Published in final edited form as:
Arch Biochem Biophys. 2008 June 1; 474(1): 72–81. doi:10.1016/j.abb.2008.02.038.

Substrate specificity of human glutamine transaminase K as an aminotransferase and as a cysteine *S*-conjugate β-lyase

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Abstract

Rat kidney glutamine transaminase K (GTK) exhibits broad specificity both as an aminotransferase and as a cysteine *S*-conjugate β-lyase. The β-lyase reaction products are pyruvate, ammonium and a sulfhydryl-containing fragment. We show here that recombinant human GTK (rhGTK) also exhibits broad specificity both as an aminotransferase and as a cysteine *S*-conjugate β-lyase. *S*-(1,1,2,2-Tetrafluoroethyl)-L-cysteine is an excellent aminotransferase and β-lyase substrate of rhGTK. Moderate aminotransferase and β-lyase activities occur with the chemopreventive agent *Se*-methyl-L-selenocysteine. L-3-(2-Naphthyl)alanine, L-3-(1-naphthyl)alanine, 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA are measurable aminotransferase substrates, indicating that the active site can accommodate large aromatic amino acids. The α-keto acids generated by transamination/L-amino acid oxidase activity of the two catechol cysteine *S*-conjugates are unstable. A slow rhGTK-catalyzed β-elimination reaction, as measured by pyruvate formation, was demonstrated with 5-*S*-L-cysteinyldopamine, but not with 5-*S*-L-cysteinyl-L-DOPA. The importance of transamination, oxidation and β-elimination reactions involving 5-*S*-L-cysteinyldopamine, 5-*S*-L-cysteinyl-L-DOPA and *Se*-methyl-L-selenocysteine in human tissues and their biological relevance are discussed.

Keywords

Cysteine *S*-conjugate β-lyase; 5-*S*-L-cysteinyl-L-DOPA; 5-*S*-L-cysteinyldopamine; glutamine transaminase K; *Se*-methyl-L-selenocysteine

Glutamine transaminase K (GTK)[1] purified from rat and bovine tissues accepts a large number of neutral, aromatic and sulfur/selenium-containing substrates [1–6]. GTK catalyzes

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[1]*Abbreviations used*: BTC, benzothiazolyl-L-cysteine; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DOPA, dihydroxyphenylalanine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GSH, glutathione; KAT, kynurenine aminotransferase; KGDHC, α-ketoglutarate dehydrogenase complex; KGM, α-ketoglutaramate; KMB, α-keto-γ-methiolbutyrate; MBTH, 3-methyl-2-benzothiazolinone hydrazone; MDH, malate dehydrogenase; MPA, metaphosphoric acid; PDHC, pyruvate dehydrogenase complex; PLP, pyridoxal 5′-phosphate; rhGTK, recombinant human glutamine transaminase K; TFEC, *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine

transamination of kynurenine to kynurenate, and is also known as kynurenine aminotransferase isozyme I (KAT I) [7]. Important biological roles include salvage of the α-keto acid analogues of several essential amino acids and closure of the last step of the methionine salvage pathway (reviewed in [5]). GTK also possesses β-lyase activity toward many cysteine *S*-conjugates that possess a strong electron-withdrawing group attached at the sulfur [5–6,8–10]. The products are aminoacrylate [CH_2=CH(NH_3^+)CO_2^-] and a sulfur-containing fragment [XSH]. The aminoacrylate is non-enzymatically converted to pyruvate [$CH_3C(O)CO_2^-$] and ammonium (Eqn. 1).

$$XSCH_2CH(NH_3^+)CO_2^- + H_2O \rightarrow CH_3C(O)CO_2^- + NH_4^+ + XSH \quad (1)$$

Rat kidney GTK catalyzes β-elimination reactions with *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC; the cysteine *S*-conjugate of trichloroethylene), *S*-(1,1,2,2-tetrafluoroethyl)-L-cysteine (TFEC; the cysteine *S*-conjugate of tetrafluoroethylene) and many other cysteine *S*-conjugates derived from halogenated alkenes [5–6,8–10]. However, when these halogenated cysteine *S*-conjugates are substrates, transamination competes with the β-lyase reaction. As a result, an α-keto acid substrate [e.g. α-keto-γ-methiolbutyrate (KMB), phenylpyruvate] or pyridoxal 5′-phosphate (PLP) must be present in the reaction mixture to ensure optimal GTK-catalyzed β-lyase activity [5–6,8–10]. Interestingly, L-selenocysteine *Se*-conjugates are more active aminotransferase and β-lyase substrates of rat kidney GTK than are the corresponding cysteine *S*-conjugates, in most cases by an order of magnitude or more [6]. As a consequence, GTK has been suggested to be a possible target for selenium-containing chemopreventive drugs [6].

The sulfur-containing fragments cleaved from the cysteine *S*-conjugates of halogenated alkenes are highly toxic in experimental animals, especially to the kidneys, and GTK has been implicated in the bioactivation process [e.g. 11,12]. Most studies implicating GTK were carried out with the intact rat, isolated rat tissues or purified rat kidney GTK. GTK/KAT 1 activity, as assessed by standard assay procedures, is widely distributed in human tissues [13–15]. However, in order to determine whether GTK might contribute in humans to bioactivation of potentially toxic cysteine *S*-conjugates and act as a possible prodrug target, it is important to determine whether human GTK has similar broad catalytic properties to those of the rat enzyme.

Human GTK was purified from human kidney tissue in 1990 [13] and its sequence obtained in 1995 [16]. More recently, recombinant human GTK (rhGTK) has become available [17, 18]. Human GTK is a homodimer (subunit M_r ~47,900) containing one PLP per monomer [17] that was previously shown to catalyze β-lyase reactions with benzothiazolyl-L-cysteine (BTC), TFEC and DCVC [13,16]. The human enzyme has an 82% amino acid homology to the rat enzyme and 90% similarity around the active site [16]. Crystallized rhGTK has the prototypical fold of an aminotransferase of subgroup I [17]. As is typical of these aminotransferases, the active site of rhGTK is open in the absence of substrate, but changes to a more closed arrangement upon covalent attachment of the amino acid substrate to the PLP-coenzyme (but see the Discussion). The enzyme has an unusual “crown” of aromatic amino acid residues in the binding pocket, which may account for the interaction with neutral amino acids, including cysteine *S*-conjugates [17].

In the present work we have extended previous studies on the specificity of rhGTK. Several large amino acids, the potential prodrug *Se*-methyl-L-selenocysteine and potentially neurotoxic 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine were shown to be substrates. We have also extended specificity studies of snake (*Crotalus adamanteus*) venom L-amino acid oxidase, which, like rat kidney GTK, has broad specificity [e.g. 19], and used this enzyme to prepare *in situ* the α-keto acids of 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine. These α-keto acids were shown to be unstable, possibly contributing to the toxicity of 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine.

Materials and Methods

Materials

Trizma base (Tris), ammediol [2-amino-2-methyl-1,3-propanediol], 2,4-dinitrophenylhydrazine, L-kynurenine, L-phenylalanine, L-DOPA, L-glutamine, Lmethionine, L-tyrosine, *Se*-methyl-L-selenocysteine, L-selenomethionine, L-3-(1-naphthyl) alanine, L-3-(2-naphthyl)alanine, 3,5,3′-triiodo-L-thyronine (sodium salt), 3-methyl-2-benzothiazolinone hydrazone (MBTH), metaphosphoric acid (MPA), dithiothreitol (DTT), semicarbazide. HCl (solutions neutralized with NaOH before use), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), β-chloro-D,L-alanine, phenylpyruvic acid, and the sodium salts of pyruvate, α-ketoglutarate and KMB were obtained from Sigma Chemical Company (St. Louis, MO). β-(Cyclohexyl)-L-alanine was obtained from NovaBiochem (San Diego, CA). The sodium salt of β-(cyclohexyl)pyruvate was made from β-(cyclohexyl)-L-alanine by the method Meister [20]. DCVC was a gift from Dr. Robert Schwarcz (University of Maryland). TFEC [21] and BTC (acetate salt) [22] were gifts from Dr. Sam Bruschi, University of Washington, Seattle, USA. 5-*S*-L-Cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine were synthesized by the method of Spencer et al. [23]. The preparations were pure as judged by UV spectroscopic analysis, HPLC analysis with electrochemical detection (see also Figure 1A), and proton NMR analysis [23]. Stock solutions of these cysteine *S*-conjugates (20 mM) in water were stored at −20°C.

Enzymes

rhGTK was obtained by the method of Han et al. [18] and stored at 4°C at a concentration of 16 mg/ml in 15 mM potassium phosphate buffer (pH 6.8) containing 20% glycerol. The specific activity in the standard L-phenylalanine-KMB aminotransferase assay (see below) was 18 U/mg. *Crotalus adamanteus* L-amino acid oxidase (7.3 U/mg) and bovine liver catalase (200,000 U/ml) were obtained from Sigma Chemical Company (St. Louis, MO).

Determination of enzyme activities

The standard GTK assay was adapted from that of Cooper [24] for 96-well plate analyses and measures transamination between L-phenylalanine and KMB. The reaction mixture (0.05 ml) contained 20 mM L-phenylalanine, 5 mM KMB, 100 mM ammediol-HCl buffer (pH 9.0) and enzyme. After incubation at 37°C the reaction was terminated by addition of 0.15 ml of 1 M NaOH and the absorbance of phenylpyruvate-enol was determined at 320 nm. Under these conditions the extinction coefficient of phenylpyruvate-enol is 16,000 $M^{-1}cm^{-1}$. In most experiments, the ammediol buffer (pH 9.0) was replaced with 100 mM potassium phosphate buffer (pH 7.4). The activity of rhGTK at pH 7.4 is 50% that at pH 9.0 in the L-phenylalanine and KMB transaminase assay. To determine the activity of rhGTK toward L-tyrosine, L-DOPA, L-3-(1-naphthyl)alanine and L-3-(2-naphthyl)alanine as aminotransferase substrates the reaction mixture (0.05 ml) contained 5 mM L-amino acid, 5 mM KMB, 100 mM potassium phosphate buffer (pH 7.4). The reaction was terminated by addition of 0.15 ml of 1 M NaOH and the absorbance at 320 nm was determined within 2 min. The extinction coefficient of the resulting enol was assumed to be the same as that of phenylpyruvate enol. When L-DOPA was the amino acid substrate the loss of α-keto acid formed from this substrate was minimized by carrying out the enzymatic reaction for no more than 5 min before addition of base. To measure GTK-catalyzed transamination of L-kynurenine, the reaction mixture (0.05 ml) contained 5 mM amino acid, 100 mM potassium phosphate buffer (pH 7.4), 5 mM KMB and enzyme. Kynurenate was measured in the reaction mixture by the method of Hartline [25] except that the volume of the final mixture was 0.2 ml.

KMB was used as α-keto acid substrate for measurements of rhGTK-catalyzed transamination reactions involving 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA. The L-

methionine generated by transamination was measured by HPLC with CoulArray (coulometric) detection. Redox-sensitive compounds particularly those that contain a thiol or other oxidizable sulfur moieties are readily detected by this procedure. The analyte does not have to be derivatized, which is a distinct advantage when measuring potentially unstable compounds such as 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA. Preliminary experiments showed that incubation of KMB with the cysteine *S*-conjugates at 37°C resulted in some loss of α-keto acid as judged by disappearance of carbonyl function. The loss was dependent on the concentration of conjugate (data not shown). H_2O_2 is one of the products derived from autoxidation of dopamine [26]. Most likely, therefore, the loss of KMB is due at least in part to formation of H_2O_2 followed by oxidative decarboxylation of the α-keto acid. This loss could be minimized by inclusion of DTT. The optimized reaction mixture (0.01 ml) contained 5 mM 5-*S*-L-cysteinyldopamine (or 5-*S*-L-cysteinyl-L-DOPA), 1 mM KMB, 1 mM DTT, 100 mM potassium phosphate buffer (pH 7.4) and 3.2 μg of enzyme. After incubation at 37°C for 1 h the samples were frozen at −80°C until analyzed for methionine formation. To the reaction mixture was added 0.05 ml of distilled water and 0.015 ml of 25% (w/v) MPA. An aliquot (0.01 ml) was injected directly onto a Bio-Sil ODS-5S, 5−μm particle size, 4.0 × 250 mm, C18 column (Bio-Rad, Life Science Research Group, Hercules, CA) and eluted with a mobile phase consisting of 50 mM NaH_2PO_4, 3% (v/v) acetonitrile (pH 2.60) at a flow rate of 1 ml/min. PEEK™ (polyetheretherketone) tubing was used throughout the HPLC system, and a 0.2 μ PEEK™ filter was placed pre- and post-column to protect both column and flow cells, respectively, from any particulate matter. A Rheodyne injection valve with a 5-μl sample loop was used to manually introduce samples. The 8-channel CoulArray detectors (ESA, Inc., Chelmsford, MA) were set at 200, 300, 400, 450, 500, 550, 600 and 700 mV, respectively. The elution times for the redox-sensitive components methionine, KMB, 5-*S*-L-cysteinyl-L-DOPA, DTT, and 5-*S*-L-cysteinyldopamine, in the reaction mixture were 5.2, 6.1, 8.1, 9.4 and 11.8 min, respectively.

To determine the specificity of rhGTK toward non-aromatic L-amino acids as aminotransferase substrates, the reaction mixture (0.05 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM L-amino acid, 0.6 mM phenylpyruvate and enzyme. After incubation at 37°C, loss of phenylpyruvate was measured by adding 0.15 ml of 1 M NaOH and determining the absorbance at 320 nm due to phenylpyruvate-enol. To ensure that the rhGTK-catalyzed transamination of phenylpyruvate is in the linear range the reaction was terminated when no more than 15% of the phenylpyruvate had been consumed.

β-Lyase activity toward cysteine *S*-conjugates other than 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine was measured by a slight modification of the procedure described previously [27]. The reaction mixture (0.02 ml) contained 5 mM cysteine *S*-conjugate, 0.1 mM KMB, 100 mM potassium phosphate buffer (pH 7.4) and enzyme. After incubation at 37°C in a small snap-top tube, the reaction was terminated by addition of 0.01 ml of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After additional incubation of the mixture for 10 min at room temperature, 0.17 ml of 1 M NaOH was added and the absorbance at 430 nm was read within 2 min. The molar extinction coefficient of pyruvate 2,4-dinitrophenylhydrazone under these conditions is 16,000 M^{-1} cm^{-1}. KMB is included in the reaction mixture to ensure optimal β-lyase activity if transamination is a competing event. An identical procedure was used to determine β-lyase activity toward *Se*-methyl-L-selenocysteine. When β-chloro-D,L-alanine was the β-lyase substrate it was not necessary to include KMB.

HPLC was used to measure rhGTK-catalyzed pyruvate formation when 5-*S*-L-cysteinyldopamine was used as β-lyase substrates. The procedure relies on the formation of an azine when pyruvate is reacted with MBTH [28,29]. The reaction mixture (0.01 ml) in a small snap-top tube contained 5 mM L-amino acid, 1 mM KMB, 1 mM DTT, and 32 μg of enzyme. After incubation at 37°C for 1 h the reaction was stopped by addition of 0.005 ml of 3 M sodium

acetate and 0.01 ml of 0.1% (w/v) MBTH. Under these conditions, pyruvate MBTH-azine formation is complete in 40 min at 37°C. To the solution containing pyruvate MBTH-azine (0.025 ml) was added 0.08 ml of 50% acetonitrile. An aliquot (0.005 ml) of the reaction mixture treated with the MBTH reagent was injected directly into an HPLC system. Chromatographic separation and elution of the MBTH-azine α-keto acid derivatives were carried out with an MD-150 analytical column (3.0 × 150 mm, 3 micron) from ESA, Inc. (Chelmsford, MA), using a mobile phase buffer (75 mM trisodium citrate, 25 mM ammonium acetate, 26% (v/v) acetonitrile, pH 7.64) at a flow rate of 0.8 ml/min. Derivatives were measured using an ESA model 5600 CoulArray module, equipped with two coulometric array cell modules, each with four working electrodes set at 300, 400, 500, 550, 650, 750, 775 and 825 mV, respectively. 5-*S*-L-Cysteinyldopamine (and 5-S-L-cytseinyl-L-DOPA), DTT and methionine elute at or close to the void volume. On the other hand, the MBTH-azine derivatives of pyruvate and KMB are retained on the column.

Solutions of the MBTH-azine of pyruvate contain a mixture of *E*-and *Z*-isomers, that can be separated on HPLC [29]. Under our HPLC conditions, the *Z*-and *E*-isomers of the MBTH-azine derivatives of pyruvate and KMB elute at 2.8 and 3.1, and 5.2 and 5.8 min, respectively. Peak areas were analyzed using ESA, Inc, software. In some cases, the two isomers were not completely resolved. Thus, the area under both peaks was summed in calculating pyruvate formation. In reaction mixtures containing 5-*S*-L-cysteinyldopamine, KMB and enzyme an additional azine doublet was detected at ~2.2 and 2.9 min. Since these peaks appear at very low oxidation potentials they are ascribed to the *E*- and *Z*-isomers, respectively, of the MBTH-azine of the α-keto acid analogue of 5-*S*-L-cysteinyldopamine. Note that our HPLC system provides three diagnostic criteria for the identification and quantification of pyruvate as the MBTH-azine, namely retention time, voltage required to generate a signal and presence of *E*- and *Z*-isomers.

L-Amino acid oxidase activity was measured in a reaction mixture (0.02 ml) containing 5 mM L-amino acid, 100 mM potassium phosphate buffer (pH 7.4), 100 U catalase and L-amino acid oxidase. After incubation at 37°C, 0.01 ml of 2 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added. After a further incubation at 37°C for 5 min, 0.17 ml of 1 M NaOH was added and the absorbance was read at 430 nm within 2 min. The 2,4-dinitrophenylhydrazones of the α-keto acid analogues of 5-*S*-L-cysteinyl-L-DOPA, 5-*S*-L-cysteinyldopamine, β-(cyclohexyl)-L-alanine, L-3-(1-naphthyl)alanine, and L-3-(2-naphthyl)alanine, β-chloroalanine, TFEC, DCVC and BTC have not been prepared previously. Thus, the extinction coefficients at 430 nm of the corresponding 2,4-dinitrophenylhydrazones in alkaline solution have not been determined. Since, in our experience the extinction coefficients of several α-keto 2,4-dinitrophenylhydrazones are close to 16,000 M^{-1} cm^{-1}, this value was used in calculating product formation for all the L-amino acids investigated here as substrates of L-amino acid oxidase. In the case of L-glutamine, owing to >99% cyclization to a lactam [30], the α-keto acid generated in the L-amino acid oxidase reaction cannot be readily quantitated with added 2,4-dinitrophenylhydrazine in an end-point assay. The product of the reaction of L-amino acid oxidase reaction, however, can be continuously trapped as the semicarbazone adduct if semicarbazide is included in the reaction mixture [31]. Typical ε_{248nm} values for α-keto acid semicarbazones are ~10,000 $M^{-1}cm^{-1}$ [32]. Thus, to measure L-amino acid oxidase-catalyzed oxidation of glutamine, reaction mixtures contained 100 mM semicarbazide, and the appearance of α-ketoglutaramate semicarbazone was continuously monitored at 248 nm (37° C).

Reaction mixtures containing either 5-*S*-L-cysteinyl-L-DOPA or 5-*S*-L-cysteinyldopamine continuously darkened with time upon incubation at pH 7.4 at 37°C. The darkening is presumably due to oxidation of 5-*S*-L-cysteinyldopamine (or 5-*S*-L-cysteinyl-L-DOPA) to an *o*-quinone followed by additional reactions. Based on mV oxidation potentials using CoulArray

electrode detection, it is apparent that both *S*-cysteinyl catecholamine conjugates have remarkably low redox potentials. These conjugates can be oxidized more readily and at lower voltages than observed for KMB and methionine (Fig. 1). In fact, based on mV oxidation potentials, both-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine are more easily oxidized than is readily oxidizable ascorbate (data not shown). The ease of oxidation of 5-*S*-L-cysteinyldopamine is in accord with the report by Li and Dryhurst [33] that this compound is even more sensitive to oxidation than is dopamine itself. The non-enzymatic darkening reaction generates products that absorb at the wavelength (430 nm) used in the measurement of α-keto acid 2,4-dinitrophenylhydrazones. Under the conditions of the assay, after incubation for 1 h, the blank increased by about 0.3 optical density unit. This blank value was taken into account when measuring the activity of L-amino acid oxidase toward 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine.

All spectrophotometric measurements were carried out with a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). For all activity determinations using purified enzymes, the blanks were complete reaction mixtures containing heat-inactivated enzyme or lack of enzyme incubated for the same period of time as reaction mixtures containing active enzyme. A unit of enzyme activity (U) is defined as that amount of enzyme that catalyzes the formation of 1 μmol of product/min at 37°C.

Results

Aminotransferase reactions catalyzed by rhGTK

Table 1 shows that rhGTK catalyzes relatively strong aminotransferase reactions with L-glutamine, L-methionine and L-leucine when phenylpyruvate is the co-substrate, and with L-kynurenine and L-phenylalanine when KMB is the co-substrate. β-Cyclohexyl-L-alanine and L-selenomethionine are relatively poor substrates, whereas *Se*-methyl-L-selenocysteine is a moderately good aminotransferase substrate of rhGTK (Table 1). Measurable activities were detected with the large aromatic amino acids L-3-(1-naphthyl)alanine and L-3-(2-naphthyl) alanine. Moderate-to-strong aminotransferase activity was noted with several L-amino acids that contain a good leaving group attached at the β-position. Thus, in addition to *Se*-methyl-L-selenocysteine, rhGTK exhibits substantial aminotransferase activity with TFEC and DCVC (Table 1). Measurable aminotransferase activity could also be detected with 5-*S*-L-cysteinyldopamine and 5-*S*-L- cysteinyl-L-DOPA, which was linear for at least an hour (Table 2). No aminotransferase activity could be detected with 3,5,3′-triiodo-L-thyronine.

The rate of rhGTK-catalyzed transamination of 5 mM 5-*S*-L-cysteinyldopamine with 1 mM KMB at pH 7.4 (~6.25 pmol/min/μg; calculated from the data in Table 2) is about 0.07% the rate at which the enzyme catalyzes transamination between 20 mM L-phenylalanine and 5 mM KMB (9.1 nmol/min/μg; Table 1). 5-*S*-L-Cysteinyl-L-DOPA is less effective than 5-*S*-L-cysteinyldopamine as an aminotransferase substrate of rhGTK (Fig. 1C; Table 2). 5-*S*-L-Cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA (Table 1) are slightly less reactive than the large aromatic amino acids L-3-(2-naphthyl)alanine and L-3-(1-naphthyl)alanine aminotransferase substrates of rhGTK (0.2% and 0.5% the rate exhibited with L-phenylalanine, respectively; Table 1).

As a result of loss of KMB at high concentrations of 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA it was not possible to accurately measure K_m values for rhGTK-catalyzed aminotransferase reactions involving these amino acids. The K_m values for the most active amino acid substrates of rhGTK (glutamine, phenylalanine, leucine, kynurenine, tryptophan, methionine, tyrosine, histidine) are between 0.7 and 6.4 mM in the presence of 16 mM α-ketobutyrate as an aminotransferase partner (pH 7.5) [18]. In addition, K_m values for rat kidney GTK-catalyzed β-lyase activity toward a large number of cysteine *S*-conjugates and

selenocysteine *Se*-conjugates are remarkably consistent, varying from 0.5 to 5 mM [6]. We estimate that the K_m values exhibited by rhGTK toward 5-*S*-L-cysteinyldopamine or 5-*S*-L-cysteinyl-L-DOPA as aminotransferase substrates are also in the mM range.

β-Lyase reactions catalyzed by rhGTK

Table 3 shows that rhGTK catalyzes β-lyase reactions with amino acids that contain a good leaving group in the β-position. In all cases, except β-chloro-D,L-alanine, maximal β-lyase activity required the presence of α-keto acid in the assay mixture. Presumably, β-chloro-L-alanine contains such a strong electron-withdrawing group that transamination cannot compete with β-elimination from the cofactor-substrate, and indeed no transamination could be detected when 0.4 mM phenylpyruvate was included in the reaction mixture. Interestingly, under the conditions employed, rhGTK-catalyzed β-lyase reactions with TFEC, DCVC and *Se*-methyl-L-selenocysteine are more favorable than the rhGTK-catalyzed aminotransferase reactions with these amino acids (compare Table 1 and Table 3).

Generation of aminoacrylate in β-lyase reactions can lead to syncatalytic inactivation. In addition, when the cysteine *S*-conjugate is derived from a halogenated alkene, the released reactive sulfur-containing fragment can also promote syncatalytic inactivation in susceptible enzymes [10]. Thus, fewer turnovers are required to inactivate mitochondrial aspartate aminotransferase in the presence of TFEC (two reactive molecules generated; aminoacrylate, reactive RSH fragment) than in the presence of β-chloroalanine (one reactive molecule generated; aminoacrylate). However, rhGTK seems to be resistant to syncatalytic inactivation. Thus, no significant inactivation was observed after at least 20,000 β-lyase turnover events when either TFEC or DCVC was used as β-lyase substrate (data not shown).

Preliminary experiments using the 2,4-dinitrophenylhydrazone procedure showed that rhGTK is able to catalyze a β-elimination reaction with 5-*S*-L-cysteinyldopamine, but the rate of pyruvate formation generated in this reaction is slow. Because of the possibility that products are formed that interfere with the 2,4-dinitrophenylhydrazone assay on prolonged incubation of 5-*S*-L-cysteinyldopamine in phosphate buffer, we used the MBTH-azine procedure coupled to HPLC to monitor this reaction (see the Materials and Methods). To check the efficacy of the procedure, several controls were run. One control (control 1) contained enzyme in phosphate buffer incubated for 1 h at 37°C prior to addition of MBTH reagent. Another control (control 2) contained complete reaction mixture plus enzyme incubated for 1 h, followed by treatment with 0.005 ml of 3 M sodium acetate and 0.01 ml water (in place of MBTH). Control 3 was identical to control 2 except that KMB was omitted. Control 4 was complete reaction mixture incubated for 4 h in the absence of enzyme and then treated with sodium acetate and MBTH. In none of these controls were interfering peaks noted in the elution profile at 2.5 to 3.0 min. The data for controls 1 – 3 are not shown; an HPLC elution profile of control 4 is shown in Fig. 2B.

Fig. 2C shows the competing transamination and β-lyase reactions when a large excess of rhGTK (32 μg) is incubated with 5 mM 5-*S*-L-cysteinyldopamine and 1 mM KMB in 0.01 ml of a reaction mixture containing 100 mM phosphate buffer (pH 7.4) for 1 h (37°C). The intensities of the peaks due to the *Z*- and *E*-isomers of the MBTH-azine derivative of KMB in Fig. 2 C indicate that >80% of the KMB in the reaction mixture was lost after incubation for 1 h. This loss is due to a competing transamination reaction that is more effective than the β-elimination. The product of the transamination reaction of 5-*S*-L-cysteinyldopamine with KMB should be an α-keto acid that can form an azine with MBTH. This azine doublet, evident at low oxidation potentials, was detected at 2.2 and 2.9 min (Fig. 2C).

The amount of pyruvate detected by the HPLC analysis of the MBTH-azine when 32 μg of rhGTK was incubated for 1 h with 1 mM KMB and 5 mM 5-*S*-L-cysteinyldopamine was 4.44

±0.84 nmol (n = 3). The subunit M_r of rhGTK is 47,900. Therefore, the turnover number for 5-*S*-L-cysteinyldopamine as a β-lyase substrate of rhGTK (pyruvate formation) is ~6.5/h/subunit. From the data in Table 2, the turnover number for transamination between 1 mM KMB and 5 mM 5-*S*-cysteinylodpamine is 17.6/h/subunit. Pyruvate could not be detected when 5-*S*-L-cysteinyl-L-DOPA was used as a substrate.

We also investigated transamination and β-elimination reactions with *Se*-methyl-L-cysteine catalyzed by rhGTK. We found that methionine could be detected by CoulArray HPLC in reaction mixtures containing *Se*-methyl-L-cysteine, KMB and rhGTK. As a result of the competing β-elimination reaction, the loss of *Se*-methyl-L-cysteine was greater than the amount of methionine generated (data not shown). The expected elimination product is methylselenol (CH_3SeH), which is predicted to be redox active. However, a redox active compound corresponding to methylselenol could not be detected by HPLC with CoulArray detection in reaction mixtures containing *Se*-methyl-L-cysteine, KMB and rhGTK. Moreover, selenols readily reduce DTNB to 5-mercapto-2-nitrobenzoate, which can be readily detected by its absorption at 412 nm (ε_{412nm} = 14,150 $M^{-1}cm^{-1}$) [35]. However, no product could be detected that reduced DTNB when this reagent was included in the reaction mixture or when it was added after the reaction had been terminated.

Oxidation of large L-amino acids by snake venom L-amino acid oxidase

A number of large hydrophobic/aromatic amino acids are excellent substrates of *C. adamanteus* L-amino acid oxidase (Table 3). Therefore, 5-*S*-L-cysteinyl-L-DOPA and 5-*S*-L-cysteinyldopamine were predicted to be substrates of L-amino acid oxidase. This prediction was confirmed (Table 4).

Stability of the α-keto acids generated from 5-S-L-cysteinyl-L-DOPA and 5-S-L-cysteinyldopamine

The α-keto acid analogue of 5-*S*-L-cysteinyldopamine expected to be generated in a transamination reaction was generated *in situ* by incubating the amino acid (2 mM) at 37°C in a reaction mixture (0.2 ml) containing 100 mM potassium phosphate buffer (pH 7.4), 20 μg of L-amino acid oxidase and 100 U of catalase. α-Keto acid formation was maximal in about one hour. Thereafter, 10-μl aliquots were withdrawn and assayed for carbonyl content by the 2,4-dinitrophenylhydrazine method. Under these conditions, “keto 5-*S*-L-cysteinyldopamine” was unstable. The half-life for loss of carbonyl function was estimated to be about 2 h. Similar experiments were carried out with 5-*S*-L-cysteinyl-L-DOPA. Under the experimental conditions, maximal α-keto acid formation occurred in about 10 min, followed by a loss of carbonyl function with a half-life of about 1 h.

Possible enzyme-catalyzed reactions leading to α-keto acid formation from 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA are shown in Fig. 3 and Fig. 4. The figures also show possible elimination products that contain a free mercapto (-SH) group located on the aromatic ring.

Discussion

The goals of the present work were to a) extend the known specificity of human GTK, b) characterize its possible role in bioactivation of halogenated cysteine *S*-conjugates, c) determine whether potentially neurotoxic 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA are substrates of GTK/L-amino acid oxidase, and d) provide evidence that human GTK may be a target for selenium-containing prodrugs.

The present study extends the findings of Han et al. [18] that phenylalanine, glutamine, methionine, leucine and kynurenine are excellent amino acid substrates of rhGTK (Table 1). Despite switching from an open to a more closed configuration upon binding of L-amino acid substrate [17], the active site of rhGTK can accommodate relatively large L-amino acids. Thus, measurable aminotransferase activity was noted with L-3-(2-naphthyl)alanine and L-3-(1-naphthyl)alanine, 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA (Table 1 and Table 2). 3,5,3′-Triiodo-L-thyronine was not a substrate, possibly as a result of the strong electronegativity of the iodine substituents or because it is too large. Interestingly, selenomethionine and cyclohexylalanine are much less active aminotransferase substrates than methionine and leucine. *Se*-Methyl-L-selenocysteine is >100 times more effective as an aminotransferase substrate than is L-selenomethionine despite the difference of only one methylene group. Thus, despite the ability of rhGTK to bind large amino acids there are subtle constraints in the geometry of amino acids that can bind most effectively at the active site.

As was previously noted for the rat enzyme [6,9], our data show that rhGTK catalyzes β-elimination more effectively than transamination when TFEC and DCVC are substrates (compare Table 1 and 3). Yamauchi et al. [9] obtained a preparation of rat kidney GTK of exceptionally high specific activity both as an aminotransferase and as a β-lyase. Comparison of our data in Table 3 with those of Yamauchi et al. [9] suggests that the specific activity of rhGTK in the standard phenylalanine-KMB aminotransferase assay is similar to that of the rat enzyme. However, the specific activity of rhGTK as a β-lyase (pH 8.5; Table 3) with DCVC and TFEC is about three times that reported previously [6,9] for the rat enzyme. Interestingly, BTC is a moderately good substrate of the human enzyme (Table 3), but is a poor substrate of the rat enzyme [21,36]. Overall, the present work shows that rhGTK exhibits similar catalytic properties to those of rat kidney GTK, but there are some subtle differences.

Mitochondria are especially vulnerable to toxic, halogenated cysteine *S*-conjugates (reviewed in [10]). As a result of alternative splicing of a single gene transcript, expressed GTK is present in the cytosolic and mitochondrial fractions of rat organs [2,37]. To our knowledge a possible mitochondrial form of human GTK has not been considered. The rhGTK used in the present studies corresponds to a cytosolic form. However, we note from inspection of the human genome that alternative splicing could theoretically generate a 94-amino addition at the N terminus that contains a mitochondrial-targeting sequence. The current work indicates that human GTK, like its rat counterpart, has the *potential* to bioactivate halogenated cysteine *S*-conjugates. However, the relative distribution of GTK in cytosolic and mitochondrial compartments, and the precise role of this enzyme in targeting of mitochondria by halogenated cysteine *S*-conjugates in human tissues remain to be determined.

The autoxidation of dopamine (DA) and L-dihydroxyphenylalanine (L-DOPA), or oxidation by superoxide [38] or peroxynitrite [39], yields *o*-quinones. These quinones react rapidly with cellular thiols, such as L-cysteine, generating 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA [40]. The quinones also react with GSH giving rise to glutathione *S*-conjugates, which may subsequently be converted to the cysteine *S*-conjugates by the action of γ-glutamyltransferase and dipeptidases [38]. The ratio of 5-*S*-L-cysteinyldopamine to DA is markedly increased in the substantia nigra of patients who died of Parkinson disease [40], as is the concentration of 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA [23]. These findings suggest that 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA, or their derivatives, may act as potential endogenous nigral toxins. In fact, 5-*S*-L-cysteinyl-catecholamines have been shown to induce neuronal apoptosis by a mechanism involving an increase in intracellular oxidative stress, increases in DNA oxidation and caspase-3 activation [41]. Moreover, mice expressing ~5% of the vesicular monoamine vesicular transporter 2 (VMAT2; SLC18A2) display age-associated nigrostriatal dopamine dysfunction that

ultimately results in neurodegeneration [42]. The changes were associated with a marked increase in striatal 5-*S*-cysteinyl-L-DOPA and 5-*S*-cysteinyl-DOPAC [42].

In the present work, we showed that rhGTK, in keeping with its proclivity for aromatic L-amino acids, can catalyze transamination of 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA (Table 2). Although by comparison with L-phenylalanine, the rate of transamination of 5-*S*-L-cysteinyldopamine catalyzed by rhGTK is slow, the physiological significance is not diminished because of the high inherent activity of GTK and wide tissue distribution of this enzyme. Moreover, other aminotransferases, such as mitochondrial aspartate aminotransferase [43], tyrosine aminotransferase [44] and α-aminoadipate aminotransferase/KAT II [25], that catalyze transamination with aromatic amino acids might also catalyze the formation of α-keto acids from 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA. In future studies it will be important to determine the extent to which 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA can undergo transamination *in vivo*.

At least two L-amino acid oxidases exist in mammalian tissues, with relatively high activity toward aromatic amino acids, namely an L-amino acid oxidase that is especially rich in kidney, but is found in other tissues including brain [45–50], and another in leukocytes [51,52]. Rat kidney L-amino acid oxidase has a remarkably similar specificity to that of *C. adamanteus* venom (compare for example [19] with [45]). Thus, if the *C. adamanteus* enzyme can generate α-keto acids from 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA, then the possibility exists that they can be generated *in vivo* in human tissues not only by an aminotransferase reaction, but also by an L-amino acid oxidase reaction. Accordingly, in the present work, we determined the ability of *C. adamanteus* L-amino acid oxidase to oxidize 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA and compared the rates of oxidation with these amino acids to rates obtained with other L-amino acids.

Hydrophobic/aromatic amino acids (methionine, leucine and phenylalanine) are the most active substrates of *C. adamanteus* L-amino acid oxidase [19]. We have extended the earlier findings [19] by showing that the enzyme also utilizes L-selenomethionine, β-(cyclohexyl)-L-alanine, 5-*S*-L-cysteinyldopamine, 5-*S*-L-cysteinyl-L-DOPA, two L-naphthylalanines and 3,5,3′-triiodo-L-thyronine (Table 4). These findings show that the active site can accommodate large hydrophobic/aromatic amino acids. Interestingly, 5-*S*-L-cysteinyl-L-DOPA is a better substrate than is 5-*S*-L-cysteinyldopamine (Table 4), possibly because the $-CH_2CH(NH_3^+)CO_2^-$ grouping of 5-*S*-L-cysteinyl-L-DOPA binds preferentially to the active site over the $-SCH_2CH(NH_3^+)CO_2^-$ grouping of 5-*S*-L-cysteinyldopamine.

We showed that the α-keto acid analogues of 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA generated by *C. adamanteus* L-amino acid oxidase are rapidly converted to compounds that lack a carbonyl function. Given the tendency of DOPA to generate H_2O_2 [27], it is likely that the cysteinyl *S*-conjugates and their α-keto acids would also generate H_2O_2. Therefore, in the absence of catalase, loss of carbonyl function may occur by oxidative decarboxylation. However, oxidative decarboxylation cannot account for the loss of carbonyl function when the cysteine *S*-conjugates are incubated with L-amino acid oxidase in the presence of catalase. Possibilities for loss of carbonyl function include aldol condensations, lactol formation between the α-keto group and ring hydroxy groups, and hemithioacetal/hemithioketal formation. Regardless of the mechanism, irreversible conversion of the α-keto acid analogue of 5-*S*-L-cysteinyldopamine (and 5-*S*-L-cysteinyl-L-DOPA) may be important biochemically.

As noted above, 5-*S*-L-cysteinyl-catechol adducts accumulate in the substantia nigra in human brain [41] and mouse brain [42]. 5-*S*-L-Cysteinyldopamine readily undergoes conversion *in vitro* to additional cysteine *S*-conjugates, two benzothiazines, and benzothiazine cysteine *S*-

conjugates. Many of these metabolites are neurotoxic by interfering with brain metabolism [53–61]. Strong evidence suggests that 5-*S*-L-cysteinyldopamine is an important precursor of the pheomelanin component of the pigment neuromelanin in the substantia nigra [54,62–64]. Given the potential neurotoxicity of 5-*S*-L-cysteinyldopamine, incorporation into neuromelanin might be neuroprotective. Additionally, neuromelanin is generally regarded as a powerful antioxidant [64]. However, neuromelanin may become deleterious in Parkinson disease as the disease progresses [65]. If 5-*S*-L-cysteinyldopamine were an aminotransferase/oxidase substrate *in vivo* then formation of the α-keto acid might be a regulatory factor in the formation of neuromelanin and an additional detoxification mechanism. However, if the products derived from the α-keto acids are toxic then the formation of the α-keto acids would be a bioactivation (toxification) event. These points need to be further investigated.

Commandeur et al. [6] showed that cysteine *S*-conjugates of the structure $ArSCH_2CH(NH_3^+)CO_2^-$, where Ar = phenyl-, benzyl-, 4-methylbenzyl- or 4-methoxybenzyl- are aminotransferase substrates of rat kidney GTK at about 5–20% the rate exhibited with DCVC and TFEC. β-Lyase activity toward phenyl- and benzyl cysteine *S*-conjugates was detectable, but very slow (a few percent of the aminotransferase activity) [6]. Inasmuch as rhGTK exhibits aminotransferase activity with 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA, we considered the possibility that this enzyme might also catalyze a competing β-elimination reaction with these amino acids. In fact, we were able to demonstrate pyruvate formation when 5 mM 5-*S*-L-cysteinyldopamine was incubated with relatively large amounts of rhGTK and 1 mM KMB (pH 7.4). Transamination was favored over elimination by a factor of four. 5-*S*-L-Cysteinyl-L-DOPA is not as effective as 5-*S*-L-cysteinyldopamine as an aminotransferase substrate of rhGTK (Table 2) and we were unable to detect rhGTK-catalyzed pyruvate formation with this amino acid.

Although we have not yet characterized the sulfur-containing species derived by β-elimination of 5-*S*-L-cysteinyldopamine, the structure is theoretically 3-mercaptodopamine (Fig. 3). The rate of rhGTK-catalyzed formation of this compound is limited compared to transamination. Nevertheless, our work demonstrates that the S-C bond in 5-*S*-L-cysteinyldopamine can be enzymatically cleaved. Moreover, it is possible that other PLP-enzymes might catalyze this reaction. If this reaction were to occur *in vivo* the formation of a dopamine analogue containing an −SH group attached to the ring might be a bioactivating event.

As noted in the introduction, a number of selenocysteine *Se*-conjugates, including *Se*-methyl-L-selenocysteine, are both aminotransferase and β-lyase substrates of rat kidney GTK [6]. The aminotransferase reaction, however, was invariably the more favorable reaction [6]. Interestingly, we found that the β-lyase reaction catalyzed by rhGTK with *Se*-methyl-L-selenocysteine was more favored than the aminotransferase reaction (compare Table 1 and Table 3). The detection of a β-elimination reaction with *Se*-methyl-L-selenocysteine catalyzed by rhGTK is important because epidemiological studies and clinical and pre-clinical trials provide strong evidence for a role of *Se*-methyl-L-selenocysteine (a natural product in garlic and other *Allium* species) in cancer chemoprevention particularly against prostate cancer [e.g. 66].

We could not detect methylselenol, the selenium-containing fragment predicted to be formed in the β-lyase reaction on *Se*-methyl-L-selenocysteine catalyzed by rhGTK. Rooseboom et al. [67] showed that *C. adamanteus* L-amino acid oxidase catalyzes a β-elimination side-reaction with *Se*-methyl-L-cysteine, but were also unable to detect the predicted product methylselenol. Methylselenol must be extremely volatile and hydrophobic or extremely unstable. As methylselenol is thought to be the proximate chemopreventive agent generated from *Se*-methyl-L-selenocysteine, our findings and those of Rooseboom et al. [67] present an interesting dilemma. Suzuki et al. [68] also noted the extreme difficulty in detecting methylselenol. These

authors showed, however, that methylselenol, generated *in situ* from reduction of methylseleninic acid with DTT, can be reductively demethylated in liver homogenates to methanol and H_2Se. Since GTK activity is present in liver, it is possible that this enzyme participates, along with the enzyme(s) involved in the reductive demethylation in the chemopreventive reactions associated with *Se*-methyl-L-selenocysteine. Further studies with naturally occurring and synthetic organoselenium compounds will address these issues.

α-Keto acid substrates of human GTK, such as KMB and phenylpyruvate, are likely of low concentration in mammalian tissues (~μM [69]). Nevertheless, the presence of α-ketoglutaramate in human cerebrospinal fluid [70] indicates that α-keto acid substrates are available for transamination of glutamine in human tissues *in vivo*. The concentration of glutamine in tissues is generally in the low mM range, which is the ~K_m for glutamine exhibited by rhGTK [18]. In conclusion, GTK is likely not saturated with glutamine in human tissues and can thus accommodate amino acids such as *Se*-methyl-L-selenocysteine, 5-*S*-L-cysteinyldopamine, 5-*S*-L-cysteinyl-L-DOPA, albeit at probably slow turnover rates. The biochemical consequences of interaction of these compounds with GTK and other PLP-containing enzymes (and L-amino acid oxidases) deserve further study.

Acknowledgements

This work was supported in part by NIH grants RO1 [ES8421] (to AJLC), [CA111842] (to JTP), [A144399] (to JL), and by a Medical Research Council (MRC) grant [G0400278/NI02] (to JPES).

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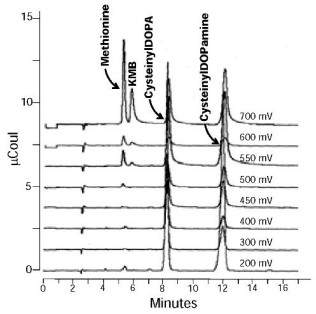
A



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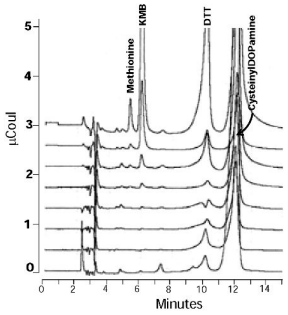

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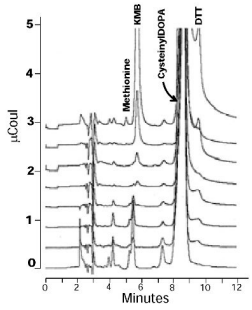
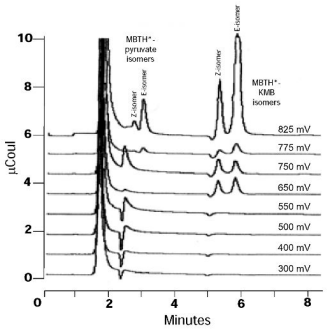


Fig. 1.

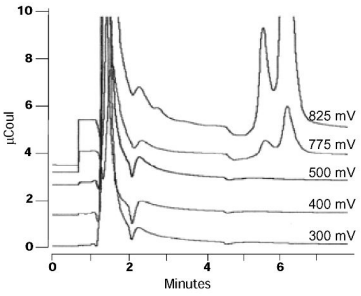
HPLC detection of a transamination product (methionine) in reaction mixtures containing rhGTK, α-keto-γ-methiolbutyrate (KMB) and 5-*S*-L-cysteinyldopamine (or 5-*S*-L-cysteinyl-L-DOPA). **A**. Standards (50 nmol/ml) of methionine, α-keto-γ-methiolbutyrate (KMB), 5-*S*-L-cysteinyldopamine (cysteinylDOPamine) and 5-*S*-L-cysteinyl-L-DOPA (cysteinylDOPA). Note the very low potentials required to oxidize 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA compared to the potentials required to oxidize methionine and KMB. **B**. Enzymatic formation of methionine by transamination of KMB with 5-*S*-L-cysteinyldopamine. The reaction mixture (0.01 ml) contained 5 mM 5-*S*-L-cysteinyldopamine, 1 mM KMB, 1 mM DTT, 100 mM potassium phosphate buffer (pH 7.4) and 3.2 µg of enzyme in a small snap-top

tube. After incubation for 1 h at 37°C, the reaction was terminated with MPA and the reaction mixture was analyzed for redox-active compounds. No methionine was detected in a blank consisting of complete reaction mixture in which the enzyme was omitted (data not shown). **C**. Enzymatic formation of methionine by transamination of KMB with 5-*S*-L-cysteinyl- L-DOPA. As for C, except that the reaction mixture contained 5-*S*-L-cysteinyl-L-DOPA in place of 5-*S*-L-cysteinyldopamine. No methionine was detected in a blank consisting of complete reaction mixture in which the enzyme was omitted (data not shown).

A



B



C

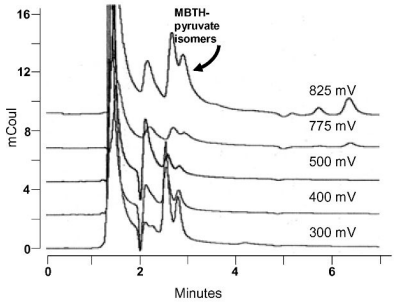


Fig. 2.

HPLC detection of pyruvate formed by a β-lyase reaction in mixtures containing rhGTK, α-keto-γ-methiolbutyrate (KMB) and 5-*S*-L-cysteinyldopamine. **A**. Pyruvate (12 nmol/ml) and KMB (100 nmol/ml) standards detected as azines with 3-methyl-2-benzothiazolinone hydrazone (MBTH). Note that the MBTH-azines exist as *E*-and *Z*-isomers. Our assignment of isomers is based on the HPLC elution profiles of α-keto acid MBTH-azines provided by Tanaka et al. [29]. **B**. Control consisting of complete reaction mixture incubated for 1 h in the absence of enzyme followed by treatment with acetate buffer and MBTH. **C**. rhGTK-catalyzed formation of pyruvate from 5-*S*-L-cysteinyldopamine. The reaction mixture (0.01 ml) contained 1 mM DTT, 1 mM KMB, 5 mM 5-*S*-L-cysteinyldopamine and 32 μg of rhGTK

incubated for 1 h at 37°C in a small snap-top tube. Pyruvate formation was detected as the MBTH-azine (doublet at ~3 min, E and Z-isomers) at high oxidation potentials. Note the doublet at low potentials at ~3 min. This doublet is ascribed to the azine of the α-keto acid derived from 5-*S*-L-cysteinyldopamine. The intensity is less than expected based on the disappearance of α-keto-γ-methiolbutyrate and reflects the instability of the keto analogue of 5-*S*-L-cysteinyldopamine. For ease of visualization some of the traces at different voltage levels have been omitted in panels B and C.

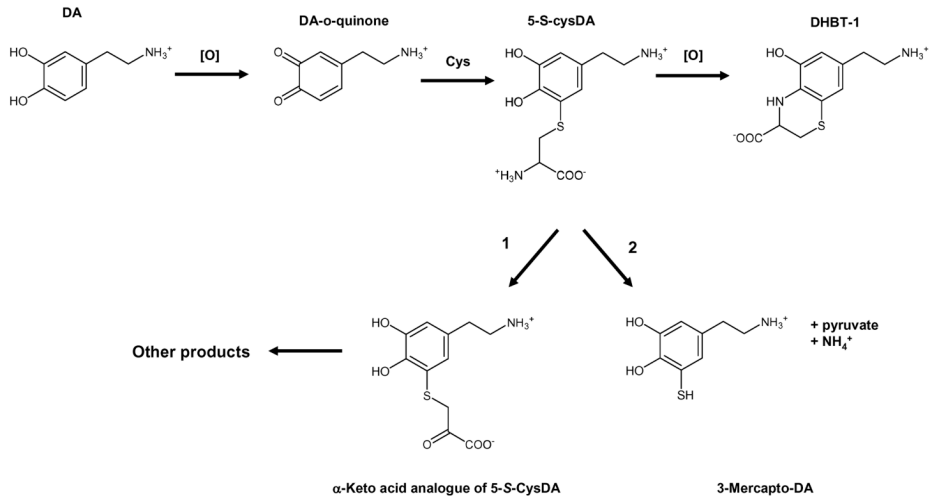


Fig. 3.

Metabolism of 5-*S*-L-cysteinyldopamine (5-*S*-CysDA). 5-*S*-L-Cysteinyldopamine is oxidized to the corresponding *o*-quinone, which undergoes intramolecular cyclization to yield a number of toxic products, but predominantly 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBT-1). The present work suggests that 5-*S*-L-cysteinyldopamine may also be converted by aminotransferases (including GTK) and possibly by L-amino acid oxidases to the corresponding α-keto acid (pathway **1**). The α-keto acid is unstable and is converted to unknown products with loss of carbonyl function. Alternatively, 5-*S*-L-cysteinyldopamine may undergo a β-lyase reaction generating pyruvate (pathway **2**). The theoretical structure of the sulfur-containing fragment generated in the lyase reaction is 3-mercaptodopamine (3-mercapto-DA). The cysteine *S*-conjugate is shown as arising by direct addition of cysteine to the *o*-quinone of DA. However, the cysteine *S*-conjugate may also arise via the glutathione *S*-conjugate followed by subsequent conversion to the cysteinylglycine *S*-conjugate and the cysteine *S*-conjugate [41]. Another possible metabolic pathway involves aminochrome formation through oxidation at the 6 position to a hydroxyl followed by cyclization to a five-membered ring by condensation with the amine. However, we are not aware of any report that documents aminochrome formation from 5-*S*-cysteinyldopamine (or from 5-*S*-cysteinyl-L-DOPA). Moreover, in the presence of excess cysteine mono-, di-and tri adducts can be formed at the 2-, 5- and 6 positions [53]. Thus, it is more likely that RS-adducts will be formed at the 6 position than an −OH.

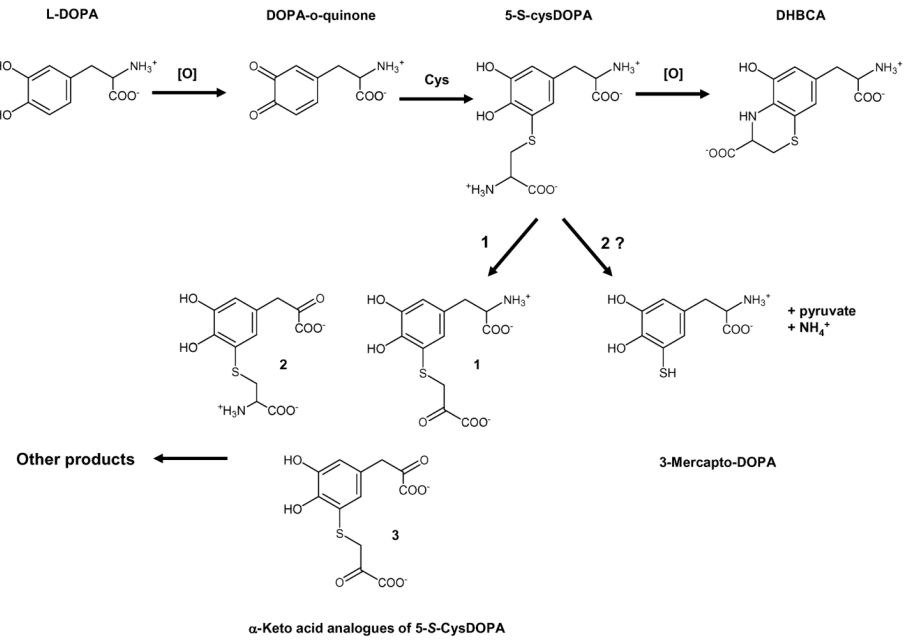


Fig. 4.

Metabolism of 5-*S*-L-cysteinyl-L-DOPA (5-S-CysDOPA). 5-*S*-L-CysteinylDOPA is oxidized to the corresponding *o*-quinone, which undergoes intramolecular cyclization to yield toxic 7-(2-amino-2-carboxyethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid (DHBCA). The present work suggests that 5-*S*-L-cysteinyl-L-DOPA may also be converted by aminotransferases (including GTK) and possibly by L-amino acid oxidases to α-keto acids (pathway **1**). Three possible α-keto acids (1–3) are shown. These α-keto acids are unstable and are converted to unknown products with loss of carbonyl function. A β-lyase reaction that converts 5-*S*-L-cysteinyl-L-DOPA to 3-mercapto-DOPA is also possible (pathway **2**), but has not yet been demonstrated. The cysteine *S*-conjugate is shown as arising by direct addition of cysteine to the *o*-quinone of DOPA. However, the cysteine *S*-conjugate may also arise via the glutathione *S*-conjugate followed by subsequent conversion to the cysteinylglycine *S*-conjugate and the cysteine *S*-conjugate [41].

Table 1

Aminotransferase substrates of human recombinant GTK (rhGTK)[a]

	mmol/min/mg
L-Phenylalanine (20 mM)[b,c]	9.1±0.2 [18.1±1.1]
L-Glutamine	5.85±0.05 [10.8±0.5]
TFEC	5.69±0.28
L-Methionine	2.66±0.87
L-Leucine	2.32±0.11
L-Kynurenine[d]	2.15±0.85
DCVC	1.19±0.13
Se-Methyl-L-selenocysteine	0.85±0.15
L-Tyrosine (1 mM)[c]	0.84±0.02
β-Cyclohexyl-L-alanine[e]	0.077±0.019
L-3-(2-Naphthyl)alanine[c]	0.051±0.008
L-DOPA[c]	0.026±0.005
L-3-(1-Naphthyl)alanine[c]	0.0177±0.0010
L-Selenomethionine[f]	0.0080±0.0011
3,5,3′-Triiodo-L-thyronine	<0.01
β-Chloro-D,L-alanine	<0.01

[a] Except where noted, the reaction mixture (0.05 ml) contained 5 mM amino acid, 0.6 mM phenylpyruvate, 100 mM potassium phosphate buffer (pH 7.4) and enzyme. After incubation at 37°C for 10 min the disappearance of phenylpyruvate was measured. The amount of enzyme added varied with the substrate, but was adjusted to ensure that the amount of phenylpyruvate consumed is a reflection of initial velocities. N = 3 or 4. The values in square brackets are rates of transamination in the presence of 100 mM ammediol buffer (pH 9.0).

[b] The α-keto acid substrate was 5 mM KMB.

[c] The enol form of the aromatic α-keto acid product was measured.

[d] Kynurenate formation was measured.

[e] (Cyclohexyl)pyruvate, the α-keto acid analogue of β-(cyclohexyl)-L-alanine is also a poor substrate. At a concentration of 5 mM it is only about 4% as effective as KMB in rhGTK-catalyzed transamination of L-phenylalanine.

[f] The possibility exists for a γ-elimination reaction generating α-ketobutyrate, but no α-keto acid product could be detected among the products by the 2,4-dinitrophenylhydrazone procedure.

Table 2

Transamination of 5-*S*-L-cysteinyldopamine and 5-*S*-L-cysteinyl-L-DOPA with α-keto-γ-methiolbutyrate (KMB) catalyzed by rhGT K[a]

Amino acid substrate	Time of incubation (min)	Methionine formed (nmol)
5-*S*-L-Cysteinyldopamine	15	0.269±0.020
5-*S*-L-Cysteinyldopamine	60	1.20±0.33
5-*S*-L-Cysteinyl-L-DOPA	30	0.083±0.034
5-*S*-L-Cysteinyl-L-DOPA	60	0.176±0.014

[a]The reaction mixture (0.01 ml) contained 5 mM amino acid, 1 mM KMB, 100 mM potassium phosphate buffer (pH 7.4) and 3.2 μg (57.6 mU) of rhGTK. At the times indicated, the reaction was stopped by the addition of 0.05 ml of water and 0.015 ml of 25% (w/v) MPA. Methionine was measured by HPLC with CoulArray detection.

Table 3

β-Lyase substrates of rhGT K[a]

	µmol/min/mg
TFEC	24.9±0.8 [40.2±1.8][b]
DCVC	8.32±0.53 [17.0±0.8][b]
BTC	2.40±0.43
β-Chloro-D,L-alanine[b]	2.10±0.16
Se-Methyl-L-selenocysteine	1.77±0.10

[a]Except where noted, the reaction mixture (0.02 ml) contained 5 mM amino acid, 0.1 mM KMB, 100 mM potassium phosphate buffer (pH 7.4) and enzyme. After incubation at 37°C for 5 min the amount of pyruvate formed was determined by the 2,4-dinitrophenylhydrazine procedure. The amount of enzyme added varied with the substrate, but was adjusted to ensure that the amount of pyruvate formed is a reflection of initial velocities. N = 3 or 4. The rates in square brackets were obtained in 100 mM Tris buffer, pH 8.5.

[b]No transamination could be detected with this amino acid (Table 1). As a result it was not necessary to include KMB in the reaction mixture.

Table 4

Oxidation of L-amino acids by *Crotalus adamanteus* L-amino acid oxidase[a]

Amino Acid	α-Keto acid produced (nmol)
L-Methionine	21.4±1.2
L-Leucine	18.1±0.8
β-Cyclohexyl-L-alanine	16.5±0.6
L-Selenomethionine	16.3±0.8
L-Phenylalanine	15.6±0.4
TFEC	13.8±0.6
L-Tyrosine (1mM)	10.3±0.3
DCVC	10.1±0.3
5-*S*-L-Cysteinyl-L-DOPA	7.83±0.43[b]
L-3-(1-Naphthyl)alanine	6.65±0.50
β-Chloro-D,L-alanine	6.62±0.48
L-3-(2-Naphthyl)alanine	3.33±0.32
BTC	2.20±0.15
L-Glutamine[c]	2.10±0.09
Se-Methyl-L-selenocysteine[d]	1.29±0.19
5-*S*-L-Cysteinyldopamine	0.57±0.01[b]
3,5,3′ -Triiodo-L-thyronine	0.42±0.08

[a] Except where noted, the reaction mixture (0.02 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM L-amino acid, 0.1 μg of L-amino acid oxidase and 100 U catalase in a final volume of 0.02 ml. After incubation at 37°C for 30 min, α-keto acid formation was determined with 2,4-dinitrophenylhydrazine. N = 3 – 6.

[b] Based on the instability of the α-keto acids generated (see the text), there may have been 10–40% loss of α-keto acid during the incubation.

[c] α-Keto acid formation was measured with semicarbazide.

[d] The product may contain some pyruvate [67].