brain has similar amounts of the soluble and mitochondrial forms of glutamine transaminase-K. Thus, the heterogenous distribution of this enzyme with a higher level in cerebellum warrants further investigation regarding its physiological role in this brain region.

5. Conclusion

This study has confirmed the identification of highly purified rat brain cytosolic tyrosine/glyoxylate transaminase as glutamine transaminase-K. This conclusion was based on multiple findings. First, purification of tyrosine/glyoxylate transaminase from a soluble fraction of rat brain resulted in a concurrent enrichment in the glutamine/phenylpyruvate transaminase activity, a known marker for glutamine transaminase-K (Cooper, 1978). In addition, during purification, a constant ratio of recoveries was noted for both tyrosine/glyoxylate and glutamine/phenylpyruvate aminotransferase activities. Secondly, the α-keto acid co-substrate specificity of the purified transaminase was consistent with published information for glutamine transaminase-K (Cooper and Meister, 1974, 1981; Van Leuven, 1976, 1984, 1985). The highest rates of tyrosine transamination occurred with the α-keto acids of tyrosine, methionine and phenylalanine which also displayed appreciable substrate inhibition (Fig. 3). In contrast, the aliphatic α-keto acid co-substrates examined did not display substrate inhibition in the range of concentrations evaluated in this study. This attribute may help explain differences in reported transamination rates for α-keto acids across published studies. With respect to amino acid specificity, the purified rat brain enzyme catalyzed transamination of tyrosine and glutamine, as well as all other aromatic amino acids tested. While

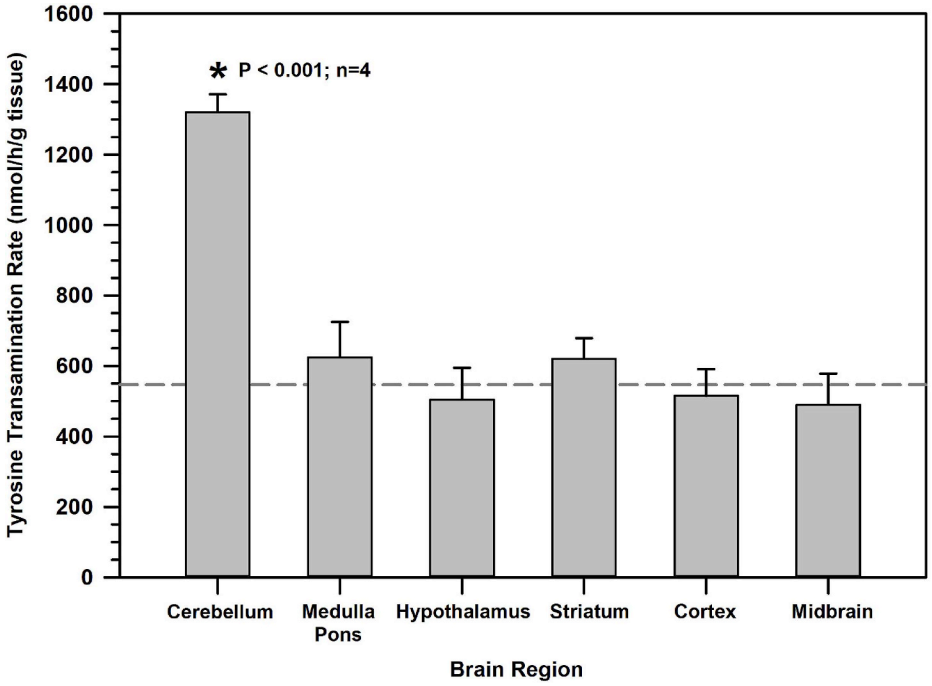

Fig. 4. Regional distribution of tyrosine/glyoxylate aminotransferase in rat brain. Brains from 4 male Wistar rats were obtained freshly, chilled in ice-cold isotonic saline, dissected anatomically, homogenized in 4 vol of 0.25 M sucrose and then centrifuged. The level of tyrosine/glyoxylate transaminase was then quantified in the resulting supernatants using the assay described in the Methods section. As shown, the *in vitro* rate of tyrosine transamination was relatively consistent across most brain regions (mean value of 547 ± 38 nmol/h/g tissue is shown as the dashed line). Unlike the other regions, the extract from cerebellum displayed a significantly higher rate of tyrosine transamination (1320 ± 51 nmol/h/g tissue) by a factor of about 2½.

aromatic amino acids are good substrates for the K-form of glutamine transaminase, they are poor substrates for the L-form of glutamine transaminase (Cooper and Meister, 1972, 1981, 1984, 1985). In this study, glutamine transaminase-L (*i.e.,* L-albizziin/glyoxylate transaminase activity) was very low in crude soluble extracts of brain and we found no evidence of an enrichment in this transaminase during purification of tyrosine/glyoxylate transaminase (data not shown). Pinto et al. (2014) reported that recombinant mKAT-3 (glutamine transaminase-L) reacts efficiently with the co-substrate pair, L-albizziin and glyoxylate. In addition, Han et al. (2010a,b) found that oxaloacetate is a good amino acceptor, while both pyruvate and phenylpyruvate are poor co-substrates for mKAT-3. More recently, Yang et al. (2016) reported on the characterization of recombinant forms of mKAT-1 and mKAT-3. While we recognize the potential for a species difference, it seems the specificity of our purified cytosolic brain enzyme is more like KAT-1, especially based on the low reactivity obtained with oxaloacetate (see Table 5). Thus, we believe the totality of biochemical and immunochemical evidence supports the conclusion that purified cytosolic Tyr/glyoxylate transaminase enzyme is GlnT-K (KAT-1) rather than being GlnT-L (KAT-3). Third, the results of the kinetic analysis were consistent with the identify of purified brain tyrosine transaminase as glutamine transaminase-K, with p-tyrosine having a K_M of 1.41 mM. Fourth, at equimolar concentrations glutamine was a potent *in vitro* inhibitor of tyrosine/glyoxylate transaminase activity of the purified brain enzyme. Fifth, biochemical characterization was consistent with the identity of the purified enzyme as glutamine transaminase-K, including pH optimum of 8.5 (Cooper and Meister, 1972, 1974) and spectrophotometric analysis revealed absorption maxima of 280 and 411 nm (Cooper and Meister, 1972; Van Leuven, 1975). Lastly, immunochemical evidence confirmed the identify of purified brain cytosolic tyrosine/glyoxylate transaminase as glutamine transaminase-K, as a specific antiserum to rat renal glutamine transaminase-K neutralized both the tyrosine/glyoxylate and glutamine/phenylpyruvate transaminase activities in the purified enzyme. Moreover, the antibody efficiently neutralized the *in vitro* tyrosine transaminase activity present in a crude soluble extract of rat brain for a number of different α-keto acid co-substrates. This finding suggests that glutamine transaminase-K should be regarded as a predominant *in vitro* activity when evaluating tyrosine transamination in rat brain tissue and other organs. In conclusion, this study has confirmed that glutamine transaminase-K represents a major *in vitro* enzyme in cytosolic extracts of rat brain that's capable of catalyzing the transamination of p-tyrosine and other aromatic amino acids, including L-dopa and 5-hydroxytryptophan.

Funding

This research project did not receive any specific grant from funding agencies, in the public, commercial or not-for-profit sectors.

CRediT authorship contribution statement

Ronald R. Bowsher: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **David P. Henry:** Conceptualization, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of competing interest

The authors have no conflict of interest to report.

Acknowledgments

We wish to thank Ms. Shanna Northrup for her assistance in helping to prepare this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104653.

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