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Fluorination at the 4 position alters the substrate behavior of L-glutamine and L-glutamate: Implications for positron emission tomography of neoplasias

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Abstract

Two 4-fluoro-L-glutamine diastereoisomers [(2*S*,4*R*)-4-FGln, (2*S*,4*S*)-4-FGln] were previously developed for positron emission tomography. Label uptake into two tumor cell types was greater with [¹⁸F](2*S*,4*R*)-4-FGln than with [¹⁸F](2*S*,4*S*)-4-FGln. In the present work we investigated the enzymology of two diastereoisomers of 4-FGln, two diastereoisomers of 4-fluoroglutamate (4-FGlu) (potential metabolites of the 4-FGln diastereoisomers) and another fluoro-derivative of L-glutamine [(2*S*,4*S*)-4-(3-fluoropropyl)glutamine (FP-Gln)]. The two 4-FGlu diastereoisomers were found to be moderate-to-good substrates relative to L-glutamate of glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase. Additionally, alanine aminotransferase was shown to catalyze an unusual γ -elimination reaction with both 4-FGlu diastereoisomers. Both 4-FGlu diastereoisomers were shown to be poor substrates, but strong inhibitors of glutamine synthetase. Both 4-FGln diastereoisomers were shown to be poor substrates compared to L-glutamine of glutamine transaminase L and α -amino adipate aminotransferase. However, (2*S*,4*R*)-4-FGln was found to be a poor substrate of glutamine transaminase K, whereas (2*S*,4*S*)-4-FGln was shown to be an excellent substrate. By contrast, FP-Gln was found to be a poor substrate of all enzymes examined. Evidently, substitution of H in position 4 by F in L-glutamine/L-

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AUTHOR CONTRIBUTIONS

AJLC, HFK and KP conceived the idea of investigating the enzymology of 4-FGlu and 4-FGln. TMJ, AJLC, EK, JAA, JTP, CS and JWE carried out the enzyme studies. HFK and KP synthesized the 4-FGlu and 4-FGln diastereoisomers used in the current study and JL provided purified samples of GTK/KAT I, AAT/KAT II and GTL/KAT III. AJLC and TMJ wrote the paper with the assistance of JTP.

The authors declare no conflict of interest

glutamate has moderate-to-profound effects on enzyme-catalyzed reactions. The present results: 1) show that 4-FGln and 4-FGlu diastereoisomers may be useful for studying active site topology of glutamate- and glutamine-utilizing enzymes; 2) provide a framework for understanding possible metabolic transformations in tumors of ^{18}F -labeled (2*S*,4*R*)-4-FGln, (2*S*,4*S*)-4-FGln, (2*S*,4*R*)-4-FGlu or (2*S*,4*S*)-4-FGlu; and 3) show that [^{18}F]FP-Gln is likely to be much less metabolically active *in vivo* than are the [^{18}F]4-FGln diastereoisomers.

Graphical Abstract

Metabolism of the 2*S*,4*S*- and 2*S*,4*R* diastereoisomers of 4-FGln and 4-FGlu The solid arrows indicate the proposed metabolic pathways for the 2*S*,4*S*- and 2*S*,4*R* diastereoisomers of 4-FGln and 4-F-Glu, while the broken arrows indicate the less favored pathways.

Keywords

Fluoroglutamine; Fluoroglutamate; Glutamine-utilizing enzymes; Glutamate-utilizing enzymes

1. INTRODUCTION

Positron emission tomography (PET) is frequently used to image aggressive neoplasias and to assess the efficacy of treatment. One important and emerging group of imaging agents for these studies, are ^{18}F -labeled analogues of glutamate and glutamine. These compounds exploit the high requirement for glutamine (glutamine addiction) exhibited by many cancer cell lines to selectively image these cells. The intracellular fate of this pool of glutamine depends on the origins of the neoplastic cells. Following cellular uptake, glutamine is deamidated to glutamate by glutaminase. This pool of glutamate is commonly thought to undergo conversion to 2-oxoglutarate and thereby enter the TCA cycle and support anaplerosis [1–12]. By contrast, Tardito et al. demonstrated the glutamine-derived pool of glutamate in glioblastoma cells is either exported or converted to glutamine by glutamine synthetase [13]. Moreover, under conditions of glutamine starvations these cells siphon 2-oxoglutarate from the TCA cycle (cataplerosis) to maintain the intracellular glutamine pools of glioblastoma cells. These observations reveal important variations in the metabolism of glutamine and glutamate among neoplastic cells of differing origins. The extremely rapid rates at which glutamate and glutamine are metabolized within cells [14], make the ^{18}F -labeled fluorinated forms of these amino acids especially attractive as PET probes for the study of tumor cell metabolism.

Kung *et al.* have developed procedures for the rapid radiochemical synthesis of all four possible ^{18}F -labeled diastereoisomers of 4-FGln (i.e., 2*S*,4*S*-, 2*S*,4*R*-, 2*R*,4*S*- and 2*R*,4*R*-4-FGln) [15–17] (^{18}F is a positron emitter with a $t_{1/2}$ of 109.8min). It was reasoned that replacement of an H with an F in the 4 position of L-glutamine will result in labeled glutamine analogues that can be used for PET imaging of glutamine-utilizing tumors [15–

17]. Our previous work with (2*S*,4*R*)-4-FGln and (2*S*,4*R*)-4-FGlu supports this hypothesis [18], but with certain caveats, namely that the rates at which the enzymes utilize (2*S*,4*R*)-4-FGln and (2*S*,4*R*)-4-FGlu as substrates relative to their non-fluorinated counterparts are different [18]. Since glutamine and glutamate serve as substrates for a wide range of enzymes, we sought to extend our earlier studies by examining the substrate behavior of *both* (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu diastereoisomers toward glutamate-utilizing enzymes and *both* (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln diastereoisomers toward glutamine-utilizing enzymes. Note that 2*S*- is equivalent to 2*L*- at carbon 2, whereas 2*R*- is equivalent to 2*D*- at carbon 2. Therefore, only the 2*S*- diastereoisomers (and not the 2*R*- diastereoisomers) of fluorinated analogues were used in the present studies as likely substrates of L-glutamate- and L-glutamine-utilizing enzymes.

Previous work showed that tumors in rodents metabolize [¹⁸F](2*S*,4*R*)-4-FGln in part to ¹⁸F⁻ [17]. Subsequently, the glutamine analogue [¹⁸F](2*S*,4*S*)-4-(3-fluoropropyl)glutamine ([¹⁸F]FP-Gln) was synthesized as a tumor PET imaging agent [19]. This compound is unlikely to undergo defluorination *in vivo*. In the present work we show that FP-Gln is likely to be metabolized very slowly *in vivo*. We have also uncovered a few remarkable differences in the enzymology between (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln. The present studies provide insights as to the possible metabolic fates *in vivo* of (2*S*,4*S*)-4-FGlu, (2*S*,4*R*)-4-FGlu, (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln. In addition, we suggest that these fluorinated compounds will be excellent probes for studying the active site topology of a number of glutamate- and glutamine-utilizing enzymes.

2. MATERIALS AND METHODS

2.1 Reagents

(2*S*,4*R*)-4-FGln, (2*S*,4*S*)-4-FGln, (2*S*,4*R*)-4-FGlu and (2*S*,4*S*)-4-FGlu were prepared using previously published methods [15–18]. These compounds were dissolved in aqueous solutions by prolonged heating at 37 °C. 2,4-Dinitrophenylhydrazine was purchased from ICN Biomedicals (Irvine, CA) while all other compounds were supplied by Sigma-Aldrich (St. Louis, MO).

2.2 Enzymes

Highly purified recombinant human glutamine transaminase K [rhGTK; also known as kynurenine aminotransferase I (KAT I)] [20], mouse α-amino adipate aminotransferase (AAT; also known as KAT II) [21] and mouse glutamine transaminase L (GTL; also known as KAT III) [22, 23] were prepared as indicated in the corresponding references. All three enzymes were stored at 4 °C in 10 mM potassium phosphate buffer (pH 7.4) at a concentration of 11.2, 11.3 and 3.7 mg/ml, respectively. GAC (0.58 mg/ml), the short form (residues 72–603) of mouse kidney-type glutaminase (GLS1), was overexpressed with an N-terminal hexahistidine tag in *Escherichia coli* and purified as described [24]. *Crotalus adamanteus* venom L-amino acid oxidase type 4 (9.8 mg/ml; 4 U/mg aqueous solution), pig heart cytosolic aspartate aminotransferase (AspAT, lyophilized powder; 100 U/mg), and pig heart alanine aminotransferase (AlaAT, lyophilized powder; 65 U/mg) were obtained from Sigma. Beef liver glutamate dehydrogenase (GDH, 100 mg/ml in 20% glycerol; 50 U/mg)

was obtained from Boehringer Ingelheim. Human glutamine synthetase (GS) was expressed and purified by the method of Listrom *et al.* [25] except that HA Ultrogel® (Pall Life Sciences) was used in place of hydroxylapatite.

A unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μmol of product per minute under standard assay conditions. In most cases the specific activities of the enzymes investigated in the present work are provided as indicated above and the number of units (U) (or mU) is included in the descriptions of the assay procedures. However, notable exceptions are GTK/KAT I, AAT/KAT II or GTL/KAT III. These enzymes have very broad substrate specificities and different investigators have used different definitions of specific activity. Thus, for these enzymes we report here relative activities toward various substrates in the presence of a defined concentration of enzyme.

2.3 Preparation of rat liver cytosol

Rat liver cytosol was prepared by the method of Krasnikov *et al.* [26]. The preparation (~10 mg protein/ml) was devoid of activity of the mitochondrial marker GDH. On the other hand, the specific activity of cystathionine γ -lyase (cytosolic marker) in the preparation was high (data not shown). The preparation was stored at $-20\text{ }^{\circ}\text{C}$.

2.3 Determination of Enzyme Activities

2.3.1 Glutaminase—The reaction mixture (0.025 ml total volume) consisted of variable concentrations L-glutamine (or 4-FGln), 100 mM potassium phosphate, 50 mM Tris-acetate buffer, pH 8.6, and 50 nM enzyme. After incubation for 2 minutes at $23\text{ }^{\circ}\text{C}$ the reaction was quenched by addition of 2.5 μl of 3 M HCl. Product formation was then measured by the appearance of absorbance at 340 nm (due to formation of NADH; $\epsilon\ 6.22 \times 10^3\ \text{M}^{-1}\text{cm}^{-1}$) after addition of 0.2 ml of a reaction mixture containing 12 U GDH, 2 mM NAD^+ , 100 mM hydrazine and 100 mM Tris-HCl buffer pH 9.2 [24]. We previously showed that (2*S*,4*R*)-4-FGlu is a substrate of GDH [18]. In the present work, (2*S*,4*S*)-4-FGlu was also shown to be a substrate of GDH (see below). Thus, the glutaminase method used herein is suitable for measurement of glutaminase activity toward both diastereoisomers of 4-FGlu. In addition to using this end-point procedure, we also measured glutaminase activity by using a continuous spectrophotometric assay in which ammonia production is coupled to the GDH reaction [18].

2.3.2 L-Amino Acid Oxidase (LOX)—Snake venom LOX catalyzes the oxidation of a large number of L-amino acids to the corresponding 2-imino acid and H_2O_2 [e.g. 27]. The 2-imino acid is non-enzymatically hydrolyzed to the corresponding 2-oxo acid and ammonia [28,29]. The assay used in the current work is a slight modification of that which we used previously [18] and makes use of the fact that the initial product of the LOX reaction (i.e. a 2-imino acid) reacts rapidly with semicarbazide to form the corresponding 2-oxo acid semicarbazone and ammonia [29]. Under the conditions used by Hafner and Wellner 4 mM semicarbazide effectively traps 50% of the imine generated from the LOX-catalyzed oxidation of L-leucine at pH 7.7 at $25\text{ }^{\circ}\text{C}$ [29]. We found that trapping is essentially complete in the presence of 100 mM semicarbazide at $37\text{ }^{\circ}\text{C}$. Several 2-oxo acid semicarbazones exhibit an extinction coefficient (ϵ) of $\sim 10,000\ \text{M}^{-1}\text{cm}^{-1}$ at 248 nm [30]. It

is assumed in the present work that the 2-oxo acid semicarbazones generated in the LOX reactions investigated here also have an ϵ of $\sim 10,000 \text{ M}^{-1}\text{cm}^{-1}$ at 248 nm. The reaction mixture (0.198 ml) contained 2 mM L-amino acid, 100 mM potassium phosphate buffer (pH 7.4), 1,000 U catalase, and 100 mM semicarbazide at a temperature of 37 °C. The reaction was initiated by addition of 2 μl of a solution of LOX in water (containing 0.1 to 2.5 μg , depending on the substrate) and the increase of absorbance at 248 nm was continuously monitored.

2.3.3 Glutamine transaminases—Glutamine transaminase activity was measured using 4-methylthio-2-oxobutanoate (α -keto- γ -methiolbutyrate) as the 2-oxo acid substrate [31]. The reaction mixture contained 0.6 mM L-amino acid, 0.6 mM 4-methylthio-2-oxobutanoate, 60 mM potassium phosphate buffer (pH 7.4) and enzyme in a final volume of 0.025 ml. After incubation at 37 °C for 30 min the reaction was terminated by addition of 0.010 ml of 25% w/v metaphosphoric acid and L-methionine product was measured by high performance liquid chromatography (HPLC). The HPLC system consisted of a liquid chromatograph equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA) [31]. The reaction mixture, after treatment with metaphosphoric acid and centrifugation at 10,000 g to remove precipitated protein, was injected directly onto a Bio-Sil ODS-5S, 5- μm particle size, 250 $\times$ 4.0 mm, C18 column (Bio-Rad, Life Science Research Group, Hercules, CA) and eluted with a mobile phase consisting of 50 mM NaH_2PO_4 , 50 μM octane sulfonic acid, and 1% (v/v) acetonitrile (pH 2.62) at a flow rate of 1 ml/min. All buffers, following preparation, were routinely degassed, filtered through a 0.2- μm Millipore nylon filter, and the pH adjusted, if necessary. PEEKTM (polyetheretherketone) tubing was used throughout the HPLC system, and 0.2- μm PEEKTM filters were 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-channels of the CoulArray detector were set at 100, 200, 300, 400, 500, 600, 700 and 800 mV, respectively. Elution times and detection potential ranges are: metaphosphoric acid (1.6 min, 100–200 mV), L-methionine (9.6 min, 600–800 mV) and 4-methylthio-2-oxobutanoate (11.1 min, 600–800 mV).

In some cases transamination between L-glutamine (or 4-FGln) and 2-oxo acid co-substrate was measured by a spectrophotometric assay. This assay was used to calculate the catalytic efficiency ($V_{\text{max}}/K_{\text{m}}$) values for rhGTK toward L-glutamine, (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln. The assay relies on the fact that phenylpyruvate enolizes more readily than do most 2-oxo acids and that phenylpyruvate is an exceptionally good 2-oxo acid substrate of GTK [32–34]. At pH 8.0 a small percentage of phenylpyruvate is in the enol form, which equilibrates with the oxo form. Nevertheless, the small amount of phenylpyruvate in the enol form at pH 8.0 ($\sim 2\%$) is easily measurable. Since the enol absorbs in the UV region and interconversion between enol and 2-oxo forms is rapid, disappearance of UV absorbance is indicative of the removal of phenylpyruvate (the 2-oxo acid substrate form of GTK) from the reaction mixture. The reaction mixture (0.2 ml) in a 96-well plate contained variable concentrations of L-glutamine (or 4-FGln), 1 mM phenylpyruvate and 100 mM Tris-HCl buffer, pH 8.0. After warming the plate to 37 °C, enzyme was added and the disappearance of phenylpyruvate was measured at 280 nm (ϵ 390 $\text{M}^{-1}\text{cm}^{-1}$) at 37 °C. To correct for a slow

non-enzymatic increase in absorbance at 280 nm blanks lacked enzyme. In the presence of rhGTK and mM concentrations of L-glutamine the disappearance of phenylpyruvate was found to be linear until >95% of the phenylpyruvate had been transaminated (i.e. concentration of phenylpyruvate at this point is < 50 μ M). Thus, the K_m exhibited by human GTK toward phenylpyruvate is in accord with previous estimates of 10 μ M or less for the K_m of this 2-oxoacid noted for the rat enzyme in the presence of glutamine as the amine donor [32–34].

2.3.4 Glutamate dehydrogenase (GDH)—The assay procedure used in the current work is as reported previously [18]. The reaction mixture (0.05 ml) consisted of 5 mM L-glutamate (or 5 mM 4-FGlu), 100 mM glycine-KOH buffer (pH 10.5), 5 mM NAD⁺ and enzyme. The increase in absorbance at 340 nm as NAD⁺ is reduced to NADH was continuously recorded at 37 °C against a blank that lacked enzyme.

2.3.5 Aspartate aminotransferase (AspAT)—The assay procedure is similar to that published previously [18]. The reaction mixture (0.2 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM L-glutamate (or 5 mM 4-FGlu), 5 mM oxaloacetic acid (prepared from freshly constructed stock solutions), 100 mM ammonium sulfate, 0.05 mM NADH and 1 U of GDH. The plate was pre-warmed to 37 °C and the reaction was initiated by addition of 5 μ l of a solution containing AspAT. The blank sample consisted of the complete reaction mixture that lacked AspAT. The disappearance of NADH at 37 °C was continuously monitored spectrophotometrically at 340 nm relative to the blank. Rates of NADH disappearance were found to be linear after a 30 minute lag period. In this procedure GDH is added as a coupling enzyme that continuously catalyzes the NADH-dependent reductive amination of the 2-oxoglutarate generated in the AspAT reaction.

2.3.6 Alanine aminotransferase (AlaAT)—The assay procedure is as described previously [18]. The reaction mixture was the same as that used for the assay of AspAT except that oxaloacetate was replaced by pyruvate and the reaction was initiated by addition of 5 μ l of a solution containing AlaAT (in place of AspAT). Production of 2-oxoglutarate was measured in the coupled reaction with GDH. The disappearance of NADH at 37 °C was continuously monitored at 340 nm relative to a blank lacking AlaAT.

2.3.7 Glutamine synthetase (GS)—GS activity was assayed by the end-point hydroxylamine-based method as modified from that of Cooper *et al.* [18] and Pamiljans *et al.* [35]. The reaction mixture (60 μ l) contained the following reagents at the indicated final concentrations: 100 mM Tris-HCl, 10 mM MgCl₂, 10 mM ATP, 100 mM hydroxylamine, 2 mM dithiothreitol, 9 μ g GS, and varying amounts of L-glutamate, (2*S*,4*S*)-4-FGlu, or (2*S*,4*R*)-4-FGlu at a final pH of 7.4. (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu were dissolved as stock solutions in water at concentrations of 71.9 mM and 34.8 mM, respectively, by warming at 37 °C for 120 min. The reactions were conducted at 37 °C and initiated by the addition of 5 μ l of enzyme solution. Reactions were terminated at 0, 5, 10, or 15 minutes by the addition of 180 μ l of a solution containing 0.37 M ferric chloride, 0.67 M HCl, and 0.2 M trichloroacetic acid. These mixtures were then centrifuged at 10,000 $\times$ g for 5 minutes at 4 °C to remove precipitated protein after which the absorbance of 200 μ l of the supernatant

fraction at 535 nm was recorded. The amount of γ -glutamylhydroxamate formed from L-glutamate under these conditions was calculated using $850 \text{ M}^{-1}\text{cm}^{-1}$ as the extinction coefficient [36]. It is assumed that the hydroxamate of the fluorinated glutamates exhibits an identical extinction coefficient to that of L-glutamate hydroxamate in the presence of acidic ferric chloride. The specific activity of purified recombinant human GS (ammonia in place of hydroxylamine as substrate) was 17.9 U/mg [37].

2.3.8 Defluorination of 4-FGlu—The assay procedure is as described previously [18]. The reaction mixture (0.02 ml) contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM 4-FGlu and purified enzyme (or liver cytosolic fraction). After incubation at 37°C the reaction was terminated by addition of 0.02 ml of 5 mM 2,4-dinitrophenylhydrazine in 1 M HCl. After a further 5 min of incubation at 37°C , 0.16 ml of 1 M NaOH was added and the absorbance due to 2-oxoglutarate 2,4-dinitrophenylhydrazone ($\epsilon \sim 16,000 \text{ M}^{-1}\text{cm}^{-1}$) was determined at 430 nm. The blank contained complete reaction mixture plus enzyme, but lacked 4-FGlu (or 4-FGln). In a separate experiment, we determined that incubation of solutions of 4-FGlu or 4-FGln in 0.1 M potassium phosphate buffer (pH 7.4) for one hour at 37°C did not generate detectable 2,4-dinitrophenylhydrazine-reactive material.

2.4 Spectroscopy and protein determination

All spectrophotometric measurements were carried out with a SpectraMax 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Protein measurements were performed by using either the Bradford method or the bicinchoninic acid (BCA) procedure using Pierce BCA Reagents.

2.5 Statistics

Except where noted, all enzyme assays were carried out in triplicates. Differences among means were analyzed using the Mann-Whitney U test or unpaired t test. A p -value of < 0.05 is considered statistically significant. Except where noted, data are presented as the mean $\pm$ SD.

3. RESULTS

3.1 Enzyme reactions studied with L-glutamine, (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln as substrates

3.1.1 Glutaminase—GAC was incubated with increasing concentrations of L-glutamine (or 4-FGln) and assayed by an end-point procedure. A hyperbolic plot of L-glutamine concentration versus rate of product formation was obtained with an estimated K_m of $\sim 2 \text{ mM}$ (Fig. 1). When (2*S*,4*S*)-4-FGln was employed as a substrate, a hyperbolic plot was not obtained and the rate increased linearly up to a concentration of about 50 mM (at which point the rate of the reaction began to approach that observed with high concentrations of L-glutamine). This finding shows that (2*S*,4*S*)-4-FGln is a substrate for GAC, but its K_m value is much higher than that exhibited with L-glutamate. An accurate K_m was not obtainable due to the limited solubility of (2*S*,4*S*)-4-FGln, but can be estimated to be approximately 25 mM. (2*S*,4*R*)-4-FGln is an even poorer substrate of GAC than is (2*S*,4*S*)-4-FGln. Saturation kinetics were not apparent, and even at a concentration of 50 mM, the rate exhibited with

this amino acid is only ~5% that exhibited with 50 mM L-glutamine. Similar results were obtained when GAC was assayed by the continuous spectrophotometric procedure in which ammonia production is coupled to the GDH reaction. In the presence of 5 mM substrate, 100 mM potassium phosphate buffer (pH 7.2) glutaminase activity at 37 °C toward L-glutamine, (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln was 5.52 ± 0.26 , 1.34 ± 0.21 , and 0.81 ± 0.16 nmol/min/μg, respectively (n = 3). Activity toward 5 mM FP-Gln could not be detected using the continuous spectrophotometric assay.

3.1.2 Snake venom L-amino acid oxidase (LOX)—Snake venom LOX was used as a surrogate for the possible oxidation of 4-FGln by endogenous mammalian LOXs. The major mammalian LOX is the B form of L-hydroxy acid oxidase [38]. The enzyme has some oxidase activity toward L-amino acids, especially L-methionine, L-leucine and L-phenylalanine; activity toward L-alanine is relatively poor [39]. With respect to substrate specificity toward L-amino acids, the mammalian LOX is remarkably similar to that exhibited by snake venom LOX. Lichtenberg and Wellner [27] noted that, under the conditions of their assay (4 mM amino acid, 40 mM Tris-HCl buffer, pH 7.5; 37 °C), L-methionine is the most active substrate among the seventeen L-amino acids tested as substrates. The relative activity with L-methionine, L-glutamine and L-glutamate was reported to be 100, 9.9 and 0.4%, respectively. Under the conditions of our assay (5 mM L-amino acid, 100 mM potassium phosphate buffer, pH 7.4, 37 °C) the relative activity was also found to be in the order: L-methionine >> L-glutamine > L-glutamate (Table 1). We reported previously that, under the conditions of our assay procedure, (2*S*,4*R*)-4-FGln is a better substrate of *C. adamanteus* LOX than is L-glutamine [18]. When we repeated this experiment, we found that (2*S*,4*R*)-4-FGln is about 2.5 times as effective a substrate as is (2*S*,4*S*)-4-FGln (Table 1) ($p < 0.0001$). Similarly, the (2*S*,4*R*)-4-FGlu diastereoisomer is a better substrate than is the (2*S*,4*S*)-4-FGlu diastereoisomer ($p = 0.0002$). However, under the conditions used in the present assay L-glutamate, L-glutamine, both diastereoisomers of 4-FGlu, both diastereoisomers of 4-FGln, and FP-Gln are all relatively poor substrates of LOX relative to L-methionine (Table 1).

3.1.3 Glutamine-utilizing aminotransferases—It was previously shown that, among the many L-amino acid substrates tested as substrates of GTK/KAT I and GTL/KAT III, the highest $k_{\text{cat}}/K_{\text{m}}$ values were obtained for L-glutamine [20, 22]. L-Glutamine is also a substrate of AAT/KAT II, but in this case the activity is modest compared to the most active L-amino acid substrates [21]. Thus, it was of considerable interest to determine the relative ability of these enzymes to catalyze transamination reactions with (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln. In previous work we showed that the enzyme-catalyzed rates with 4.5 mM (2*S*,4*R*)-4-FGln as an amine donor relative to 10 mM L-glutamine (10 mM 4-methylthio-2-oxobutanoate as 2-oxo acid substrate; 100 mM potassium phosphate buffer, pH 7.4; 37 °C) were ~8, 43 and 20% for GTK/KAT I, AAT/KAT II and GTL/KAT III, respectively [18]. In the present work the ability of GTK/KAT I, AAT/KAT II and GTL/KAT III to catalyze transamination of L-glutamine and (2*S*,4*R*)-4-FGln was reinvestigated, but at considerably lower concentrations of L-amino acid and 4-methylthio-2-oxobutanoate substrates than that was used in the previous study. Under the present conditions (2*S*,4*R*)-4-FGln is a much poorer substrate of GTK/KAT I, AAT/KAT II and GTL/KAT III than is L-glutamine (Table

2). Activities relative to that exhibited with L-glutamine were noted to be ~3.2, ~2.0 and ~3.7% for the three enzymes, respectively. When similar studies were conducted with (2*S*, 4*S*)-4-FGln this diastereoisomer was also shown to be a poor substrate of AAT/KAT II and GTL/KAT III - rates relative to that exhibited with L-glutamine were ~4.1 and 2.3 %, respectively (Table 2). Thus, under the conditions of the assay, both (2*S*,4*S*)-4-FGln and (2*S*, 4*R*)-4-FGln are much poorer substrates of AAT/KAT II and GTL/KAT III than is L-glutamine. However, under the conditions noted in Table 2 (2*S*,4*S*)-4-FGln was found to be at least as good a substrate of GTK/KAT I as is L-glutamine and ~35 times better than is (2*S*,4*R*)-4-FGln.

Following the discovery of the remarkable selectivity of GTK/KAT I toward the two diastereoisomers of 4-FGln (4-methylthio-2-oxobutanoate as 2-oxo acid substrate) we investigated the kinetic properties of this enzyme in more detail. We determined the relative rates of disappearance of 1.0 mM phenylpyruvate (2-oxo acid substrate) in the presence of 2.53 μ g human GTK/KAT I (0.0528 nmol based on the molecular weight of the monomer; active human GTK/KAT I is a homodimer with a monomer M_r ~48,000), various concentrations of L-Gln, (2*S*,4*R*)-4-FGln, and (2*S*,4*S*)-4-FGln at 37 °C in 100 mM Tris buffer (pH 8.0). From Lineweaver-Burk plots it was determined that the $V_{\max}$ values exhibited by human GTK/KAT I toward L-Gln and (2*S*,4*S*)-4-FGln were 568 and 316 min^{-1} , respectively. K_m values were found to be 10 and 5 mM, respectively. Thus, catalytic efficiencies (k_{cat}/K_m) were calculated to be 56.8 and 63.2 $\text{min}^{-1}\text{mM}^{-1}$ for L-Gln and (2*S*, 4*S*)-4-FGln, respectively. Under the conditions of the assay, 5 mM (2*S*,4*R*)-4-FGln could not be detected as a substrate (< 2% activity relative to that exhibited with 5 mM L-glutamine; phenylpyruvate as co-substrate). The possibility was considered that (2*S*,4*R*)-4-FGln is, nevertheless, an inhibitor of hGTK/KAT I. However, no significant inhibition of human GTK-catalyzed transamination of 5 mM L-Gln with 1.0 mM phenylpyruvate could be detected in the presence of 5 mM (2*S*,4*R*)-4-FGln (data not shown). Finally, it should be noted that FP-Gln is a very poor substrate of GTK/KAT I and AAT/KAT II, and cannot be detected as a substrate of GTL/KAT III (Table 2).

3.2 Enzyme reactions studied with L-glutamate, (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu as substrates

3.2.1 Glutamate dehydrogenase (GDH)—At physiological pH values (pH 7.2–7.4) L-glutamate formation is thermodynamically favored over glutamate oxidation in the GDH reaction *in vitro*. Thus, in order to measure the forward reaction (*i.e.*, oxidation of L-glutamate to 2-oxoglutarate), high levels of L-glutamate, alkaline pH, or continuous removal of 2-oxoglutarate (e.g. with hydrazine) are procedures commonly employed. In the present work, the reaction was investigated at pH 10.5. The initial rate of increase of absorbance at 340 nm was followed at 37 °C after addition of 5 mU of GDH to the reaction mixture. It was previously shown that (2*S*,4*R*)-4-FGlu is an effective substrate of GDH [18]. Here we show that (2*S*,4*S*)-4-FGlu is also an effective substrate. Thus, the rate NADH formation in the presence of 10 mM L-glutamate, (2*S*,4*R*)-4-FGlu and (2*S*,4*S*)-4-FGlu was found to be 225 ± 31 , 162 ± 15 and 163 ± 8 nmol/min/ μ g GDH, respectively ($n = 3$ for all three substrates). Under the conditions of the assay, L-glutamate is a slightly better substrate than are the two 4-FGlu diastereoisomers ($p = 0.05$). No activity toward FP-Gln could be detected.

3.2.2 Cytosolic aspartate aminotransferase (AspAT)—In previous work it was shown that, under the conditions of the assay employed, (2*S*,4*R*)-4-FGlu is ~57% as effective as L-glutamate as a substrate of highly purified pig heart AspAT [18]. In the present work we verified this result and showed that the (2*S*,4*S*)-4-FGlu diastereoisomer is also an effective substrate of this enzyme. Thus, under the conditions of the present assay (5 mM L-amino acid substrate), specific activities with L-glutamate (*n* = 9), (2*S*,4*R*)-4-FGlu (*n* = 3) and (2*S*,4*S*)-4-FGlu (*n* = 3) were found to be 149 ± 5 , 74 ± 4 , and 155 ± 9 nmol/min/μg, respectively [*p* < 0.0001 for activity with (2*S*,4*R*)-4-FGlu versus both L-glutamate and (2*S*,4*S*)-4-FGlu]. No activity toward FP-Gln could be detected.

3.2.3 Alanine aminotransferase (AlaAT)-catalyzed transaminase reactions—Relative rates of pig heart AlaAT-catalyzed transamination with 5 mM L-glutamate, (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu were found to be 20.6 ± 2.4 , 29.6 ± 1.7 , 30.8 ± 2.0 nmol/min/μg. Thus, as reported previously [18] (2*S*,4*R*)-4-FGlu is a better substrate of AlaAT than is glutamate (*p* = 0.05). In the present work, (2*S*,4*S*)-4-FGlu was also shown to be a better substrate than is L-glutamate (*p* = 0.05). Activities toward the two 4-FGlu diastereoisomers were not significantly different from each other. No activity toward FP-Gln could be detected.

3.2.4 Alanine aminotransferase-catalyzed γ -elimination reactions—In previous work it was shown that pig heart AlaAT catalyzes an unusual γ -lyase side reaction with (2*S*,4*R*)-4-FGlu generating 2-oxoglutarate [18]. Here we compared the ability of AlaAT to catalyze a γ -elimination reaction with *both* (2*S*,4*R*)-4-FGlu and (2*S*,4*S*)-4-FGlu (Table 3). The findings indicate that under the conditions of the assay there is no significant difference in the ability of AlaAT to generate 2-oxoglutarate from either the 2*S*,4*R* or 2*S*,4*S* diastereoisomer of 4-FGlu. The greater production of 2-oxoglutarate when a small amount of 2-oxoglutarate is present in the initial reaction mixture is presumably due to a priming effect. In the presence of only amino acid substrate much of the active pyridoxal 5'-phosphate (PLP)-enzyme will be converted largely to the pyridoxamine 5'-phosphate (PMP) form, which cannot support a γ -lyase reaction. The presence of 2-oxoglutarate at the beginning of the reaction ensures relatively more enzyme in the PLP form, which is capable of catalyzing a γ -elimination reaction. Aminooxyacetate (AOAA) is often used as an inhibitor of PLP enzymes, including aminotransferases. Table 3 shows that the γ -lyase activity of the enzyme is very strongly inhibited by 5 mM AOAA, indicating the importance of PLP in the catalysis of the γ -lyase reaction. L-Glutamate also strongly inhibits the γ -lyase reaction (Table 3). Presumably, L-glutamate acts as a strong competitive inhibitor to the binding of the 4-FGlu diastereoisomers at the active site. Moreover the presence of a high glutamate concentration (50 mM) in this experiment ensures that almost all the enzyme will be in the PMP form, which, as noted, cannot support a γ -lyase reaction, further inhibiting the γ -lyase reaction.

3.2.5 γ -Elimination reaction with (2*S*,4*R*)-4-FGlu and (2*S*,4*S*)-4-FGlu catalyzed by rat liver cytosol—The experiment described in the previous paragraph was repeated except that purified AlaAT was replaced with liver cytosol (2 μl; 20 μg protein). After a one hour incubation, the amount of 2-oxoglutarate generated (no addition of 2-oxoglutarate at

the start of the reaction) from (2*S*,4*R*)-4-FGlu and (2*S*,4*S*)-4-FGlu was 9.00 ± 0.73 and 12.4 ± 0.9 nmol, respectively. The two values are significantly different ($p = 0.05$). No 2-oxoglutarate could be detected when 5 mM AOAA was included in the reaction mixture. This finding indicates that γ -elimination catalyzed by the rat liver cytosol is dependent on PLP-enzyme(s). The finding that γ -elimination is more favored with the 2*S*,4*S* diastereoisomer than it is with the 2*S*,4*R* diastereoisomer indicates that enzymes other than AlaAT might contribute to the γ -elimination reaction in the rat liver cytosol. However, the reactions described in the previous paragraph were carried out with purified pig heart AlaAT and the possibility that this enzyme may have slightly different catalytic properties to that of the AlaAT in the rat liver cytosol cannot be ruled out.

3.2.6 Glutamine synthetase (GS)—The progress curves for the first five minutes indicate that both (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu are substrates of human GS (Fig. 2). However, the fluoro compounds are much less active than is L-glutamate. Of the two diastereoisomers (2*S*,4*S*)-4-FGlu is a better substrate than is (2*S*,4*R*)-4-F-Glu. The fact that the progress curves for L-glutamate are non-linear is readily explained by considering the likely K_m value for L-glutamate. Pamiljans *et al.* [35] reported that sheep brain GS exhibits a K_m value for L-glutamate of 2.5 mM (hydroxamate assay). The K_m value for the human enzyme (hydroxamate assay) has not yet been determined, but clearly 10 mM is not saturating (Fig. 2). The volume of the GS assay mixture is 0.06 ml. Thus, the amount of L-glutamate present at a concentration of 2.5 mM in the reaction mixture is 150 nmol. However, by 15 min the amount of hydroxamate formed in the reaction mixture containing 2.5 mM L-glutamate is ~50 nmol. Thus, due to the relatively high K_m value for L-glutamate, and the removal of ~one third of the substrate it is not surprising that the reaction begins to slow by 15 minutes. Similar arguments hold for the progress curves for L-glutamate as a substrate at concentrations of 5 and 20 mM. Clearly, however, such arguments cannot hold for the (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu diastereoisomers. Inspection of Fig. 2 suggests that initially the enzyme is active with both diastereoisomers, but that the enzyme is slowly inactivated, so that by 15 minutes the enzyme is no longer turning over substrate to product.

4. DISCUSSION

4.1. General comments on the use of ^{18}F -labeled glutamine analogues as PET tumor imaging agents

For a radiotracer to be a good tumor imaging agent, label associated with the tracer must accumulate in the tumor. In this context, it was previously shown that label associated with both $[^{18}\text{F}](2\text{S},4\text{R})\text{-4-FGln}$ and $[^{18}\text{F}](2\text{S},4\text{S})\text{-4-FGln}$ accumulated in 9L tumor cells and in SF188-Bcl-xL tumor cells in a transport-dependent manner [15]. Over a period of two hours more label accumulated in the tumor cells exposed to $[^{18}\text{F}](2\text{S},4\text{R})\text{-4-FGln}$ than with $[^{18}\text{F}](2\text{S},4\text{S})\text{-4-FGln}$ [15]. Evidence was presented that uptake of $[^{18}\text{F}](2\text{S},4\text{R})\text{-4-FGln}$ was via the N transporter (a major transporter for glutamine) and via the ASC system (a Na^+ -dependent neutral amino acid transporter) [15]. The uptake of $[^{18}\text{F}](2\text{S},4\text{R})\text{-4-FGln}$ by SF188 cells was comparable to that with L- $[^3\text{H}]\text{glutamine}$ [16]. PET imaging showed a much higher uptake of label derived from $[^{18}\text{F}](2\text{S},4\text{R})\text{-4-FGln}$ into tumors than into background muscle in F344 rats carrying 9L tumor cells and in transgenic mice with M/

tomND spontaneous mammary gland tumors [16]. Moreover, it was shown that [^{18}F](2*S*,4*R*)-4-FGln is metabolized *in vivo* in part to [^{18}F](2*S*,4*R*)-4-FGlu and to $^{18}\text{F}^-$ in both tumor types [16]. In other experiments it was shown that uptake of [^{18}F]FP-Gln by amino acid transporters into 9L tumor cells in F344 9L rats was even more effective than that noted with L-[^3H]Gln [19]. The results of a dual labeling experiment with L-[^3H]Gln and [^{18}F]FP-Gln showed that the label associated with administered L-[^3H]Gln was largely incorporated into macromolecules in 9L tumor cells, whereas label associated with [^{18}F]FP-Gln was not appreciably incorporated into macromolecules [19].

Thus, there is ample evidence that [^{18}F](2*S*,4*R*)-4-FGln, [^{18}F](2*S*,4*S*)-4-FGln and [^{18}F]FP-Gln are avidly taken up into tumors via neutral amino acid/glutamine transporters and that these labeled compounds are excellent PET tumor-imaging agents. In order to model the disposition of label after the uptake of [^{18}F](2*S*,4*R*)-4-FGln, [^{18}F](2*S*,4*S*)-4-FGln or [^{18}F]FP-Gln it will be necessary to understand the possible metabolic fates of these labeled compounds. With this in mind the following sections provide a brief discussion of our findings on the enzymology of unlabeled (2*S*,4*R*)-4-FGln, (2*S*,4*S*)-4-FGln and FP-Gln.

4.2 4-FGln diastereoisomers are poor substrates of GAC

Lieberman *et al.* [16] demonstrated that [^{18}F](2*S*,4*R*)-4-FGln is converted to [^{18}F](2*S*,4*R*)-4-FGlu in two tumors, indicative of the actions of a glutaminase or glutaminase-like enzymes. In support of this idea, we previously demonstrated that rat kidney cytosol effectively deamidated (2*S*,4*R*)-4-FGln *in vitro* [18]. As reported here, however, (2*S*,4*R*)-4-FGln and (2*S*,4*S*)-4-FGln are poor substrates of GAC. One explanation for the apparently contradictory results is that the glutaminase present in tumors investigated by Lieberman *et al.* [16] is of the liver type and that this enzyme effectively utilizes (2*S*,4*R*)-4-FGln as a substrate, whereas kidney type does not. It is also possible that (2*S*,4*R*)-4-FGln is a substrate of an amidase that is neither the kidney type nor the liver type glutaminase. γ -Glutamyltransferase has the requisite activity [40–42] but is a membrane-bound enzyme and presumably poorly represented in kidney cytosol. Thus, the enzyme(s) responsible for the conversion of [^{18}F](2*S*,4*R*)-4-FGln to [^{18}F](2*S*,4*R*)-4-FGlu in tissues and tumors awaits further study.

4.3 The ability of various aminotransferases catalyze transamination of (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln

Both (2*S*,4*S*)-4-FGln and (2*S*,4*R*)-4-FGln are poor substrates of AAT/KAT II and GTL/KAT III as compared to L-glutamine (Table 2). Remarkably, however, this is not the case with GTK/KAT I where chirality of the C-F bond at position 4 strongly dictates the rate of catalysis. In particular, the (2*S*,4*S*)-4-FGln diastereoisomer is at least as good a substrate of GTK/KAT I as is L-glutamine, whereas the (2*S*,4*R*)-4-FGln diastereoisomer is a very poor substrate (Table 2, 4-methylthio-2-oxobutanoate as 2-oxo acid substrate). This was confirmed by studies in which the catalytic efficiency of the GTK-catalyzed transamination of L-glutamine [or (2*S*,4*S*)-4-FGln] with phenylpyruvate as 2-oxo acid substrate was determined. The catalytic efficiency with (2*S*,4*S*)-4-FGln as a substrate was found to be similar to that exhibited with L-glutamine. These observations suggest that [^{18}F](2*S*,4*R*)-4-

FGln (but not the [^{18}F](2*S*,4*S*)-4-FGln diastereoisomer) is much less likely than is L-glutamine to be metabolized *in vivo* by L-glutamine-utilizing aminotransferases.

Thus, there are remarkable differences in the utilization of L-glutamine, (2*S*,4*R*)-4-FGln and (2*S*,4*S*)-4-FGln by L-glutamine-utilizing aminotransferases. The amide moiety of the fluorinated substrates presumably serves to anchor these compounds within the active site, which in turn orients the C-F bond into environments that either favor catalysis or not. van der Waals and atomic radii of the F atom (1.47 Å, 0.42 Å, respectively) are somewhat different from those of the H atom (1.2 Å, 0.53 Å, respectively) (data from Bondi [43]). Moreover, unique reactions between the C-F bond and neighboring amino acid residues in the active site are likely given the very large difference in relative electronegativity (E) between the F atom (E = 4) and H atom (E = 2.1) and this may play a role in short-range interactions between fluorinated ligands and host proteins [44]. Substantial evidence exists for the occurrence in organic compounds of halogen (X) bonds where X = Cl, Br, I, but not F [45]. Therefore, it is unlikely that halogen bonding plays a substantial role in explaining the remarkable substrate specificity of GTK/KAT I toward the 4-FGln diastereoisomers. Fluorine atoms in C-F linkages are theoretically capable of forming strong hydrogen bonds, whereas the hydrogen atoms in C-H linkages do not readily form such bonds. The formation of hydrogen bonds by fluorine atoms, however, has been a matter of debate centering on whether the atomic distances between juxtaposed F and H atoms favor van der Waals interactions or hydrogen bonding [46, 47]. Nevertheless, there is now strong evidence that, in the case of protein-ligand interactions, hydrogen bonding can occur between a fluorinated ligand and HX (where X = O or N) of a protein amino acid residue [48 and references quoted therein]. Thus, the length of the C-F bond and its ability to form hydrogen bonds or strong van der Waals interactions will strongly influence the ability of the two 4-FGln diastereoisomers to react with discreet amino acid moieties within enzyme active sites relative to that exhibited by the natural substrate glutamine. We hypothesize that these interactions generally lead to much lower rates of turnover of the 4-FGln diastereoisomers compared to L-glutamine. The one exception is (2*S*,4*S*)-4-FGln, which, as noted above, is at least as good a substrate of GTK/KAT I as is L-glutamine.

4.4 (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu as substrates of glutamate dehydrogenase (GDH), aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT)

As noted above, [^{18}F](2*S*,4*S*)-4-FGln and [^{18}F](2*S*,4*R*)-4-FGln are converted to [^{18}F](2*S*,4*S*)-4-FGlu and [^{18}F](2*S*,4*R*)-4-FGlu in tumors *in vivo* [16]. (2*S*,4*R*)-4-FGlu undoubtedly undergoes further metabolism, since this compound is a substrate of GDH, AspAT, AlaAT and various L-glutamate-utilizing aminotransferases [18 and the present study]. Significant differences are noted, however, in the ability of these enzymes to utilize L-glutamate as substrate compared to the two diastereoisomers of 4-FGlu. However, in general, the differences are not as marked as those noted for the interaction of the two diastereoisomers of 4-FGln with L-glutamine-utilizing aminotransferases or with GAC. An additional metabolic transformation that [^{18}F](2*S*,4*S*)-4-FGlu and [^{18}F](2*S*,4*R*)-4-FGlu may undergo includes γ -elimination to produce the ^{18}F observed *in vivo* [16]. Evidence for this pathway is supported by our prior finding that not only is unlabeled (2*S*,4*R*)-4-FGlu a transaminase substrate of purified AlaAT, but the enzyme also catalyzes a γ -elimination reaction as a

competing side reaction with (2*S*,4*R*)-4-FGlu [18]. As shown here, (2*S*,4*S*)-4-FGlu also acts as a γ -lyase substrate of purified AlaAT.

Examples of elimination reactions involving F⁻ are rare [49]. The high electronegativity of F should favor the ability of F⁻ to act as a leaving group. However, the high bond strength of C-F and low polarizability of this bond ensures a relatively high energy of activation for elimination of F⁻. Nonetheless, the present work shows that AlaAT can catalyze γ -elimination of F⁻ from both (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu, albeit at a much slower rate than the rate at which the enzyme catalyzes a canonical transamination reaction. However, because the elimination of F⁻ is irreversible the accumulation of [¹⁸F] (2*S*,4*S*)-4-FGlu and [¹⁸F](2*S*,4*R*)-4-FGlu may be appreciable *in vivo* [16].

4.5 (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu as potential tight binding inhibitors of recombinant human glutamine synthetase (GS)

The progress curves for (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu as substrates of human GS (Fig. 2) are reminiscent of those shown previously for inhibition of this enzyme by L-methionine-*S*,*R*-sulfoximine (MSO) [37]. The inhibition of human GS by MSO was shown to be biphasic – an initial reversible competitive inhibition ($K_i = 1.19$ mM) is followed by rapid irreversible inactivation [37]. This inactivation is due to phosphorylation of the imine nitrogen, such that the resulting MSO-phosphate binds very tightly (and essentially irreversibly) at the active site as a transition state mimetic [50]. We suggest that the 4-FGlu diastereoisomers behave similarly to MSO except that they act initially as a substrate rather than as pure competitive inhibitors. However, as noted for MSO, with time a stable species is formed that strongly inhibits the enzyme activity. At present the mechanism is unknown and will require further study.

4.6 Metabolic transformations of (2*S*,4*S*)-4-(3-fluoropropyl)-L-glutamine (FP-Gln)

As noted above [¹⁸F]FP-Gln has been developed as an *in vivo* imaging agent for L-glutamine transport [19]. An advantage of this ¹⁸F-labeled compound is that it is unlikely to be as readily de-fluorinated *in vivo* as are the ¹⁸F-labeled 4-FGlu diastereoisomers or the 4-FGln diastereoisomers (following conversion to the 4-FGlu diastereoisomers). In most cases, elimination reactions involving an L-amino acid occur via β - or γ -eliminations and require PLP enzymes. Although it is possible that FP-Gln can bind to PLP enzymes, there is no possibility of double bond migration in the active site of the PLP-amino acid condensation product (a Schiff base) that will lead to an elimination of F⁻. Another advantage of [¹⁸F]FP-Gln is that it is much less likely than are the ¹⁸F-labeled 4-FGlu diastereoisomers or the 4-FGln diastereoisomers to be metabolized by alternative pathways. Consistent with this hypothesis the finding that unlabeled FP-Gln is a very poor substrate of GDH, AlaAT, and AspAT. This finding is perhaps not surprising since FP-Gln bears minimal structural analogy to L-glutamate. However, since FP-Gln is an L-glutamine analogue (albeit highly branched at the γ -position), and GTK/KAT I, AAT/KAT II and GTL/KAT III possess wide substrate specificity and utilize glutamine as a substrate, it was considered instructive to determine whether FP-Gln is a substrate of these aminotransferases. FP-Gln, however, was found to be a very poor substrate of GTK/KAT I and AAT/KAT II and could not be detected as a substrate of GTL/KAT III (Table 2). Moreover FP-Gln could not be detected as a substrate

of GAC. Presumably, the bulky fluoropropyl attachment at the γ -position hinders effective binding to the active site of the glutamine-utilizing aminotransferases and to the active site of GAC. Finally, FP-Gln is a poor substrate of LOX. Taken together these observations indicate the [18]FP-Gln is likely to accumulate in cells that exhibit accelerated glutamine uptake, but is unlikely to be extensively metabolized.

4.7 Possible relevance of the present findings to imaging of brain tumors

Because [2*S*,4*S*]-4-FGlu [2*S*,4*R*]-4-FGlu and are structurally similar to L-glutamate they are potentially excitotoxic if they enter the brain in substantial quantities from the blood after tissue deamidation of [2*S*,4*S*]-4-FGln and [2*S*,4*R*]-4-FGln, respectively. However, very little glutamate normally enters the brain from the blood [51] and glutamate is of relatively high concentration in the blood (typically 50 – 100 μ M). These factors, together with the relatively small amounts of unlabeled [2*S*,4*S*]-4-FGlu [2*S*,4*R*]-4-FGlu expected to be present in the blood after injection of [18 F][2*S*,4*S*]-4-FGln and [18 F][2*S*,4*R*]-4-FGln [15–17], suggest that uptake of [2*S*,4*S*]-4-FGlu and [2*S*,4*R*]-4-FGlu into the brain will be minimal. On the other hand, circulating glutamine has been shown to enter the mouse and human brain [52]. However, this uptake contributes maximally 20% to the brain astrocytic glutamine pool [52]. Moreover, the high concentration of glutamine in the blood (typically ~0.5 mM) will compete with the 4-FGln diastereoisomers at the blood-brain barrier. Finally, only a very small amount of label was shown to enter the brain after injection of [18 F][2*S*,4*R*]-4-FGln into rats [16].

In summary, administration of [18 F][2*S*,4*R*]-4-FGln to rodents is unlikely to cause an excitotoxic response. However, the possibility should be considered that exclusion of [18 F][2*S*,4*R*]-4-FGln/[18 F][2*S*,4*R*]-4-FGlu at the blood-brain barrier may be advantageous for imaging of brain tumors where the blood-brain barrier may be eroded, providing good imaging contrast between tumor and brain.

5. CONCLUSIONS

A major finding of the present work is that both diastereoisomers of 4-FGln are relatively poor substrates of AAT/KAT II, GTL/KAT III and GAC. On the other hand, (2*S*,4*S*)-4-FGln is an excellent substrate of GTK/KAT I, whereas, (2*S*,4*R*)-4-FGln is a relatively poor substrate. It is suggested that (2*S*,4*R*)-4-FGln and (2*S*,4*S*)-4-FGln may be useful probes for studying the detailed topology of the active sites of glutamine-utilizing enzymes such as glutaminase and glutamine transaminases. These compounds may also serve to identify differential *in situ* responses of tumor cells to glutamine metabolites, for example kidney vs. liver cancer.

In general, the substrate specificities of GDH, AlaAT and AspAT toward (2*S*,4*S*)-4-FGlu are similar to those published previously for (2*S*,4*R*)-4-FGlu [18], including the ability of AlaAT to catalyze a unique γ -elimination reaction with this diastereoisomer. However, differences in the specific activities of these enzymes toward L-glutamate, (2*S*,4*S*)-4-FGlu, and (2*S*,4*R*)-4-FGlu, suggest that the 4-FGlu diastereoisomers might also be useful probes for the active sites of glutamate-utilizing enzymes. A remarkable finding of the present work is that both diastereoisomers of 4-FGlu are relatively weak substrates of GS and the enzyme

is eventually inactivated by these compounds. The mechanism of this inhibition requires further study, but suggests a potentially new avenue for the design of GS inhibitors. A summary of the present findings regarding the enzymology of the two diastereoisomers of 4-FGln and that of the two diastereoisomers of 4-FGlu is shown in Fig. 3.

Finally, using a selection of purified enzymes we showed that FP-Gln is much less likely to be metabolized *in vivo* than are the 4-FGln and 4-FGlu diastereoisomers. This metabolic “inertness” provides a distinct advantage in modeling metabolic fates when [^{18}F]FP-Gln rather than ^{18}F -labeled 4-FGlu or 4-FGlu is used as an *in vivo* tumor imaging agent.

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Abbreviations

AAT	L- α -aminoadipate aminotransferase
AlaAT	alanine aminotransferase
AOAA	aminooxyacetate
AspAT	aspartate aminotransferase
FP-Gln	(2 <i>S</i> ,4 <i>S</i>)-4-(3-fluoropropyl)-L-glutamine
4-FGln	L-4-fluoroglutamine (chirality at the 4 position not specified)
(2<i>S</i>,4<i>R</i>)-4-FGln	(2 <i>S</i> ,4 <i>R</i>)-4-fluoroglutamine
(2<i>S</i>,4<i>S</i>)-4-FGln	(2 <i>S</i> ,4 <i>S</i>)-4-fluoroglutamine
4-FGlu	L-4-fluoroglutamate (chirality at the 4 position not specified)
(2<i>S</i>,4<i>R</i>)-4-FGlu	(2 <i>S</i> ,4 <i>R</i>)-4-fluoroglutamate
(2<i>S</i>,4<i>S</i>)-4-FGlu	(2 <i>S</i> ,4 <i>S</i>)-4-fluoroglutamate
HPLC	high performance liquid chromatography
GDH	glutamate dehydrogenase
GAC	isoform C of mouse kidney-type glutaminase (GLS1)
GS	glutamine synthetase
GTK	glutamine transaminase K
GTL	glutamine transaminase L

KAT	kynurenine aminotransferase
LOX	L-amino acid oxidase
MSO	L-methionine- <i>S,R</i> -sulfoximine
PET	positron emission tomography
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate
rhGTK	recombinant human GTK;TCA, tricarboxylic acid
TCA	tricarboxylic acid

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Highlights

- Activity with glutamine transaminase K: $\text{Gln} \approx (2S,4S)\text{-4-FGln} \gg (2S,4R)\text{-4-FGln}$
- Activity with glutamine transaminase L: $\text{Gln} \gg (2S,4R)\text{-4-FGln} \approx (2S,4S)\text{-4-FGln}$
- Activity with glutaminase (short form): $\text{Glu} > (2S,4S)\text{-4-FGln} \gg (2S,4R)\text{-4-FGln}$
- Activity with glutamine synthetase: $\text{Glu} > (2S,4S)\text{-4-FGlu} \approx (2S,4R)\text{-4-FGlu}$
- $(2S,4S)\text{-4-F-(3-Fluoropropyl)glutamine}$ is likely largely metabolically inert in vivo

Fig. 1. L-Glutamine (Gln), (2S,4S)-4-FGln and (2S,4R)-4-FGln as substrates of GAC. For details see the text.

Fig. 2.

Progress curves for hydroxamate formation catalyzed by human GS with L-Glu, (2*S*,4*S*)-4-FGlu and (2*S*,4*R*)-4-FGlu as substrates. Each point represents the mean $\pm$ SEM of three independent experiments. Each of the independent experiments was performed in duplicate. In many cases, the SEM is less than the height of the symbols representing the mean values. The points on each curve were fitted by a mono-exponential rise function, according to pseudo first-order reaction kinetics. All of the fitted progress curves have *R* values of 0.99 with *P* < 0.05. Note that the three ordinate axes are all shown to the same scale to facilitate

direct comparison among the glutamate and the glutamate analogues. For details of the assay used see the Methods section.

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Fig. 3.

Enzyme-catalyzed reactions with the 2*S*,4*S*- and 2*S*,4*R* diastereoisomers of 4-FGln and 4-FGlu. The solid arrows indicate enzyme-catalyzed reactions with the fluorinated analogues uncovered in the present work that are favorable compared to those with glutamine/glutamate. The broken arrows indicate enzyme-catalyzed reactions that could be detected, but which are unfavorable compared to those with glutamine/glutamate. The figure also indicates the discovery of an unusual γ -elimination reaction with the two diastereoisomers of 4F-Glu catalyzed by AlaAT. Transaminases investigated in the present work with 4-FGln as substrates included GTK/KAT I, AAT/KAT II and GTL/KAT II. Transaminases investigated with 4-FGlu as substrates included AlaAT and AspAT. Abbreviations: Ala, alanine aminotransferase; AspAT, aspartate aminotransferase; GTK, glutamine transaminase K, GTK; GTL, glutamine transaminase L; KAT, kynurenine aminotransferase; GS, glutamine synthetase; GDH, glutamate dehydrogenase.

Table 1Activity of *C. adamanteus* L-amino acid oxidase (LOX) toward various L-amino acids ^a

L-Amino acid	Activity (nmol/min/mg)	Relative Activity (%)
Methionine	2050 ± 340	100
Glutamine	21.1 ± 0.8	1.03
(2 <i>S</i> ,4 <i>R</i>)-4-FGln	54.7 ± 8.9	2.67
(2 <i>S</i> ,4 <i>S</i>)-4-FGln	21.2 ± 6.3	1.04
Glutamate	14.5 ± 3.6	0.71
(2 <i>S</i> ,4 <i>R</i>)-4-FGlu	58.3 ± 1.6	2.85
(2 <i>S</i> ,4 <i>S</i>)-4-FGlu	27.7 ± 1.8	1.35
FP-Gln	20.3 ± 2.7	0.99

^aThe reaction mixture (0.2 ml) contained 5 mM L-amino acid, 100 mM potassium phosphate buffer (pH 7.4), 100 mM semicarbazide and 0.1 µg (0.4 mU) to 2.5 µg (10 mU) of LOX (depending on the substrate). The rate of 2-oxo acid semicarbazone formation was monitored at 248 nm at 37 °C (n = 3 – 6).

Table 2Relative rates of transamination catalyzed by glutamine-utilizing aminotransferases ^a

Substrate	Enzyme		
	GTK/KAT I	AAT/KAT II	GTL/KAT III
L-Glutamine	100 ± 25	100 ± 69	100 ± 6
(2 <i>S</i> ,4 <i>S</i>)-4-FGln	111 ± 34	4.10 ± 1.70	2.25 ± 0.21
(2 <i>S</i> ,4 <i>R</i>)-4-FGln	3.2 ± 0.8	2.05 ± 0.48	3.67 ± 0.92
FP-Gln	0.53 ± 0.30	2.44 ± 1.57	< 1.0

^aThe reaction mixture (0.025 ml) contained enzyme, 0.6 mM L-amino acid, 0.6 mM KMB and 60 mM potassium phosphate buffer (pH 7.4) incubated at 37 °C. At intervals, reaction mixtures were quenched with 25% metaphosphoric acid and the production of L-methionine was determined by HPLC with electrochemical detection as described in the text. The activity exhibited by AAT/KAT II toward various substrates was determined in triplicate. In the case of GTK/KAT I and GTL/KAT III the results represent the pooled data from 3 or 4 separate determinations each carried out in triplicate. The amount of enzyme added to each reaction mixture was 2.8 µg (GTK/KAT I), 2.8 µg (AAT/KAT II) and 0.93 µg (GTL/KAT III). Activities with L-glutamine as substrate were ~0.43, 0.015 and 0.56 nmol/min/µg, respectively. All rates for the fluorinated analogs [except that for (2*S*,4*S*)-4-FGln] are significantly different from that observed with glutamine (p = 0.05 to 0.01).

Table 3

2-Oxoglutarate (nmol) formation from 4-FGlu diastereoisomers catalyzed by purified pig heart alanine aminotransferase ^a

Addition	(2 <i>S</i> ,4 <i>R</i>)-4-FGlu	(2 <i>S</i> ,4 <i>S</i>)-4-FGlu
None	25.5 ± 1.0	25.8 ± 1.0
50 mM L-Glutamate	<1.0 ^b	<1.0 ^b
5 mM AOAA	<1.0 ^b	<1.0 ^b
0.25 mM 2-Oxoglutarate	35.5 ± 1.3 ^{b,c}	38.2 ± 2.2 ^{b,c}

^aThe reaction mixture (0.02 ml) contained 100 mM potassium phosphate buffer (pH 7.2), 5 mM 4-FGlu, 0.65 U (10 µg, 650 mU) alanine aminotransferase and the indicated addition. After incubation for four hours at 37 °C the amount of 2-oxoglutarate formed was determined by the 2,4-dinitrophenylhydrazine procedure. Blanks lacked 4-FGlu.

^bSignificantly different from the no addition value (p = 0.05).

^cThe value is corrected for 2-oxoglutarate present in the reaction mixture at the start of the incubation.