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Comparative enzymology of (2S,4R)4-fluoroglutamine and (2S,4R)4-fluoroglutamate

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Abstract

Many cancer cells have a strong requirement for glutamine. As an aid for understanding this phenomenon the ¹⁸F-labeled 2S,4R stereoisomer of 4-fluoroglutamine [(2S,4R)4-FGln] was previously developed for *in vivo* positron emission tomography (PET). In the present work, comparative enzymological studies of unlabeled (2S,4R)4-FGln and its deamidated product (2S,4R)4-FGlu were conducted as an adjunct to these PET studies. Our findings are as follows: Rat kidney preparations catalyze the deamidation of (2S,4R)4-FGln. (2S,4R)4-FGln and (2S,4R)4-FGlu are substrates of various aminotransferases. (2S,4R)4-FGlu is a substrate of glutamate dehydrogenase, but not of sheep brain glutamine synthetase. The compound is, however, a strong inhibitor of this enzyme. Rat liver cytosolic fractions catalyze a γ -elimination reaction with (2S,4R)4-FGlu, generating α -ketoglutarate. Coupling of a deamidase reaction with this γ -elimination reaction provides an explanation for the previous detection of ¹⁸F⁻ in tumors exposed to [¹⁸F](2S,4R)4-FGln. One enzyme contributing to this reaction was identified as alanine aminotransferase, which catalyzes competing γ -elimination and aminotransferase reactions with (2S,4R)4-FGlu. This appears to be the first description of an aminotransferase catalyzing a γ -elimination reaction. The present results demonstrate that (2S,4R)4-FGln and (2S,4R)4-FGlu are useful analogues for comparative studies of various glutamine- and glutamate-utilizing enzymes in normal and cancerous mammalian tissues, and suggest that tumors may metabolize (2S,4R)4-FGln in a generally similar fashion to glutamine. In plants, yeast and bacteria a major route for ammonia assimilation involves the consecutive action of glutamate synthase plus glutamine synthetase (glutamate synthase cycle). It is suggested that (2S,4R)4-FGln and (2S,4R)4-FGlu will be useful probes in studies of ammonia assimilation by the glutamate synthase pathway in these organisms. Finally, glutamine transaminases are conserved in mammals, plants and bacteria, and probably serve to close the methionine salvage pathway, thus linking nitrogen metabolism to sulfur

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metabolism and one-carbon metabolism. It is suggested that (2*S*,4*R*)-4-FGln may be useful in studies of the methionine salvage pathway in a variety of organisms.

Keywords

Elimination of fluoride from 4-fluoroglutamate; 4-fluoroglutamine; 4-fluoroglutamate; glutamate; glutamine; transamination of 4-fluoroglutamate; transamination of 4-fluoroglutamine

1. Introduction

In the 1920s Otto Warburg and colleagues showed that cancer cells take up considerably more glucose and produce more lactate than comparable non-cancerous cells, even in the presence of ambient oxygen (the Warburg Effect) (Levine and Puzio-Kuter, 2010). Cancer cells rely heavily on glycolysis, but this process does not provide enough energy for the growing and dividing cancer cells. With the development of novel advances in understanding regulatory signaling and metabolic control, L-glutamine has recently been “rediscovered” as an important energy source and nitrogen supply for many cancer cells, a phenomenon referred to as “glutamine addiction” (e.g. Szeliga and Obara-Michlewska, 2009; Wang et al., 2010; Erickson and Cerione, 2010; Levine and Puzio-Kuter, 2010; Suzuki et al., 2010; Hu et al., 2010; Chatterjee et al., 2011; Dang et al., 2011; Koppenol et al., 2011).

As a result of this addiction, it should be possible to metabolically “image” many types of tumors in patients with a suitably labeled L-glutamine or analogue of glutamine (Qu et al. 2011a). One strategy is to label glutamine with a positron-emitting isotope such as ^{11}C ($t_{1/2}$ 20.4 min) or ^{13}N ($t_{1/2}$ 9.96 min) and follow the incorporation of label into tissues and/or tumors by positron emission tomography (PET). Methods for introducing ^{11}C into amino acids have mostly relied on the Strecker synthesis, which results in the formation of a D,L-racemic mixture of the desired [^{11}C] amino acid, from which the L-enantiomer must be laboriously resolved (Vaalberg et al., 1992). In part, as a result of this limitation, amino acids labeled with ^{11}C in the α position via the Strecker synthesis have not been systematically reported as PET tracers *in vivo*. However, [^{11}C -*methy*] methionine and several ^{18}F -labeled amino acids (^{18}F , $t_{1/2}$ 109.8 min) have been extensively used in PET studies (Laverman, 2011).

It is relatively straightforward to enzymatically synthesize [^{13}N -*amide*]glutamine and [^{13}N]glutamate in the L-configuration (Gelbard and Cooper, 1995). Indeed, some earlier studies have used L-[^{13}N]glutamate to image sarcomas in cancer patients (Gelbard et al., 1979; Reiman et al., 1981, 1982). However, although L-[*amide*- ^{13}N]glutamine and L[*amine*- ^{13}N]glutamine have provided useful metabolic information on the metabolism of glutamine in rat liver *in vivo* (Cooper et al., 1988) these labeled forms of glutamine have not been systematically used in human or rodent PET imaging studies, presumably in part as a result of the short half of ^{13}N .

A strategy for metabolically imaging glutamine is to prepare glutamine labeled in such a way that it can be imaged by magnetic resonance spectroscopy (MRS). For example, Qu et al. (2011a) synthesized L-[5- ^{13}C -4- $^2\text{H}_2$]glutamine and used MRS imaging to show that when this compound is injected into human glioma cells (myc upregulated SF188-Bcl-x_L tumor cells) it is rapidly converted to L-[5- ^{13}C -4- $^2\text{H}_2$]glutamate presumably by endogenous glutaminase. In addition, studies with L-[3,4- ^3H (N)]glutamine showed uptake of label that was even greater than that demonstrated with [^{18}F]fluorodeoxyglucose ([^{18}F]FDG), a traditionally useful PET imaging agent for tumors (Qu et al., 2011a).

Due in part to the relatively long half life of ^{18}F (relative to that of other positron-emitting isotopes such as ^{13}N and ^{11}C), ^{18}F -labeled imaging agents are now being produced under standardized synthetic procedures for a wide variety of biomedical PET imaging purposes (Clanton, 2002). ^{18}F -labeled 4-fluoroglutamine ($[^{18}\text{F}]4\text{-FGln}$) was recently added to this list. Thus, Qu et al. (2011b) have devised methods for the rapid radiochemical synthesis of all four ^{18}F -labeled diastereoisomers of 4-FGln. It was reasoned that although introduction of a fluorine atom into L-glutamine results in the formation of a non-physiological L-amino acid, replacement of an H with an F in the 4 position of glutamine will result in a glutamine analogue with similar biological properties to those of L-glutamine

Uptake and inhibitor studies using 9L tumor cells and SF188Bcl-xL tumor cells (glutamine-addicted tumor cells derived from a glioblastoma) provided strong evidence for the potential application of $[^{18}\text{F}]$ fluoroglutamines in conjunction with PET for *in vivo* imaging of tumors that use glutamine as a major energy source (Qu et al. 2011b). Of the four ^{18}F -labeled diastereoisomers studied, label accumulation was most pronounced with (2*S*,4*R*)[^{18}F]4-FGln [i.e. L-glutamine containing a C-F bond in the 4 (or γ) position with an *R* configuration and an *S* (or L-) configuration at the 2 (or α) position]. The uptake of ^{18}F label into 9L tumor cells after administration of (2*S*,4*R*)[^{18}F]4-FGln was comparable to that observed with $[^{18}\text{F}]$ FDG, but was considerably greater in SF188-Bcl-xL tumor cells (Qu et al., 2011b).

In order to understand processes contributing to the metabolic imaging of administered (2*S*,4*R*)[^{18}F]4-FGln, it is important to understand the metabolic fate of (2*S*,4*R*)[^{18}F]4-FGln and to ascertain whether (2*S*,4*R*)4-FGln truly is a surrogate for L-glutamine *in vivo*. To this end we have investigated the enzymatic transformations of unlabeled (2*S*,4*R*)4-FGln and (2*S*,4*R*)4-FGlu [a major product previously shown to arise from the metabolism of (2*S*,4*R*)4-FGln (Qu et al., 2011b; Lieberman et al., 2011; Ploessl et al., 2012)]. The results are reported here. In general, the results suggest that replacing a C-H with a C-F bond in the 4 position of glutamine and glutamate has only minor effects on the ability of glutamine- and glutamate-utilizing enzymes to bind the fluorinated analogues and process them as substrates (with the notable exception of glutamine synthetase). These findings are consistent with the similar van der Waal and atomic radii of F (1.47 Å, 0.42 Å, respectively) and H (1.2 Å, 0.53 Å, respectively) (data from Bondi, 1964). On the other hand, there is a very large difference in relative electronegativity (F, 4; H, 2.1), but it appears that this difference does not generally alter the ability of (2*S*,4*R*)4-FGln or (2*S*,4*R*)4-FGlu to interact with glutamine- or glutamate-utilizing enzymes, respectively.

During the course of these studies we uncovered a novel γ -elimination reaction with 4-FGlu catalyzed by alanine aminotransferase. We also showed that this compound is a strong inhibitor of sheep brain glutamine synthetase. Finally, we suggest that (2*S*,4*R*)4-FGln and (2*S*,4*R*)4-FGlu may be useful probes for studying nitrogen metabolism in plants, yeast and bacteria, and that (2*S*,4*R*)4-FGln may also be a useful probe in studying the ubiquitous methionine salvage pathway.

2. Materials and methods

2.1. Reagents

Ammonium sulfate, dithiothreitol (DTT), glycine, L-glutamine, L-glutamate, L-phenylalanine, L-homoserine, sodium α -keto- γ -methiolbutyrate (KMB), sodium pyruvate, oxaloacetic acid, NADH, NAD^+ , magnesium chloride, ATP, potassium chloride, the trisodium salt of phosphoenolpyruvate (PEP), semicarbazide.HCl, hydroxylamine.HCl, aminooxyacetic acid hemi HCl (AOAA), ferric chloride and trichloroacetic acid were obtained from Sigma, St. Louis, MO. All solutions containing HCl salts were neutralized

with NaOH before use. 2,4-Dinitrophenylhydrazine was purchased from ICN Biomedicals, Irvine, CA. Stock solutions (180 mM) of α -ketoglutaramate (KGM) were prepared by the method of Krasnikov et al. (2009a). (2*S*,4*R*)4-FGln (hereinafter simply referred to as 4-FGln) was prepared by the method of Qu et al. (2011b) [98.5% purity; 97% enantiomeric purity (~3% (2*S*,4*S*)4-FGln)]. (2*S*,4*R*)4-FGlu (hereinafter simply referred to as 4-FGlu) was prepared as follows: Fully protected (2*S*,4*R*)4-FGln [(2*S*,4*R*)-*tert*-butyl 2-(*tert*-butoxycarbonylamino)-4-fluoro-5-oxo-5-(2,4,6-trimethoxybenzyl amino)pentanoate (Qu et al., 2011b)] (0.150 g, 0.3 mmol) was dissolved in 6 M aqueous HCl (5 mL). The solution was heated to 60 °C for 2.5 h. After cooling to room temperature, the mixture was diluted with water (5 mL) and washed with ether (2 × 10 mL). The aqueous phase was dried under vacuum. The resulting residue was dissolved in a small amount of 95% ethanol. Upon cooling in an ice bath, excess of propylene oxide (7 mL) was added. After stirring at 0 °C for 30 min, the mixture was filtered, the precipitated white solid was collected and dried under vacuum to yield (2*S*,4*R*)4-FGlu ((0.043 g, 86%); [α]_D²⁵ = + 29.1 (*c* = 1.0, 1.0 M aqueous HCl) ([α]_D²³ = + 36.8 (*c* = 0.9, 1.0 M HCl) (Konas and Coward, 2001)); HPLC (performed on an HPLC Agilent Technologies® 1200 Series with solid phase Phenomenex® Chirex 3126 (D)-penicillamine (150 × 4.6 mm), UV detector set at 254 nm; mobile phase: 2.0 mM CuSO₄ solution, flow rate: 1.0 mL/min, column temperature 25 °C) of 4-FGLU: 98.0%, major peak: Rt = 20.9 min; 2.0%, minor peak: Rt = 23.3 min; ¹H NMR (Bruker® DPX 200 MHz, D₂O) δ 5.29 (dd, 0.5 H, *J*₁ = 10.0 Hz, *J*₂ = 3.0 Hz), 5.04 (dd, 0.5 H, *J*₁ = 10.0 Hz, *J*₂ = 3.0 Hz), 4.10 (t, 1 H, *J* = 6.4 Hz), 2.78-2.48 (m, 1 H), 2.44-2.24 (m, 1 H); ¹³C NMR (50 MHz, D₂O + trace CD₃OD) δ 176.2, 175.8, 173.8, 91.4, 87.8, 53.0, 34.7, 34.3; HRMS (Agilent Technologies® LC/MS TOF) calcd for C₅H₈FNO₄ (M + H)⁺: 166.0516; found: 166.0516. Stock solutions of 9 mM 4-FGln and 10 mM 4-FGlu were made up in doubly distilled water. These concentrations are close to saturation in water at 25 °C. These concentrations are lower than saturating levels of L-glutamine (246 mM) and L-glutamic acid (57 mM) in water at 25 °C.

2.2. Enzymes

Highly purified recombinant human glutamine transaminase K [rhGTK; also known as kynurenine aminotransferase I (KAT I)] (Han et al. 2004), mouse α -amino adipate aminotransferase (also known as KAT II) (Han et al., 2008) and mouse glutamine transaminase L (GTL; also known as KAT III) (Han et al., 2009) were prepared as indicated. All three enzymes were stored at 4 °C in 20% glycerol, 10 mM potassium phosphate buffer (pH 7.4) at a concentration of 11.3, 11.3 and 3.7 mg/mL, respectively. Rat liver mitochondrial aspartate aminotransferase [1.35 mg/mL in 20 mM Tris HCl buffer, pH 8.3, containing 0.1 mM EDTA, 150 mM NaCl and 0.2% (w/v) sodium azide; 410 U/mg at 37 °C], expressed in *Escherichia coli* and purified as described previously (Mattingly et al., 1993) was a generous gift from Dr. Ana Ariarte (University of Missouri, Kansas City). *Crotalus adamanteus* venom L-amino acid oxidase type 4 (9.8 mg/mL; 4 U/mg aqueous solution), *E. coli* glutaminase (grade II; lyophilized powder, 6.1 U/mg), beef liver catalase (18,600 U/mg), sheep brain glutamine synthetase (lyophilized powder; 9.1 U/mg), pig heart cytosolic aspartate aminotransferase (lyophilized powder; 100 U/mg), pig heart alanine aminotransferase (lyophilized powder; 65 U/mg), rabbit muscle pyruvate kinase (470 U/mg, lyophilized powder) and rabbit muscle lactate dehydrogenase (720 U/mg in 50% glycerol) were obtained from Sigma. ω -Amidase (specific activity 11 U/mg; 1 mg/mL), an enzyme that catalyzes the hydrolysis of KGM to α -ketoglutarate and ammonia, was purified from the cytosolic fraction of rat liver by the method of Hersh (1971) as modified by Krasnikov et al (2009b), except that the last step was omitted. [KGM is the α -keto acid analogue of L-glutamine, which is generated from L-glutamine by a transamination reaction or by an L-amino acid oxidase reaction.] Beef liver glutamate dehydrogenase (100 mg/mL in 20% glycerol; 50 U/mg) was obtained from Boehringer Ingelheim. A unit (U) of enzyme activity

is defined as the amount of enzyme that produces 1 μmol of product per min under standard assay conditions.

Note that the specific activities of most of the enzymes used in the present work are provided above, and the number of units (U) (or millunits, mU) is included in the descriptions of the assay procedures. The notable exceptions are GTK/KAT I, AAT/KAT II or GLT/KAT III). These enzymes have very broad specificities and different investigators have used very different definitions of specific activity. Thus, for these enzymes we have preferred to use the amount of enzyme rather than units in comparative studies of various substrates.

2.3. Preparation of rat tissue

Except where stated all procedures were carried out at 0 – 4°C.

Rat liver was obtained from an adult male Sprague Dawley rat and homogenized in five volumes of 10 mM potassium phosphate buffer (pH 7.4) by means of a glass to Teflon hand held 50-mL Potter homogenizer. Insoluble material was removed by centrifugation at 10,000 g for ten min. The resulting supernatant (cytosolic fraction) (~10 mg protein/mL) was stored frozen at –20 °C. Several enzymes of interest to the current work, including glutamine synthetase, ω -amidase, cystathionine γ -lyase, aspartate aminotransferase and alanine aminotransferase are stable to repeated freeze-thawing of the cytosolic fraction.

In order to detect the activity of the mitochondrial enzyme glutaminase in rat kidney preparations an adult female Sprague-Dawley rat was sacrificed. The kidneys were removed and stored at –20 °C for five days. The kidneys were then thawed on ice, homogenized in an excess of 10 mM potassium phosphate buffer (pH 7.4), and centrifuged at 1,000 g for 10 min to remove cellular debris and membranes. Lubrol was then added to the 1,000 g supernatant fraction to a final concentration of 0.05% (to solubilize the glutaminase in the mitochondria). The suspension in 0.05% lubrol, 10 mM potassium phosphate buffer (pH 7.4) was then homogenized and insoluble material was removed by centrifugation at 10,000 g for 10 min. The resulting supernatant was labeled 'kidney supernatant' and stored at –20 °C. Glutaminase activity (section 2.4.5.) was assayed in the thawed supernatant.

2.4. Determination of enzyme activities

2.4.1. L-Amino acid oxidase—L-Amino acid oxidase (EC 1.4.3.2) catalyzes the oxidation of a large number of L-amino acids to the corresponding α -imino acid and H_2O_2 . The α -imino acid non-enzymatically hydrolyzes within a few seconds to the corresponding α -keto acid and ammonia (Hafner and Wellner, 1971). The assay used in the current work makes use of the finding that the initial product of the L-amino acid oxidase reaction (i.e. the α -imino acid) reacts rapidly with semicarbazide to form the corresponding α -keto acid semicarbazone and ammonia (Hafner and Wellner, 1979). Under the conditions used by Hafner and Wellner (1979) about 4 mM semicarbazide effectively trapped 50% of the imine generated from the L-amino acid oxidase-catalyzed oxidation of L-leucine at pH 7.7 at 25 °C. We found that the trapping is essentially complete in the presence of 100 mM semicarbazide at 37 °C. Several α -keto acid semicarbazones exhibit an extinction coefficient (ϵ) of 10,000 $\text{M}^{-1}\text{cm}^{-1}$ at 248 nm (Olson, 1959). It is assumed in the present work that the α -keto acid semicarbazones generated in the L-amino acid oxidase reactions investigated here also have an ϵ of ~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 L-amino acid oxidase solution (8 mU) and the rate of increase of absorbance was continuously monitored at 248 nm.

2.4.2. ω -Amidase (EC 3.5.1.3)—The assay procedure was that of Krasnikov et al. (2009a). The assay mixture contained 5 mM KGM, 100 mM Tris-HCl buffer (pH 8.5) and enzyme in a final volume of 0.05 mL. After incubation for 10 min at 37 °C the reaction was stopped by addition of 0.02 mL of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After incubation at 37 °C for 5 min, 0.13 mL of 1 M NaOH was added. The absorbance was read at 430 nm within 2 min against blanks lacking KGM or enzyme (ϵ α -ketoglutarate 2,4-dinitrophenylhydrazone, $16 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$). In some cases the volume of the assay mixture was reduced to 0.02 mL. It should be noted that in strong acid, KGM is fully cyclized to a lactam that masks the carbonyl function at the α carbon. Thus, the product of the ω -amidase reaction, α -ketoglutarate, reacts with the carbonyl reagent 2,4-dinitrophenylhydrazine whereas KGM does not.

2.4.3. Glutamate dehydrogenase (EC 1.4.1.2)—The reaction mixture (0.05 mL) consisted of 5 mM L-glutamate (or 5 mM 4F-Glu), 100 mM glycine-KOH buffer (pH 10.5), 5 mM NAD⁺ and enzyme. The rate of increase in absorbance at 340 nm as NAD⁺ is reduced to NADH (ϵ $6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) was continuously recorded at 37 °C against a blank that lacked enzyme. The blank takes into account possible contaminating ammonia.

2.4.4. Glutamine synthetase (EC 6.3.1.2)—Two methods were employed. The first method relied on the fact that hydroxylamine can replace ammonia in the reaction catalyzed by glutamine synthetases purified from animal and plant sources (Pamijans et al., 1962). The resulting γ -glutamylhydroxamate forms a relatively stable brown colored solution with acidic ferric ions. The hydroxamate assay used here is essentially that of Pamijans et al. (1962) modified for 96-well plate analysis. The reaction mixture (0.05 mL) contained 100 mM Tris HCl buffer (pH 7.4), 5 mM glutamate, 10 mM MgCl₂, 10 mM ATP, 100 mM hydroxylamine, 2 mM DTT and enzyme. After incubation at 37 °C for 1 h the reaction was terminated by addition of 0.15 mL of a solution containing 0.37 M ferric chloride, 0.67 M HCl and 0.2 M trichloroacetic acid (ferric chloride reagent). Precipitated protein was removed by centrifugation (10,000 g) for five min. The absorbance of the supernatant was read at 535 nm against a blank (reaction mixture lacking L-glutamate/4-FGlu). The extinction coefficient of γ -glutamylhydroxamate under these conditions is $850 \text{ M}^{-1}\text{cm}^{-1}$ (Ronzio et al. 1969).

The second method relied on the measurement of the glutamine synthetase product ADP by a coupled assay using pyruvate kinase and lactate dehydrogenase. The reaction mixture was modified from that of Wellner et al. (1966) for well plate analysis. The reaction mixture (0.2 mL) contained 75 mM potassium chloride, 20 mM magnesium chloride, 3.5 mM PEP, 10 mM ATP, 5 mM L-glutamate, 50 mM ammonium sulfate, 1 mM NADH, 3 U lactate dehydrogenase and 5 U pyruvate kinase in 100 mM Tris-HCl buffer (final pH 8.0). The reaction mixture was incubated at 37 °C for 10 min to remove any contaminating ADP. Thereafter, the reaction was initiated by addition of a solution (5 μ L) containing glutamine synthetase and the rate of disappearance of NADH was measured at 340 nm. The blanks lacked glutamate or added glutamine synthetase.

2.4.5. Glutaminase—Glutaminase (EC 3.5.1.2) activity in the kidney supernatant was determined by the procedure of Kvamme et al. (1970) modified for well plate analysis. The assay utilized glutamate dehydrogenase as coupling enzyme to continuously monitor ammonia production. The reaction mixture (final concentrations) contained 20 mM α -ketoglutarate, 0.2 mM NADH, 100 mM potassium phosphate buffer (pH 8.0), 0.2 mM EDTA, 2.5 U of glutamate dehydrogenase and 0.01 mL of tissue cytosolic fraction. Covered reaction mixtures in the well plate were incubated for one hour at 37 °C to remove endogenous ammonia. Thereafter, the cover was removed and the reaction was initiated by

the addition of freshly prepared L-glutamine (or 4-FGln) such that the final volume of the reaction mixture was 0.2 mL and final concentration of L-glutamine or 4-FGln was 5 mM. The blank contained complete reaction mixture and cytosolic fraction except that L-glutamine/4-FGln was omitted. The rate of disappearance of absorbance at 340 nm was continuously measured relative to the blank.

Since the pH optimum of *E. coli* glutaminase is about 4.5–5.0 (Meister et al. 1955) and glutamate dehydrogenase has low activity at this pH value, a different strategy was used to measure the activity of *E. coli* glutaminase. The reaction mixture (0.05 mL) contained 5 mM L-glutamine (or 4-FGln), 100 mM sodium acetate buffer (pH 5.0) and enzyme. After incubation for one hour at 37 °C the reaction was quenched by addition of 0.15 mL of 167 mM glycine-NaOH buffer (pH 10.5) containing 2.5 U of glutamate dehydrogenase and 5 mM NAD⁺. The blank contained complete reaction mixture to which enzyme was added prior to addition of the glycine buffer. After a further incubation for one hour at 37 °C the increase in absorbance at 340 nm relative to the blank was recorded. The blank takes into account the small amount of contaminating ammonia in the reaction mixture (typically <1 mM). A standard curve consisting of glutamate or 4-FGlu standards (0, 50, 100 nmoles) added to assay mixtures lacking glutaminase were analyzed simultaneously. In a separate experiment it was shown that 4-FGlu is a good substrate of glutamate dehydrogenase (see section 3.5.).

2.4.6. Aspartate aminotransferase (EC 2.6.1.1)—The reaction mixture (0.2 mL) contained 100 mM potassium phosphate buffer (pH 7.4), 5 mM L-glutamate (or 5 mM 4-FGlu), freshly prepared 5 mM oxaloacetic acid, 100 mM ammonium sulfate, 0.05 mM NADH and 1 U of glutamate dehydrogenase. The plate was pre-warmed to 37 °C and the reaction was initiated by addition of 5 µl of a solution containing aspartate aminotransferase. The blank was complete reaction mixture lacking aspartate aminotransferase. The rate of disappearance of NADH at 37 °C was continuously monitored spectrophotometrically at 340 nm relative to the blank. In this procedure glutamate dehydrogenase is added as a coupling enzyme that continuously catalyzes the NADH-dependent reductive amination of the α-ketoglutarate generated in the aspartate aminotransferase reaction. Inasmuch as 4-FGlu is a good substrate of glutamate dehydrogenase (section 3.5.) and the reaction is reversible, the 4-F α-ketoglutarate generated by transamination was expected to also be a substrate of glutamate dehydrogenase. This expectation was verified by conversion of 100 nmol of 4-F-Glu to the corresponding α-keto acid analogue with excess L-amino acid oxidase. The product was found to be an excellent substrate of glutamate dehydrogenase. In this regard, it is worth noting that the closely related positional isomer of 4-F α-ketoglutarate, namely 3-F α-ketoglutarate, is also an excellent substrate of glutamate dehydrogenase (Grissom and Cleland, 1988).

2.4.7. Alanine aminotransferase (EC 2.6.1.2)—The reaction mixture was the same as that used for the assay of aspartate aminotransferase (section 2.4.6.) except that oxaloacetate was replaced by pyruvate and the reaction was initiated by addition of 5 µl of a solution containing alanine aminotransferase. The rate of disappearance of NADH at 37 °C was continuously monitored at 340 nm relative to a blank lacking alanine aminotransferase.

2.4.8. Cystathionine γ-lyase (EC 4.4.1.1)—The assay is based on the ability of the enzyme to catalyze conversion of L-homoserine to α-ketobutyrate and ammonia (Washtien et al., 1977). The standard reaction mixture (0.05 mL) contained 20 mM L-homoserine and 100 mM Tris HCl buffer (pH 8.5). After incubation at 37 °C α-ketobutyrate was measured as its 2,4-dinitrophenylhydrazone (Cooper and Pinto, 2005).

2.5. HPLC measurement of L-methionine

Transamination reactions involving L-glutamine or 4-FGln were assayed by utilizing KMB as the α -keto acid substrate and measuring formation of L-methionine. HPLC measurement of methionine was determined as described by Bridges et al. (2011). The HPLC system consisted of a liquid chromatograph equipped with an 8-channel coulometric array (CoulArray) detector (ESA, Inc., Chelmsford, MA) (Pinto et al., 2005, 2009). The enzyme activity in the reaction mixtures (0.05 mL) was terminated by addition of 0.015 mL of 25% w/v metaphosphoric acid (MPA). The resulting solutions containing 5% w/v MPA were injected directly onto a Bio-Sil ODS-5S, 5- μ m 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 NaH₂PO₄, 50 μ M octane sulfonic acid, and 1% (v/v) acetonitrile (pH 2.52) at a flow rate of 1 mL/min. All buffers, following preparation, were routinely degassed, filtered through a 0.2- μ m Millipore nylon filter, and the pH adjusted, if necessary. PEEKTM (polyetheretherketone) tubing was used throughout the HPLC system, and a 0.2- μ m PEEKTM 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- μ L 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 (min) and detection potential ranges (mV) are: MPA (1.6, 100–200 mV), L-methionine (9.6, 600–800 mV) and KMB (11.1, 600–800 mV).

2.6. Defluorination reactions

The assay procedure is predicated on the fact that γ -elimination reactions involving amino acids containing a good leaving group in the γ position generate an α -keto acid product, leaving group and ammonia (Churchich, 1986). Thus, elimination of F[−] (leaving group) from 4-FGlu should result in the formation of α -ketoglutarate and ammonia. In the case of 4-FGln, the product is predicted to be KGM, which, in the presence of ω -amidase will be converted to α -ketoglutarate. The reaction mixture (0.02 mL) contained 100 mM buffer (phosphate, pH 7.4, or Tris-HCl, pH 8.5), 5 mM 4-FGln (or 4-FGlu) and purified enzyme (or liver cytosolic fraction). (The liver cytosolic fraction contains considerable amounts of ω -amidase, which will convert KGM to α -ketoglutarate at pH 8.5.) After incubation at 37 °C for a predetermined time (see section 3.8) 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 α -ketoglutarate 2,4-dinitrophenylhydrazone was read at 430 nm (see the ω -amidase assay procedure, section 2.4.2.). The blank contained complete reaction mixture plus enzyme, but lacked 4-FGlu (or 4-FGln). In a separate experiment it was shown that incubation of solutions of 4-FGlu or 4-FGln in phosphate (pH 7.4) or Tris buffer (pH 8.5) for one hour did not generate any detectable 2,4-dinitrophenylhydrazine-reactive material.

2.7. Determination of the non-enzymatic conversion of 4-FGln to 4-fluoro-5-oxoproline

The stability of 4-Gln in a 3 mM saline solution (section 3.9) at various temperatures was monitored by HPLC (performed on an HPLC Agilent Technologies® 1200 Series with solid phase Phenomenex® Chirex 3126 (D)-penicillamine (150 $\times$ 4.6 mm), UV detector set at 254 nm; mobile phase: 1.0 mM CuSO₄ solution, flow rate: 1.0 mL/min, column temperature 10 °C (HPLC I)). The retention time for 4-FGln is ~ 1 min. During incubation in the saline solution at 37 °C, only a single decomposition product was observed. The peak amplitude of this new product became larger with time (retention time ~ 8 min) and coincided with the disappearance of the 4-FGln peak. The identity of the decomposition product was determined by LC-MS Agilent LC/MSD TOF (ESI-pos), Agilent 1100 Series with solid phase Phenomenex® 2.6 μ C18 100 Å, 100 $\times$ 4.6 mm and mobile phase of acetonitrile/0.1% formic acid 1/9, flow rate 0.3 mL/min, UV detector set at 210 nm and ambient temperature

(HPLC II). The retention time for 4-FGln was ~ 4 min [calc for $C_5H_{10}FN_2O_3$ ($M + H$)⁺ 165.0675, found 165.0690; mass accuracy $\Delta = -8.8$ ppm]. The new peak eluted at 6 min with an m/z value of 148.0422 ($C_5H_7FNO_3$ ($M + H$)⁺; calc 148.0410). This molecular weight corresponds to 4-fluoro-5-oxoproline ($C_5H_6FNO_3 + H$)⁺ ($M + H$)⁺ ($\Delta = -8.1$ ppm). Details on the rates of conversion of 4-FGln to 4-fluoro-5-oxoproline are given in the Results section (section 3.9.).

2.8. 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 by the Bradford procedure or bicinchoninic acid (BCA) procedure using Pierce BCA Reagents.

2.9. Statistics

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

3. Results

3.1. 4F-Gln is a substrate of *E. coli* glutaminase and of glutaminase-like activity in rat kidney supernatant

We first determined that 4-FGln is a substrate of a commercially available purified glutaminase, namely *E. coli* glutaminase. When 1 mU of enzyme was incubated with L-glutamine or 4-FGln under the conditions outlined in section 2.4.5, the amounts of L-glutamate and 4-FGlu formed after one hour were 40.5 ± 0.9 and 37.6 ± 6.6 nmoles, respectively. This finding established proof of principle that 4-FGln is capable of enzymatic conversion to 4-FGlu. However, the pH optimum of the *E. coli* enzyme (4.5 – 5.0) is considerably different from that of the mammalian enzymes (~8.0). Because most tumors possess the kidney type glutaminase we investigated the glutaminase reaction in a rat kidney preparation (section 2.3). We found that the glutaminase specific activity with L-glutamine and 4-FGln was 3.42 ± 0.51 ($n = 15$) and 8.13 ± 0.84 ($n = 5$) nmol/min/mg, respectively ($p = 0.00001$). The glutaminase reaction with 4-FGln and other reactions related to the metabolism of 4-FGln and 4-FGlu uncovered in the present work are shown in Fig. 1.

The ecto-enzyme γ -glutamyltransferase can catalyze a glutaminase reaction (Tate and Meister, 1974), and under certain circumstances this enzyme may be a major source of glutamine-derived ammonia (Curthoys and Kuhlenschmidt, 1975). However, since γ -glutamyltransferase is membrane-bound most of the enzyme is removed from the rat kidney preparation used in the present experiment by the 1000 g centrifugation step.

3.2. F-Gln is a substrate of snake venom L-amino acid oxidase

A possible route for 4-FGln metabolism involves transamination. However, prior to investigating 4-FGln as an aminotransferase substrate it was necessary to understand some of the properties of the expected α -keto acid product. The α -keto acid product derived from transamination of 4-FGln should have the structure 4-F α -ketoglutamamate (4-FKGM). However, to our knowledge 4-FKGM has not previously been prepared. L-Amino acid oxidase was therefore used to generate an *in situ* standard of this α -keto acid. It was previously reported that at a concentration of 4.0 mM (pH 8.0), among the amino acids investigated, L-methionine is the best substrate of *C. adamanteus* L-amino acid oxidase (Lichtenberg and Wellner, 1968). Relative activities using L-phenylalanine as standard were reported to be in the order: L-methionine (214), L-phenylalanine (100), L-glutamine (19), L-

glutamate (0.9) (Lichtenberg and Wellner, 1968). In the present work we determined the relative rates of the *C. adamanteus* L-amino acid oxidase-catalyzed oxidation of various L-amino acids at a concentration of 2 mM (pH 7.4) by the semicarbazone procedure (see section 2.4.1.). The results are shown in Table 1. As expected from the findings of Lichtenberg and Wellner (1968), the data showed that under the conditions of our assay the activity was in the order: L-methionine >> L-glutamine > L-glutamate. Interestingly, 4-FGln is a much better substrate than L-glutamine, and 4-FGlu is a somewhat better substrate than is L-glutamate. It should be noted that the α -keto acid products of KGM and FKGM cyclize to lactam structures masking the carbonyl function (see section 3.3.). However, under the assay conditions employed, the linear α -imino acid products generated by the action of L-amino acid oxidase on L-glutamine and 4-FGln can be trapped as the corresponding semicarbazone in the presence of a high (100 mM) concentration of semicarbazide before cyclization can occur. On the other hand, semicarbazide addition after the L-amino acid oxidase reaction with L-glutamine and 4-FGln is complete, results in no detectable semicarbazone formation.

3.3. 4-FKGM cyclizes to a lactam – inability to detect reversibility of lactam formation

KGM exists predominantly as a lactam (2-hydroxy-5-oxoproline) at physiological pH values (see section 3.4.). However, the reaction is reversible so that the open-chain form acts as a substrate for ω -amidase. Therefore, it was important to determine whether 4-FKGM cyclizes to a lactam in a similar fashion to that exhibited by KGM and, if so, whether the lactam is in equilibrium with an open-chain form that has the potential to act as a substrate of ω -amidase. Accordingly, a 0.02 ml solution containing 5 mM 4-FGln, 100 mM potassium phosphate buffer (pH 7.4), 80 mU of L-amino acid oxidase and 1,000 U catalase was incubated at 37 °C in a small snap-top tube for 2 h. Measurement of ammonia (by coupling to the glutamate dehydrogenase reaction) showed that ~35 nmol of 4-FGln was oxidized under these conditions. In a separate experiment, the reaction was terminated by the addition of 0.02 ml of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After a further 10 min incubation at 37 °C, 0.16 ml of 1 M KOH was added and the absorbance was read at 430 nm against a blank consisting of reaction mixture lacking enzyme carried through the same procedure. Under these conditions a typical α -keto acid 2,4-dinitrophenylhydrazone exhibits an ϵ of ~16,000 M⁻¹ cm⁻¹. No α -keto acid could be detected (< 0.5 nmol). In control experiments in which 4-FGln was replaced by 5 mM L-glutamine or 5 mM L-methionine, 0.5 and ~80 nmol of α -keto acid were detected, respectively. The data are consistent with the hypothesis that 4-FKGM, in a similar fashion to KGM, cyclizes to a lactam that is resistant to ring opening to α -keto acid under acidic conditions.

The question then arises as to whether 4-FKGM in the lactam form is in equilibrium with a small amount of the open-chain form at neutral or slightly basic pH values and whether this open-chain form is a substrate of ω -amidase. To answer this question, the previous experiment was repeated, except that the phosphate buffer was replaced by 100 mM Tris-HCl buffer (final pH 8.5). After a 2 h incubation most of the 4-FGln (~70 nmol) in the 0.02 ml reaction mixture was shown to be oxidized (as determined by ammonia formation). In a separate experiment, after the 2 h incubation, 2 μ l of ω -amidase preparation (22 mU) was added. After a further incubation for 1 h at 37 °C α -keto acid formation was determined by the 2,4-dinitrophenylhydrazine procedure. No α -keto acid could be detected (< 0.5 nmol). As a positive control, in a parallel experiment in which 4-FGln was replaced by L-glutamine, ~68 $\pm$ 2 nmol of α -ketoglutarate was detected. (The maximum expected yield was 100 nmol.)

In separate experiments we showed that 5 mM L-glutamine, 5 mM 4-FGln and ~ 4 mM 4-FKGM (produced by the action of L-amino acid oxidase) had no effect on the activity of purified ω -amidase in the standard reaction mixture containing 5 mM KGM and 22 mU of

ω -amidase. Conceivably, 4-F KGM (open-chain form) is not a substrate of ω -amidase, but it seems more likely that the equilibrium between lactam and open-chain form favors little, if any, open-chain (potential ω -amidase substrate) form. Thus, transamination of 4-FGln (section 3.4.) will result in the formation of the “dead-end” metabolite 4-FKGM lactam.

3.4. 4-FGln is an aminotransferase substrate

Two aminotransferases have been reported earlier in the literature to catalyze the transamination of glutamine, namely GTK/KAT I and GTL/KAT III (Cooper, 1988, 2004; Cooper and Meister, 1972, 1974, 1981). [Note that the older name transaminase has been largely replaced by the name aminotransferase in the more recent literature with the exception of enzymes that catalyze transamination of glutamine.] As noted in section 3.3., transamination of glutamine yields KGM (Eq. 1) which exists predominantly as a lactam. Under physiological conditions, 99.7% is in the form of the lactam (2-hydroxy-5-oxoproline) (Hersh 1971). As also noted in section 3.3, ω -amidase catalyzes the conversion of the open-chain form of KGM to α -ketoglutarate and ammonia (Eq. 2) (Meister 1953; Otani and Meister, 1957; Meister et al., 1952, 1953; Hersh, 1971, 1972). The combined action of these two enzymes (the glutaminase II reaction) is shown in Eq. 3. Interconversion between the open-chain and cyclized forms of KGM is specific base-catalyzed and is rapid at pH > 8.0. At pH 8.5 the rate of interconversion between open-chain and cyclized forms is rapid and not rate limiting for amounts of ω -amidase present in typical assay mixtures (Hersh, 1971). However, at physiological pH (pH 7.2 – 7.4) the rate of interconversion may limit the rate at which ω -amidase can catalyze conversion of KGM to α -ketoglutarate. As a result, steady state levels of KGM in rat tissues and human CSF are in the μ M range (Duffy et al., 1974) and KGM is a natural metabolite in human urine (Kuhara et al., 2011).

Owing to its similarity to glutamine and the wide substrate specificities of GTK/KAT I and GTL/KAT III (Cooper and Meister, 1972, 1974, 1981; Han et al, 2004, 2009), 4-FGln was predicted to be a substrate of these enzymes and therefore would be a source of 4-FKGM lactam. In addition, we predicted that other aminotransferases might also catalyze transamination of 4-FGln. For example, although α -amino adipate aminotransferase/KAT II has been studied most extensively as an α -amino adipate and kynurenine aminotransferase, the enzyme also has a relatively broad amino acid specificity (Hartline 1985; Han et al., 2008). Although human α -amino adipate aminotransferase is most active with α -amino adipate, some activity is observed with glutamine (~5% that exhibited with α -amino adipate) (Han et al. 2008). Thus, the possibility was considered that this enzyme might also catalyze the transamination of 4-FGln. Finally, mitAspAT has some activity toward amino acids other than aspartate and glutamate (Miller and Litwak, 1971; Shrawder and Martinez-Carrion, 1972). This enzyme has some activity with kynurenine (Guidetti et al., 2007) and is classified by some researchers as KAT IV (Han et al., 2009).

Therefore, we determined whether 4-FGln is a substrate of the aminotransferases mentioned in the previous paragraph. We chose KMB as the α -keto acid co-substrate in these studies because KMB is a good substrate of both GTK/KAT I and GTL/KAT III (Cooper and Meister, 1972, 1974, 1981; Han et al, 2004, 2009) and of α -amino adipate aminotransferase/

KAT II (Han et al., 2008). Furthermore, the product of the transamination reaction with KMB (i.e. L-methionine) can be readily quantitated by HPLC with CoulArray detection (see section 2.5.). Table 2 shows that transamination of 4-FGln with KMB is catalyzed by GTK/KAT I, GTL/KAT III and α -aminoadipate aminotransferase/KAT II. Transamination between L-glutamine (or 4-FGln) and KMB in the presence of mitochondrial aspartate aminotransferase (5 μ g; 2 U) was detectable, but the reaction was too slow for an accurate determination of specific activity.

3.5. 4-FGlu is a substrate of beef liver glutamate dehydrogenase

After determining that 4-FGln can be hydrolyzed to 4-FGlu by the action of glutaminase, the next step was to determine whether 4-FGlu can be further metabolized by glutamate-utilizing enzymes. We began our survey with glutamate dehydrogenase.

At physiological pH values (pH 7.2–7.4) the glutamate dehydrogenase reaction (Eq. 4) is drawn overwhelmingly toward L-glutamate formation. In order to measure the forward reaction, high levels of L-glutamate and/or a high pH (low $[H^+]$) are required. Because of limited amounts and solubility of 4-FGlu the reaction was investigated at high pH values (pH 10.5; see section 2.4.3.). The initial rate of increase of absorbance was followed at 37 °C after addition of 5 mU of glutamate dehydrogenase. The rate of NAD^+ reduction to NADH in the presence of L-glutamate and 4-FGlu was 3.39 ± 0.27 and 2.72 ± 0.37 nmol/min/mU glutamate dehydrogenase, respectively. Under the conditions of the assay, L-glutamate is a slightly better substrate ($p = 0.05$) than is 4-FGlu.

It is possible that contaminating ammonia in the reaction mixture inhibits the forward direction of Eq. 4. However, this seems unlikely as the contaminating ammonia is difficult to measure because its concentration in the assay mixture is low (<1.0 mM) and the K_m exhibited by glutamate dehydrogenase is relatively high, typically ~10 mM or higher (Chee et al., 1979).

3.6. 4-FGlu is an aminotransferase substrate of three glutamate-utilizing aminotransferases

The ability of 4-FGlu to replace L-glutamate as an aminotransferase substrate of three highly purified glutamate-utilizing aminotransferases (rat liver mitochondrial aspartate aminotransferase, pig heart cytosolic aspartate aminotransferase and pig heart alanine aminotransferase) was investigated (Table 3). As discussed below (section 3.8.), rat liver cytosolic fractions catalyze defluorination of 4-FGlu to α -ketoglutarate. If this reaction competes with the aminotransferase reaction then the rate of α -keto acid formation from 4-FGlu shown in Table 3 will be the sum of competing defluorination and aminotransferase reactions. However, no α -ketoglutarate could be detected in the absence of α -keto acid co-substrate when pig heart cytosolic aspartate aminotransferase and rat liver mitochondrial aspartate aminotransferase were incubated with 4-FGlu. Thus, for these two enzymes, the rate of α -ketoglutarate formation is a true reflection of the aminotransferase reaction. From the data in Table 3, at a concentration of 5 mM, 4-FGlu is almost as good a substrate as is 5 mM L-glutamate in aminotransferase reactions catalyzed by rat liver mitochondrial and pig heart cytosolic aspartate aminotransferases.

Because pig heart alanine aminotransferase can catalyze a γ -elimination reaction with 4-FGlu (see section 3.8.) it is possible that the rate of α -keto acid production from 4-FGlu catalyzed by this enzyme involves both a transamination component (production of 4-F α -ketoglutarate) and a γ -elimination component (production of α -ketoglutarate). However,

partitioning between transamination and γ -elimination greatly favors transamination (see section 3.8. and the Discussion). Thus, the rate of α -keto acid production shown in Table 3 is a reflection of the true transamination rate. Interestingly, under the conditions of the assay, 4-FGlu appears to be a better aminotransferase substrate of pig heart alanine aminotransferase than is L-glutamate (Table 3).

3.7. 4-FGlu is an inhibitor of sheep brain glutamine synthetase

Glutamine synthetase assay mixture 1 (see the Methods, section 2.4.4.) containing 5 mM L-glutamate and 100 mM hydroxylamine was incubated for one hour at 37 °C in the presence of 4 mU of sheep brain glutamine synthetase. γ -Glutamylhydroxamate formation was then measured by the ferric chloride procedure. The amount of product formed was 169 ± 10 nmoles. Several glutamate analogues that contain a substitution in the γ position are substrates of sheep brain glutamine synthetase and the color yield of the resulting hydroxamates are the same as that noted for γ -glutamylhydroxamate (Kagan and Meister, 1966). Thus, we considered the possibility that if 4-FGlu is a substrate, the resulting hydroxamate would be detectable by the ferric chloride procedure. However, when 5 mM L-glutamate was replaced by 5 mM 4-FGlu no hydroxamate formation could be detected by the ferric chloride procedure. Similar results were obtained with the rat liver cytosolic fraction. The specific activity of the glutamine synthetase reaction in the liver cytosolic fraction in the presence of 5 mM glutamate or 5 mM 4-FGlu was 3.14 ± 0.10 and <0.5 $\mu\text{mol/h/mg}$, respectively, as determined by the hydroxamate assay. [Our value for the specific activity of glutamine synthetase in the rat liver cytosolic fraction is of the same order as that reported by Tate et al., (1972) of 1.88 $\mu\text{mol/h/mg}$.] Thus, either the fluoride in the γ position of 4-FGlu interferes with the ferric chloride assay or 4-FGlu is not a substrate.

In order to distinguish between the two possibilities we also assayed the glutamine synthetase reaction by measuring ADP formation (assay mixture 2). The rate of ADP formation in the presence of 5 mM L-glutamate and 2.5 mU glutamine synthetase was 1.49 ± 0.07 nmol/min. No ADP formation could be detected when the L-glutamate was replaced by 4 mM 4-FGlu (<0.1 nmol/min). We next measured the activity in the presence of 10 mM L-glutamate and presence or absence of 4 mM 4-FGlu. The initial rates of ADP formation were 1.96 ± 0.08 and 0.65 ± 0.05 nmol/min, respectively (significantly different with $p = 0.05$).

3.8. Rat liver cytosolic fractions and purified alanine aminotransferase catalyze the defluorination of 4-FGlu

$^{18}\text{F}^-$ was previously shown to be a metabolite of [^{18}F]4-FGln in two tumor lines (Qu et al., 2011b). Since F is highly electronegative F^- is expected to be an excellent leaving group. Thus, we hypothesized that the production of F^- from 4-FGln is most likely due to an enzyme-catalyzed γ -elimination reaction. Such a reaction would be analogous to γ -elimination reactions catalyzed by cystathionine γ -lyase. For example, cystathionine γ -lyase catalyzes the γ -elimination of cysteine from L-cystathionine and water from L-homoserine (e.g. Washtien et al., 1977). In both cases α -ketobutyrate and ammonia are generated. A similar γ -elimination reaction with 4-FGln would theoretically generate KGM (Eq. 5).

Therefore, we investigated the ability of a rat liver cytosolic fraction (an abundant source of cystathionine γ -lyase) to catalyze this reaction. Because the rat liver cytosolic fraction also contains appreciable amounts of ω -amidase we assumed that if the rat liver cytosolic fraction contained an enzyme capable of converting 4-FGln to KGM, incubation of a

reaction mixture containing 4-FGln at pH 8.5 should generate appreciable amounts of α -ketoglutarate. [pH 8.5 was chosen as the buffer pH because, as noted in section 3.3., the rate of ring opening of KGM lactam to the open-chain form (substrate form of KGM for ω -amidase) is rapid at this pH and not likely to be rate limiting at enzyme levels used in assay of this enzyme (Hersh 1971).] Moreover, cystathionine γ -lyase has an alkaline pH optimum (Washtien et al., 1977).

However, incubation of 5 mM 4-FGln with a freeze-thawed rat liver cytosolic fraction containing 50 μ g of protein for 30 min at 37 °C in the presence of an assay mixture (0.02 ml) containing either 100 mM potassium phosphate buffer (pH 7.4) or 100 mM Tris-HCl buffer (pH 8.5) failed to generate any detectable α -ketoglutarate (as determined by the 2,4-dinitrophenylhydrazine procedure). On the other hand, incubation of 5 mM 4-FGlu for 30 min under these conditions in the presence of Tris-HCl buffer (pH 8.5) generated considerable α -keto acid (15.5 ± 0.9 nmol) as determined by the 2,4-dinitrophenylhydrazine procedure. The amount of α -keto acid formed at pH 7.4 was somewhat less (8.20 ± 0.40 nmol) than that formed at pH 8.5 ($p = 0.05$). The product formed at both pH values was found to be a substrate of glutamate dehydrogenase. Since no L-amino acid oxidase activity could be detected in the cytosolic fraction, the finding confirms the hypothesis that the α -keto acid generated from 4-FGlu by a γ -elimination reaction is α -ketoglutarate (Eq. 6).

It is possible that the freeze-thawed rat liver cytosolic fraction a) does not contain an enzyme capable of catalyzing a γ -elimination reaction with 4-FGln, b) γ -lyase activity toward 4-FGln is destroyed by freeze-thawing, or c) the product of the reaction rapidly inactivates ω -amidase. However, no α -keto acid production from 4-FGln could be detected with a fresh (unfrozen) liver cytosolic fraction, eliminating possibilities a) and b). To investigate possibility c), 2 μ l of rat liver cytosolic fraction (20 μ g of protein) was incubated with 5 mM 4-FGln in 0.01 ml of 100 mM Tris-HCl buffer (pH 8.5) for 30 min at 37 °C. Thereafter, ω -amidase activity (10-min incubation at 37 °C) of the 4-FGln-treated cytosolic fraction was compared to that in 2 μ l of cytosolic fraction that had not been treated with 4-FGln (see section 2.2.). The amount of α -ketoglutarate formed from KGM in the 4-FGln treated cytosolic fraction and control cytosolic fraction was 32.3 ± 1.7 and 30.5 ± 1.5 nmol, respectively. Thus, 4-FGln, or a product of 4-FGln, is not an irreversible inhibitor of ω -amidase in the rat liver cytosolic fraction.

We then asked the question “what enzymes catalyze the γ -elimination reaction with 4-FGlu?” γ -Elimination reactions involving amino acids in biological systems are generally catalyzed by pyridoxal 5'-phosphate (PLP)-containing enzymes, the archetypical example of which in mammals is cystathionine γ -lyase (Churchich, 1986). Therefore, we hypothesized that the formation of α -ketoglutarate from 4-FGlu by a γ -elimination reaction is due to the action of a PLP-containing enzyme in a reaction similar to that catalyzed by cystathionine γ -lyase. Evidence in favor of this hypothesis was obtained by utilizing AOAA – a widely used non-specific inhibitor of PLP-containing enzymes. When 5 mM 4-FGlu was incubated for one hour at 37 °C in a reaction mixture (0.02 ml) containing 100 mM Tris HCl buffer (pH 8.5) and 5 mM AOAA no α -keto acid product could be detected (<0.5 nmol) (Table 4). Thus, we considered the possibility that PLP-containing cystathionine γ -lyase catalyzes a γ -elimination reaction with 4-FGlu. The amount of α -ketobutyrate formed when a rat liver cytosolic fraction (20 μ g protein) was incubated for 5 min at 37 °C in the standard reaction mixture containing 20 mM homoserine was 26.2 ± 0.7 nmoles. When 50 mM L-glutamate or 50 mM L-glutamine was included in the reaction mixture the amount of product formed was 26.8 ± 1.0 and 27.1 ± 2.7 nmoles, respectively. Given the structural similarity of 4-FGlu to

L-glutamate, this finding almost certainly eliminates cystathionine γ -lyase as contributing to the formation of α -ketoglutarate from 4-FGlu.

We next considered aminotransferases. Aminotransferases are known to catalyze β -elimination side reactions with amino acids containing a good leaving group in the β -position (see the Discussion, section 4.). However, to our knowledge these enzymes have not been shown to catalyze γ -elimination reactions. Nevertheless, we obtained indirect evidence that an aminotransferase is responsible for the catalysis of the γ -elimination of F^- from 4-FGlu. First, aminotransferases contain PLP as a cofactor and are inhibited by AOAA. Secondly, many aminotransferases utilize L-glutamate/ α -keto acceptor pair, and glutamate was found to strongly inhibit the γ -lyase reaction with 4-FGlu catalyzed by a rat liver cytosolic fraction (Table 4). Finally, if 4-FGlu binds to a PLP-containing glutamate-utilizing aminotransferase the possibility exists that in the absence of added α -keto acid substrate the γ -elimination reaction will be slowed due to a competing half transamination reaction between 4-FGlu and cofactor PLP. This reaction will result in accumulation of the enzyme in the pyridoxamine 5'-phosphate (PMP) form and formation of an equivalent amount of 4-fluoro α -ketoglutarate. The PMP enzyme cannot support a γ -lyase reaction. Table 4 shows that addition, at the start of the reaction, of a small amount of α -ketoglutarate (which promotes conversion of PMP back to PLP) significantly stimulates the further production of α -ketoglutarate.

The question was then asked "which aminotransferase can catalyze a γ -elimination reaction with 4-FGlu?" Accordingly, we checked the ability of various purified aminotransferases to catalyze a γ -elimination reaction with 4-FGlu. We incubated different purified aminotransferases in 0.02 ml of a reaction mixture containing 5 mM 4-FGlu (100 nmoles) and 100 mM Tris-HCl buffer (pH 8.5) in a small snap top tube for 30 min at 37 °C. At the end of the incubation the amount of α -ketoglutarate formed was measured by the 2,4-dinitrophenylhydrazine procedure relative to a blank containing enzyme, but lacking 4-FGlu. The following highly purified aminotransferases – rat liver mitochondrial aspartate aminotransferase (1 μ g, 0.41 U), pig heart cytosolic aspartate aminotransferase (1 μ g, 100 mU), rhGTK (KAT I) (~2.7 μ g), mouse α -amino adipate aminotransferase (KAT II) (1.8 μ g) and mouse GTL (KAT III) (3.7 μ g) – did not generate any detectable α -ketoglutarate (<0.5 nmol).

On the other hand, pig heart alanine aminotransferase (10 μ g, 650 mU) was found to generate significant quantities of α -ketoglutarate from 5 mM 4-FGlu (Table 5). As noted for the activity in freeze-thawed cytosolic fractions of rat liver (Table 4), the activity was strongly inhibited by glutamate and AOAA, but stimulated by priming with small amounts of α -ketoglutarate (Table 5).

3.9. Non-enzymatic cyclization of 4-FGln

It has long been known that intramolecular attack of the amine nitrogen on the γ -carboxamide carbon of glutamine results in cyclization to 5-oxoproline (2-pyrrolidone-5-carboxylate, pyroglutamate) accompanied by elimination of ammonia (Gilbert et al., 1949). The reaction occurs readily at only moderately elevated temperatures and is accelerated in the presence of phosphate, acidic pH values and alkaline pH values (Gilbert et al., 1949). For example a solution of 25 mM glutamine is almost completely deamidated at 47 °C in the presence of 80 mM veronal-acetate buffer, pH 8.0 in 24 hours (Gilbert et al., 1949). In the present work we have found that 4-FGln cyclizes to 4-fluoro-5-oxoproline (2-pyrrolidone-4-fluoro-5-carboxylate, 4-fluoro-pyroglutamate). The half life for conversion of 4-FGln to 4-fluoro-5-oxoproline (in a 3 mM saline solution) is about 10 months, 5 days and < 1 h at 4 °C, 37 °C and 80 °C, respectively. At 4 °C and 37 °C the only product of 4-FGln decomposition noted was 4-fluoro-5-oxoproline. However, at higher temperatures (80 °C)

some 4-FGlu could also be detected by HPLC I as estimated by its retention time (about 50 min).

4. Discussion

Detailed enzyme kinetics of 4-FGln and 4-FGlu are not presented in the present study. Owing to the relatively low solubilities of 4-FGln and 4-FGlu detailed kinetic studies with these amino acids may prove difficult with some enzymes that typically exhibit high K_m values toward natural amino acid substrates (mM or higher). Nevertheless, the present work should provide a useful background for more detailed studies of these fluorinated amino acids in biomedical/biochemical research.

Recent work has shown that [^{18}F]4-FGln in conjunction with PET is useful for the imaging of various tumors in experimental animals (Qu et al. 2011b; Lieberman et al., 2011; Ploessl et al., 2012). Amino acid transport system ASC (in particular, its subtype ASCT2 (SLC1A5)) and system X_c⁻ (SLC7A11) were shown to play an important role in transporting [^{18}F]4F-Gln into cells. Label derived from [^{18}F]4-FGln (but not from [^{18}F]4-FGlu) was shown to be incorporated into cellular proteins (Ploessl et al., 2012). In studies with tumor bearing mice, label derived from [^{18}F]4-FGln was also shown to rapidly accumulate in the tumors. Once accumulated into these cells, $^{18}\text{F}^-$ and [^{18}F]4-FGlu were detected as metabolites (Lieberman et al., 2011). However, the possible occurrence of other labeled metabolites was not investigated, providing only a limited window on the possible metabolic fate of [^{18}F]4-FGln. Therefore, we undertook a detailed analysis of enzymatic reactions involving 4-FGln and 4-FGlu that may possibly occur *in vivo*.

The finding that under the conditions of our assay 4-FGln is a better substrate of glutaminase in rat kidney supernatant (Fig. 1) is interesting. The increased rate may be ascribed to facilitated nucleophilic attack by the thiol anion of an active site cysteine residue on the carbonyl of the carboxamide of 4-FGln. The electron-withdrawing properties and negative inductive effect of the F atom on the C-F bond adjacent to the carboxamide will result in a greater δ^+ charge on the carbonyl carbon, resulting in more effective nucleophilic attack by active site cysteine compared to the case with L-glutamine.

The finding that 4-FGln is a glutaminase substrate in the rat kidney supernatant fraction is important when considering the metabolic fate of label derived from [^{18}F]4-FGln in different tumors. Mammalian tissues express two different glutaminase isozymes – a liver form (glutaminase L, GLS2), a more widely distributed kidney form (glutaminase K, GLS1), and an alternatively spliced variant of the kidney form (GAC). Most cancer cells appear to express the kidney form (GLS1) (Dang et al., 2011), but some express GLS2 (Hu et al., 2010) and SAC (Delabarre et al., 2011). Although we did not investigate the liver form, it is likely that most tumors will be able to metabolize [^{18}F]4-FGln to [^{18}F]4-FGlu.

We also showed that 4-FGln is a substrate of two glutamine aminotransferases (rhGTK, GTL) and also of α -amino adipate aminotransferase (Table 2). We previously showed that GTK is present in human prostate cancer and breast cancer cells (Lee et al., 2009; Pinto et al., 2011). The expected α -keto acid product generated from 4-FGln is 4-FKGM. However, because there is little if any open-chain form, 4-FKGM cannot be converted to 4-fluoro- α -ketoglutarate by ω -amidase (section 3.3.) and [^{18}F]4-FKGM is likely to be a dead-end metabolite of [^{18}F]4-FGln in tumor cells *in vivo*.

The finding that 4-FGln can be enzymatically hydrolyzed to 4-FGlu raises the possibility that the 4-FGlu generated from 4-FGln will be metabolized further by glutamate-utilizing enzymes. One possibility is that 4-FGlu can be converted back to 4-FGln via a reaction catalyzed by glutamine synthetase. However, 4-FGlu could not be detected as a substrate of

purified sheep brain glutamine synthetase or in rat liver cytosolic fractions that possess considerable synthetase activity with L-glutamate. Moreover, 4-FGlu was found to be a strong inhibitor. In the synthetase reaction, ATP reacts with L-glutamate at the active site forming a γ -carboxy phosphate intermediate and ADP. Nucleophilic attack by ammonia (or hydroxylamine) on the γ -carboxy phosphate releases inorganic phosphate with concomitant formation of an amide (or hydroxamate) moiety at the γ position (Wellner et al., 1966). Sheep brain glutamine synthetase also catalyzes a competing intramolecular attack of the α -amino group of the γ -carboxy phosphate enzyme intermediate at the γ carbon, resulting in the formation of 5-oxoproline (Wellner et al., 1966). This reaction is relatively slow. As a result, 5-oxoproline formation is normally only a minor side reaction compared to the glutamine synthesis reaction (synthetase reaction). Possibly, intramolecular attack on the phosphorylated intermediate may be more favorable with 4-FGlu than with glutamate. However, the reaction requires formation of ADP and this was not detected.

There is precedent for strong enzyme inhibition by a compound possessing a halogen adjacent to a carboxylate. For example, 2-bromopalmitate is a strong inhibitor of enzymes that catalyze palmitoylation of N-myristoylation proteins. Four such enzymes containing a canonical DHHC cysteine-rich region are strongly, but slowly, irreversibly inhibited by 2-bromopalmitate (Jennings et al., 2009). It is possible that glutamine synthetase is inhibited by a similar mechanism. However, the detailed mechanism by which 4-FGln inhibits the glutamine synthetase reaction must await further study. Nevertheless, the results suggest that non-productive cycling between hydrolysis and synthesis of 4-FGln is unlikely to occur *in vivo*.

In order for glutamine to be utilized as an efficient energy source after its conversion to glutamate, the glutamate must be converted to α -ketoglutarate either by the action of glutamate dehydrogenase (Eq. 4) or by aminotransferase reactions. These reactions have been well documented to occur in tumors. For example, $^1\text{H}/^{15}\text{N}$ NMR studies have shown that the glutamate dehydrogenase reaction is important for the production of α -ketoglutarate in glucose-deprived hybridoma and myeloma cells (Martinelle et al., 1998). Metabolically important aminotransferases include aspartate and alanine aminotransferases, which catalyzes the reaction shown in Eq. 7 and 8, respectively.

The mitochondrial and cytosolic aspartate aminotransferase isoenzymes are important components of the malate-aspartate shuttle for the transport of reducing equivalents from cytosol to mitochondrion (Lowenstein, 1967; Fitzpatrick et al., 1983). The central importance of the aspartate aminotransferase reaction in energy and nitrogen metabolism and for the utilization of glutamine carbon and nitrogen is underscored by the fact that the enzyme activity is well represented in tumor cells (e.g. Mazurek et al., 1999). In addition to aspartate aminotransferase(s) tumor tissues are known to possess additional glutamate/ α -ketoglutarate-linked aminotransferases including alanine aminotransferase, an enzyme that may be a potential target for cancer chemotherapy (Gallagher et al., 2011; Beuster et al., 2011).

Given the prevalence of glutamate dehydrogenase and glutamate-utilizing aminotransferases in tissues and tumors, and the formation of [^{18}F]4-FGlu from [^{18}F]4-FGln in tumors *in vivo* (Qu et al., 2011; Lieberman et al., 2011), we considered the possibility that 4-FGlu will be a substrate of glutamate dehydrogenase, both aspartate aminotransferase isozymes and alanine

aminotransferase. This prediction was verified (section 3.5.; Table 3). Notably, 4-FGlu proved to be an exceptionally good substrate of pig heart alanine aminotransferase (Table 3). We previously showed that equilibration of labeled nitrogen among glutamate, aspartate and alanine in rat liver after administration of [^{13}N]ammonia or [^{13}N]amino acids via the portal vein is extremely rapid (seconds) attesting to the rapidity of N exchange among the substrates of glutamate dehydrogenase and glutamate-utilizing aminotransferases (Cooper et al., 1987, 1988). Thus, given the presence of glutamate dehydrogenase and aspartate and alanine aminotransferase activities in cancer cells we expected that after conversion of [^{18}F]4-Gln to [^{18}F]4-Glu label will quickly appear as [^{18}F]4-fluoro- α -ketoglutarate. This prediction has not yet been verified *in vivo*, but is currently under investigation.

One hour after intravenous injection of [^{18}F]4-FGln into transgenic mice bearing M/tomND spontaneous tumors about 9% of the recovered dose of radioactivity in the tumors was in the form of $^{18}\text{F}^-$ ion (Lieberman et al., 2011). Similar findings were also noted for F344 rats carrying 9L tumor xenographs (Lieberman et al., 2011). What is the origin of the $^{18}\text{F}^-$? As discussed in section 3.8., a possibility is that the formation of $^{18}\text{F}^-$ from [^{18}F]4-FGln involved a PLP-dependent enzyme that catalyzes a γ -elimination reaction. We first considered the possibility cystathionine γ -lyase that might catalyze a γ -elimination of F^- from 4-FGln. However, as discussed in the section 3.3., no evidence could be found that 4-FGln and 4-FGlu are substrates of this enzyme in the rat liver cytosolic fraction (a rich source of cystathionine γ -lyase).

Although we did not measure F^- directly, a γ -elimination reaction involving 4-FGln or 4-FGlu should produce α -keto acids (KGM from 4-FGln and α -ketoglutarate from 4-FGlu). However, no evidence was found that a liver cytosolic fraction can catalyze the conversion of 4-FGln to KGM. On the other hand, we showed that the rat liver cytosolic fraction can catalyze the conversion of 4-FGlu to α -ketoglutarate. This reaction was a) inhibited by AOAA (a general antagonist of PLP enzymes), b) inhibited by glutamate, and c) primed by trace amounts of α -ketoglutarate in the assay mixture (Table 4). These findings pointed to the involvement of a PLP-dependent aminotransferase.

Aminotransferases have long been known to catalyze non-physiological β -elimination reactions with amino acids containing a good leaving group in the β position (e.g. Ueno et al., 1982; Cooper et al., 2011 and references cited therein). Aminotransferases are also known to catalyze β -elimination reactions with α -keto acids containing a good leaving group in the β position. For example, cytosolic aspartate aminotransferase was reported to catalyze a β -elimination reaction with fluorooxaloacetate (Goldstein et al., 1978). α -Keto- β -mercaptoglutarate and α -keto- β -methylmercaptoglutarate were shown to be β -lyase substrates of cytosolic and mitochondrial isozymes of aspartate aminotransferase (but not of alanine aminotransferase) (Plaut et al., 1986). In these cases, the reaction requires the presence of an amino acid co-substrate to generate the PMP form of the enzyme that can then react with the β -substituted α -keto acid to facilitate β -elimination. In the presence of glutamate, α -keto- β -fluoroglutarate (2-oxo-3-fluoroglutarate) – a positional isomer of 4-F α -ketoglutarate – is a β -lyase substrate of various PLP-containing aminotransferases, including pig heart cytosolic aspartate-, pig heart alanine- and *Pseudomonas fluorescens* GABA aminotransferases (Grissom and Cleland, 1988). In addition, *E. coli* glutamate decarboxylase was shown to catalyze a β -elimination reaction after a portion of α -keto- β -fluoroglutarate was reductively aminated to L- β -fluoroglutarate with glutamate dehydrogenase.

The findings that aminotransferases catalyze β -elimination reactions with amino acids/ α -keto acids containing a good leaving group in the β position indicate that the covalent adduct formed between amino acid and PLP at the active site facilitates electron movement toward

the β substituent that allows β elimination to compete effectively with transamination. But could the electrons be drawn even further – i.e. toward a γ substituent in the covalent PLP-adduct at the active site of an aminotransferase when the substrate has an exceptionally good leaving group in the γ position in the form of F⁻? No evidence could be found that GTL, rhGTK, human α -aminoadipate aminotransferase, pig heart cytosolic aspartate aminotransferase and mitochondrial aspartate aminotransferases can catalyze the formation of α -ketoglutarate from 4-FGlu at a detectable rate.

However, highly purified pig heart alanine aminotransferase was shown to be able to catalyze the formation of α -ketoglutarate from 4-FGlu (Table 5). Pig heart alanine aminotransferase was previously shown to catalyze a very efficiently exchange of both the α and β protons of alanine with deuterium when the enzyme is incubated with L-alanine in the presence of pyruvate in D₂O (Cooper, 1976; Babu and Johnston, 1976). For every exchange of alanine amine with pyruvate, as expected, an α proton was observed to be exchanged. Unexpectedly, however, *all three* β protons on average were also exchanged. The mechanism for this non-productive exchange has not been fully elucidated, but may provide a clue as to the mechanism of the γ elimination observed with 4-FGlu. Other aminotransferases, such as cytosolic aspartate aminotransferase are capable of catalyzing proton exchange at the β position of the substrate glutamate, but the relative rate is considerably slower than the β -exchange of alanine protons catalyzed by alanine aminotransferase (Luthe et al., 1975; Cooper, 1976; Babu and Johnston, 1976). Thus, the propensity of suitably positioned catalytically active site residues may favor electron movement away from the α C-H bond toward the C-F bond in 4-FGlu more readily in the active site of alanine aminotransferase than in the active site of other aminotransferases. However, the exact mechanism of alanine aminotransferase-catalyzed γ elimination of F⁻ from 4-FGlu must await further studies.

The rate of alanine aminotransferase-catalyzed formation of α -ketoglutarate from 4-FGlu is relatively slow. The specific activity of the pig heart enzyme (as measured by pyruvate production) under optimal concentrations of L-alanine and α -ketoglutarate and optimal pH (~8.0) is about 65 $\mu\text{mol}/\text{min}/\text{mg}$ or about 3.9 $\text{mmol}/\text{h}/\text{mg}$ (Hopper and Segal, 1962). This value is more than three orders of magnitude greater than the amount of α -ketoglutarate generated by γ -elimination with 5 mM 4-FGlu as substrate in the 1 h incubation when calculated for 1 mg of enzyme (0.6 to 1.58 μmol ; Table 5). Although we did not determine optimal conditions for the γ -elimination reaction it is highly likely that partitioning between γ -elimination and transamination reactions greatly favors the latter. Nevertheless, a substantial amount of substrate 4-FGlu in the assay mixture (100 nmoles at the start of the reaction) was converted to product α -ketoglutarate (6–16 nmoles) (Table 5), indicating that despite the much more favorable transamination, the irreversibility of the γ -lyase reaction can drive the reaction toward α -ketoglutarate formation.

Data of Gatehouse et al. (1967) and Hopper and Segal (1962) indicate that the specific activity of purified rat liver alanine aminotransferase in the presence of 5 mM L-glutamate is ~33 $\mu\text{mol}/\text{min}/\text{mg}$ or 1.98 $\text{mmol}/\text{h}/\text{mg}$ of protein. Assuming that the pig heart enzyme behaves similarly to the rat liver enzyme then the rate of γ -elimination from 5 mM 4-FGlu catalyzed by rat liver alanine aminotransferase should be <1.98 $\text{mmol}/\text{h}/\text{mg}$. Data in Table 5 suggest a maximal γ -elimination reaction rate with 5 mM 4-FGlu of about 1.6 $\mu\text{mol}/\text{h}/\text{mg}$. Thus, it seems likely that a major enzyme contributing to the γ -elimination reaction in rat liver cytosolic fractions is alanine aminotransferase. However, we cannot rule out the possibility that other PLP-containing enzymes can also contribute to the γ elimination of F⁻ from 4-FGlu in the rat liver cytosolic fractions and in tumors.

Most of the present work has centered on mammalian enzymes, but the work should have wider relevance. For example, we showed that 4-FGln is a good substrate of *E. coli* and kidney glutaminases. Thus, 4-FGln may be useful in studies of glutaminases from a variety of organisms. 4-FGln (and 4-FGlu) may also be useful in other non-mammalian systems. For example, plants, yeasts and bacteria assimilate ammonia by a glutamate synthase cycle. The cycle consists of a glutamine synthetase reaction (Eq. 9) coupled to a glutamate synthase (GOGAT) reaction (Eq. 10). The net reaction (Eq. 11) results in the reductive amination of α -ketoglutarate to L-glutamate. The reaction is highly favorable due to the hydrolysis of ATP. In plants there are at least three GOGATs, which utilize NADPH, NADH or reduced ferredoxin as electron donor in Eq. 10. The relative distribution of the three enzymes depends on species and tissue. For a review see, for example, Suzuki and Knaff (2005). We suggest that both 4-FGln and 4-FGlu may be useful probes in the study of the glutamate synthase cycle in plants, yeasts and bacteria.

During polyamine synthesis 5'-methylthioadenosine is generated from methionine via *S*-adenosyl methionine. The 5'-methylthioadenosine is converted to methionine via the methionine salvage pathway. In this pathway the original methyl and sulfur in methionine are retained, but carbons 1 to 4 are formed anew from the ribose moiety of 5'-methylthioadenosine. The pathway must be of fundamental importance because it is of ancient lineage and represented in, for example, mammals, plants and bacteria, with only minor variations. For a recent review see Albers (2009). The last step of the salvage pathway requires the transamination of KMB. In mammals the preferred amino donor in this reaction is glutamine (reviewed by Cooper, 2004). Use of glutamine presumably ensures that the reaction is drawn in the direction of methionine formation by cyclization of KGM and/or action of ω -amidase (Cooper, 2004). Amine donors other than glutamine may be used in certain bacteria that possess aminotransferases of very broad specificity (Sekowska et al., 2004). Nevertheless, orthologues of GTK and ω -amidase are well represented in many bacteria and often the genes are associated with operons that contain other components of the methionine salvage pathway (Andrew Hanson; personal communication). We suggest that 4-FGln may be a useful glutamine analogue in the study of the methionine salvage pathway in many organisms.

5. Conclusions

The present work shows that both 4-FGln and 4-FGlu react with amino acid-utilizing enzymes generally in a fashion similar to that of glutamine and glutamate, respectively. For example, 4-FGln is a substrate of the kidney type glutaminase and three glutamine-utilizing transaminases (GTK, GTL and α -amino adipate aminotransferase); 4-FGlu is a substrate of glutamate dehydrogenase and three glutamate-utilizing aminotransferases (mitochondrial aspartate-, cytosolic aspartate- and alanine aminotransferases). These results should prove useful in modeling the disposition of label derived from [^{18}F]4-FGln in PET imaging studies of tumors. In addition, the present work revealed some unexpected findings: 4-FGlu could not be detected as a glutamine synthetase substrate, but instead was a strong inhibitor. The previous observation that $^{18}\text{F}^-$ is a metabolite of [^{18}F]4-FGln in tumors is

consistent with conversion of [^{18}F]4-FGln to [^{18}F]4-FGlu followed by a PLP-enzyme-dependent γ -elimination reaction. Unexpectedly, this reaction was shown to be catalyzed by alanine aminotransferase. Thus, 4-FGlu may prove to be a useful reagent in the study of glutamine synthetase and alanine aminotransferase. Finally, it is suggested that both 4-FGln and 4-FGlu may be useful probes in the study of glutamine- and glutamate-utilizing enzymes not only in normal and cancerous mammalian tissues, but also in a variety of non-mammalian organisms.

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Abbreviations used

AOAA	aminooxyacetate
DTT	dithiothreitol
FDG	fluorodeoxyglucose
4-FGln	(2S,4R)4-fluoroglutamine
4-FGlu	(2S,4R)4-fluoroglutamate
4-FKGM	4-fluoro- α -ketoglutaramate
KAT	kynurenine aminotransferase
KGM	α -ketoglutaramate
KMB	α -keto- γ -methiolbutyrate
GTK	glutamine transaminase K
GTL	glutamine transaminase L
MPA	metaphosphoric acid
MRS	magnetic resonance spectroscopy
PEP	phosphoenolpyruvate
PET	positron emission tomography
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate

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Figure 1.

Enzymology of 4-fluoroglutamate (4-FGln). Enzyme reactions: **1**, glutaminase; **2**, glutamate dehydrogenase; **3**, various glutamate-linked aminotransferases; **4**, various glutamine utilizing aminotransferases (transaminases); **5**, rapid non-enzymatic cyclization of 4-FKGM to a lactam; **6**, defluorination reaction (γ -elimination) catalyzed by alanine aminotransferase; **7**, slow non-enzymatic cyclization of 4-FGln to 4-fluoro-5-oxoproline and ammonia. Thus far only reactions 1 and 6 have been demonstrated *in vivo*. The remaining reactions are based on *in vitro* studies reported here on the stability of 4-FGln and on the interaction of 4-FGln and 4-FGlu with purified enzymes. HPLC methods are under

development to ascertain the contribution to the various pathways in normal and cancerous tissues.

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

L-Amino acid	Activity (nmol/min/mg)	Relative Activity (%)
Methionine	1260 ± 40	[100]
4-FGln	77 ± 4	6.1
Glutamine	9.5 ± 0.8	0.75
4-FGlu	18 ± 2	1.43
Glutamate	6.2 ± 0.2	0.49

^aThe reaction mixture (0.2 ml) contained 2 mM L-amino acid, 100 mM potassium phosphate buffer (pH 7.4), 100 mM semicarbazide and 30 µg (120 mU) of L-amino acid oxidase. The rate of α-keto acid semicarbazone formation was monitored at 248 nm at 37 °C. n = 3.

Table 2Transamination of L-glutamine (Gln) and 4-FGln by various aminotransferases ^a

Enzyme	Rate (nmol/min/μg)		4-FGln/Gln
	Gln	4F-Gln	
GTK/KAT I (1.13 μg)	0.94 ± 0.14	0.08 ± 0.02	0.08
AAT/KAT II (1.13 μg)	0.45 ± 0.01	0.20 ± 0.01	0.43
GTL/KAT III (0.37 μg)	2.00 ± 0.26	0.04 ± 0.02	0.02

^aThe reaction mixture (50 μl) contained 100 mM potassium phosphate buffer (pH 7.4), 10 mM KMB, 10 mM Gln (or 4.5 mM 4-FGln) and the indicated amount of enzyme. Duplicate samples were incubated for 15 min or 30 min at 37 °C. The reaction was then terminated by addition of 15 μl of 25% (w/v) MPA and methionine was determined by HPLC with CoulArray detection. Rates were calculated by combining the results from the two sets of duplicates. Thus, n = 4; AAT, α-aminoacidate aminotransferase; GTK, glutamine transaminase K; GTL; glutamine transaminase L; KAT, kynurenine aminotransferase.

Table 3Transamination of L-glutamate (Glu) and 4-FGlu catalyzed by various aminotransferases ^a

Enzyme	Rate (nmol/min/μg)		4-FGlu/Glu
	Glu	4F-Glu	
Pig heart cytosolic aspartate aminotransferase (0.1 μg, 10 mU)	20.9 ± 2.1	12.0 ± 1.2	0.57
Rat liver mitochondrial aspartate aminotransferase (0.1 μg, 41 mU)	48.8 ± 1.7	47.3 ± 3.4	0.97
Pig heart alanine aminotransferase (0.2 μg, 13 mU)	15.5 ± 1.5	87.0 ± 7.0	5.6

^aThe reaction mixture (0.2 ml) contained 5 mM α-ketoglutarate (or 5 mM 4-FGlu) and 5 mM oxaloacetate (aspartate aminotransferase-catalyzed reactions) or 5 mM pyruvate (alanine aminotransferase-catalyzed reaction) and the indicated amount of enzyme. Product formation from L-glutamate (i.e. α-ketoglutarate) or from 4-FGlu (i.e. 4-F α-ketoglutarate) was continuously monitored at 37 °C by coupling to the glutamate dehydrogenase reaction.

Table 4 **α -Ketoglutarate formation from 4-FGlu catalyzed by a rat liver cytosolic fraction^a**

Addition	α -Ketoglutarate generated (nmol)
None	12.1 $\pm$ 0.4
60 mM L-Glutamate	2.95 $\pm$ 0.95 ^b
5 mM AOAA	<0.5 ^b
0.25 mM α -Ketoglutarate	17.4 $\pm$ 0.9 ^{b,c}

^aThe reaction mixture (0.02 ml) contained 100 mM Tris-HCl buffer (pH 8.5), 5 mM 4-FGlu, 2 μ l of a rat liver cytosolic fraction (20 μ g protein) and the indicated addition. After incubation for one hour at 37 °C the amount of α -ketoglutarate 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 α -ketoglutarate present in the reaction mixture at the start of the incubation.

Table 5 **α -Ketoglutarate formation from 4-FGlu catalyzed by purified pig heart alanine aminotransferase ^a**

Addition	α -Ketoglutarate generated (nmol)
None	6.38 $\pm$ 1.38
60 mM L-Glutamate	<0.5 ^b
5 mM AOAA	<0.5 ^b
0.25 mM α -Ketoglutarate	15.8 $\pm$ 2.5 ^{b,c}

^aThe reaction mixture (0.02 ml) contained 100 mM Tris-HCl buffer (pH 8.5), 5 mM 4-FGlu, 0.65 U (10 μ g, 650 mU) alanine aminotransferase and the indicated addition. After incubation for one hour at 37 °C the amount of α -ketoglutarate 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 α -ketoglutarate present in the reaction mixture at the start of the incubation.