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REVIEW

ω-Amidase and Its Substrate α-Ketoglutaramate (the α-Keto Acid Analogue of Glutamine) as Biomarkers in Health and Disease

Arthur J. L. Cooper[1,a#] and Travis T. Denton[2,3,4,5,b,c*]

[1]*Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA*

[2]*LiT Biosciences, 120 N Pine ST, Ste 242, Spokane, WA 99202-5029, USA*

[3]*Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University Health Sciences Spokane, Spokane, WA, USA*

[4]*Department of Translational Medicine and Physiology, Elson S. Floyd College of Medicine, Washington State University Health Sciences Spokane, Spokane, WA, USA*

[5]*Steve Gleason Institute for Neuroscience, Washington State University Health Sciences Spokane, Spokane, WA, USA*

[a]*e-mail: arthur_cooper@nymc.edu; ORCID 0000-0002-9143-8504*

[b]*e-mail: travis.denton@litbiosciencesllc.com; ORCID 0000-0002-1222-2538* [c]*e-mail: travis.denton@wsu.edu*

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Abstract—A large literature exists on the biochemistry, chemistry, metabolism, and clinical importance of the α-keto acid analogues of many amino acids. However, although glutamine is the most abundant amino acid in human tissues, and transamination of glutamine to its α-keto acid analogue (α-ketoglutaramate; KGM) was described more than seventy years ago, little information is available on the biological importance of KGM. Herein, we summarize the metabolic importance of KGM as an intermediate in the glutamine transaminase – ω-amidase (GTωA) pathway for the conversion of glutamine to anaplerotic α-ketoglutarate. We describe some properties of KGM, notably its occurrence as a lactam (2-hydroxy-5-oxoproline; 99.7% at pH 7.2), and its presence in normal tissues and body fluids. We note that the concentration of KGM is elevated in the cerebrospinal fluid of liver disease patients and that the urinary KGM/creatinine ratio is elevated in patients with an inborn error of the urea cycle and in patients with citrin deficiency. Recently, of the 607 urinary metabolites measured in a kidney disease study, KGM was noted to be one of five metabolites that was most significantly associated with uromodulin (a potential biomarker for tubular functional mass). Finally, we note that KGM is an intermediate in the breakdown of nicotine in certain organisms and is an important factor in nitrogen homeostasis in some microorganisms and plants. In conclusion, we suggest that biochemists and clinicians should consider KGM as (i) a key intermediate in nitrogen metabolism in all branches of life, and (ii) a biomarker, along with ω-amidase, in several diseases.

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DEDICATION

This article is dedicated to my long-time friend and mentor, Dr. Arthur J. L. Cooper. We suddenly lost Dr. Cooper on May 30, 2024, at the age of 78, during the time he and I were in the process of submitting this article together. Arthur was, undeniably, one of the world's foremost experts in glutamine metabolism, as well as countless other areas of biochemistry and neuroscience. Arthur spent much time in the final years of his life seeking to raise scientific awareness

* To whom correspondence should be addressed.
# Deceased.

about the Glutamine Transaminase-omega-Amidase (GTωA) pathway. It is in Arthur's memory that I present this final publication highlighting the GTωA pathway, co-authored with Arthur J. L. Cooper. You will never be forgotten, my friend!

INTRODUCTION. THE DISCOVERY OF ENZYME-CATALYZED TRANSAMINATION

A discussion of the discovery of enzyme-catalyzed transamination provides a background for the discovery of glutamine transamination. Enzyme-catalyzed transamination [Eq. (1)] was discovered by Alexander E. Braunstein and coworkers in the 1930s. For a review of this work and its metabolic importance see [1]. Braunstein coined the word "umaminierung" (i.e., transamination) to describe this process. Enzymes that catalyze transamination reactions were originally referred to as transaminases. However, currently, the more common term for transaminases is aminotransferases. In this review we will use the original term transaminase where appropriate. The first transamination reactions discovered by Braunstein and colleagues were between L-glutamate and pyruvate [Eq. (2)] and between L-glutamate and oxaloacetate [Eq. (3)]. The enzymes that catalyze these reactions are often referred to as glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT), respectively, especially among clinicians. The recommended Enzyme Commission terms for these enzymes are alanine aminotransferase and aspartate aminotransferase, respectively, where it is understood that L-glutamate is the amino acid substrate in the forward direction. Here, we use the abbreviations AlaAT and AspAT, respectively, for these enzymes.

$$\text{L-Amino acid (1)} + \text{α-keto acid (2)} \leftrightarrows \text{α-keto acid (1)} + \text{L-amino acid (2)} \quad (1)$$

$$\text{L-Glutamate} + \text{pyruvate} \leftrightarrows \text{α-ketoglutarate} + \text{L-alanine} \quad (2)$$

$$\text{L-Glutamate} + \text{oxaloacetate} \leftrightarrows \text{α-ketoglutarate} + \text{L-aspartate} \quad (3)$$

Mammalian tissues contain two isozymes of AspAT – a mitochondrial form (mitAspAT) and a cytosolic form (cytAspAT). Each is a homodimer. The pig heart cytosolic and mitochondrial isozyme monomers contain 412 and 401 residues, respectively ([1] and references cited therein). In 1973, after enormous effort, Braunstein and coworkers sequenced pig heart cytosolic AspAT monomer [2] – at that time, it was the third largest protein to have been sequenced! Braunstein was the first to emphasize the importance of glutamate/α-ketoglutarate-linked aminotransferases [Eq. (4)] coupled to the glutamate dehydrogenase (GDH) reaction [Eq. (5)] in generating ammonia from many amino acids, or for assimilating ammonia nitrogen into many amino acids in mammalian issues. He termed these processes transdeamination and transreamination, respectively [3]. The transdeamination reaction [forward direction of Eq. (6)] is especially important in directing nitrogen from the catabolism of many of the common amino acids toward ammonia to be used for urea synthesis in the liver [4]. Esmond Snell (e.g., [5, 6]), Alton Meister (e.g., [7, 8]) and their colleagues established pyridoxal 5′-phosphate (PLP) as the cofactor involved in transamination reactions.

$$\text{L-Amino acid} + \text{α-ketoglutarate} \leftrightarrows \text{α-keto acid} + \text{L-glutamate} \quad (4)$$

$$\text{L-Glutamate} + H_2O + NAD^+ \leftrightarrows \text{α-ketoglutarate} + NADH + {}^+NH_4 \quad (5)$$

$$\text{Net: L-Amino acid} + H_2O + NAD^+ \leftrightarrows \text{α-keto acid} + NADH + {}^+NH_4 \quad (6)$$

Following the pioneering work of Braunstein and colleagues it was soon established that transamination in mammals is a major step in the metabolism of, for example, aspartate, branched chain amino acids, γ-aminobutyric acid (GABA), tyrosine and phosphoserine. However, discussion of transaminases involved in the metabolism of these amino acids is beyond the scope of the present review.

DISCOVERY AND PROPERTIES OF L-GLUTAMINE TRANSAMINASES

Discovery. In the late 1940s, Alton Meister and colleagues at the National Institutes of Health discovered transamination of L-glutamine to α-ketoglutaramate (KGM) [Eq. (7)]. They also discovered an enzyme that they named ω-amidase, which catalyzes the hydrolysis of KGM to α-ketoglutarate and ammonia [Eq. (8)]. The net reaction resulting from the combination of glutamine transaminase and ω-amidase [Eq. (9)] was named the glutaminase II pathway [9-12].

$$\text{L-Glutamine} + \text{α-keto acid} \leftrightarrows \text{α-ketoglutaramate (KGM)} + \text{L-amino acid} \quad (7)$$

$$KGM + H_2O \rightarrow \text{α-ketoglutarate} + {}^+NH_4 \quad (8)$$

$$\text{Net: L-Glutamine} + \text{α-keto acid} + H_2O \rightarrow \text{α-ketoglutarate} + \text{L-amino acid} + {}^+NH_4 \quad (9)$$

For a description of the background leading to the discovery of the glutaminase II pathway see [13].

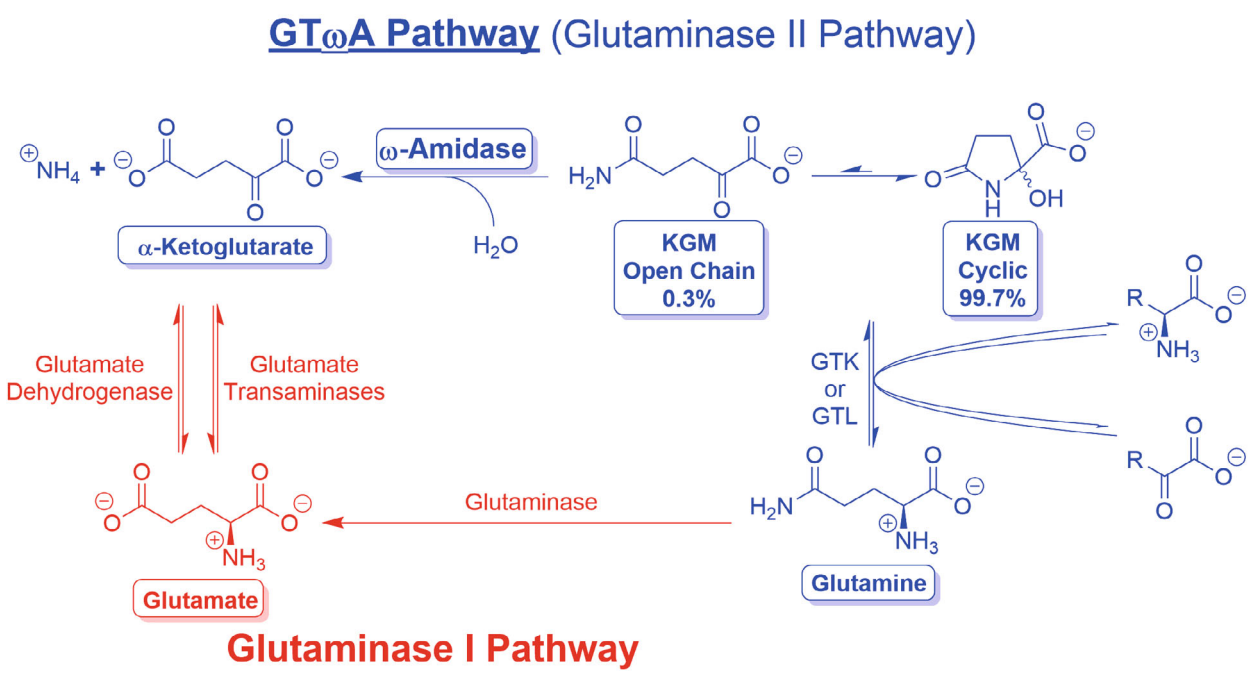

Fig. 1. The glutamine transaminase-ω-amidase (GTωA) pathway. Glutamine transamination with a suitable α-keto acid substrate is catalyzed by at least two glutamine transaminases in mammalian tissues – glutamine transaminase K (GTK) and glutamine transaminase L (GTL). The resulting α-keto acid (α-ketoglutaramate; KGM) is in equilibrium with a cyclic lactam form (2-hydroxy-5-oxoproline) [16, 17]. Under physiological conditions, the lactam predominates (~99.7%) over the open chain form (~0.3%; ω-amidase substrate) [16, 17]. [Throughout this review, the term KGM is understood to be the sum of open-chain form plus lactam, unless specified otherwise.] Note that although the transamination of glutamine is freely reversible (see [13]), the pathway is irreversibly pulled in the direction of glutamine transamination by cyclization of KGM and by the action of ω-amidase. Note also that not all enzyme reactants and products are depicted.

In order to prevent confusion between the name glutaminase II and the "true" glutaminase (GLS2) we have recently renamed the glutaminase II pathway as the GTωA pathway [13]. The GTωA pathway is depicted in Fig. 1.

Purification of glutamine transaminases. Meister and colleagues partially purified a glutamine transaminase from rat liver and showed that the enzyme exhibits wide specificity toward α-keto acids [9, 10]. Subsequently, Cooper and Meister purified the rat liver enzyme to homogeneity and showed that it is a homodimer, and PLP dependent [14]. The enzyme was also shown to exhibit broad L-amino acid specificity [14]. Cooper and Meister later showed that rat tissues contain an additional glutamine transaminase that is prominent in kidney and that they named glutamine transaminase K, to distinguish it from glutamine transaminase L (GTL) – the predominant form in rat liver [15]. Like the K-isozyme the L-isozyme is a homodimer and also has broad amino acid and α-keto acid specificity.

These authors also noted that the activities are present in cytosolic and mitochondrial fractions [15]. This was verified for GTK by Malherbe et al. [18] who showed that the N-terminus of the rat enzyme possesses a 32 amino acid mitochondrial targeting sequence that allows entry into the mitochondria; removal of this sequence ensures a competing cytosolic location [18]. The biological basis for the evolution of two glutamine transaminases with such overlapping specificity is currently unknown.

Nomenclature of glutamine transaminases and subunit composition. As noted in [13], owing to the broad substrate specificity of the L-glutamine transaminases, it is probable that some preparations of phenylalanine, serine, histidine and aminoadipate transaminases reported in the literature can be ascribed to L-glutamine transaminases. Of special note is the finding that two kynurenine aminotransferase (KAT) isozymes that have been described in the scientific literature, namely KAT1 and KAT3, are identical to GTK, and GTL, respectively [13, 19-21]. In addition, KAT2 (aminoadipate aminotransferase) has some glutamine transaminase activity [22].

Human GTK, which is annotated as KYAT1 in UniProt, is a homodimer [23]. Three isoforms of human GTK/KYAT1, produced by alternative splicing, are predicted in this database, namely Q16773-1 (canonical form; 422 amino acid residues; subunit molecular mass 47,875 Da), Q16773-2 (canonical form missing residues 68-117), and Q16773-3 (different sequence from the canonical form at residues 230-250 and missing

residues 251-422). The biological roles of Q16773-2 and 3 are unknown.

Human GTL, which is annotated as KYAT3 in UniProt, is also a homodimer [24] and is predicted to exist as three alternatively spliced isoforms. Isoform 1 (Q6YP21-1) is the canonical isoform (length 454 amino acid residues; subunit molecular mass 51,400 Da) and contains a 34 amino acid mitochondrial targeting sequence. Isoform 3 (Q6YP21-3) lacks residues 1-34 of the canonical isoform. Isoforms 1 and 3 presumably account for the presence of the enzyme in the mitochondria and cytosol, respectively. In addition, a third isoform (Q6YP21-2) is greatly truncated (missing residues 168-454) and contains an altered sequence 152-167. The function of this polypeptide is unknown.

A relatively large amount of work has been carried out on the four KATs described in the scientific literature (i.e., KATs 1, 2, 3, and 4) in part because the product of kynurenine transamination (i.e., kynurenate) is a neuromodulator (e.g., [25] and references cited therein). According to [25], kynurenate is "an endogenous antagonist of α7 nicotinic acetylcholine and excitatory amino acid receptors, regulates glutamatergic, GABAergic, cholinergic and dopaminergic neurotransmission in several regions of the rodent brain". The contribution of GTK/KAT1 and GTL/KAT3 to the formation of cerebral neuromodulator kynurenate is debatable and KAT2 is now regarded as the probable main contributor to brain kynurenate formation [26]. Moreover, among the amino acids tested as substrates for both GTK/KAT1 and GTL/KAT3, the highest catalytic efficiency (V_{max}/K_m) occurs with L-glutamine. The catalytic efficiency is much lower for kynurenine [19, 20]. In addition, L-glutamine is the most abundant amino acid in human tissues and its concentration is estimated to be 70-80 g per 70 kg individual ([27] and references cited therein). Given the molecular mass of L-glutamine [147.13 AMU] and assuming 80% water content in the body, the average concentration of L-glutamine in human tissues is about 9 mM. On the other hand, the concentration of kynurenine in mammalian tissues is much lower. The concentration of kynurenine is reported to be 20 nmol/g (~22 μM) in rat liver, with much lower concentrations in the brain, lung, and spleen [28]. Thus, the transamination of L-glutamine catalyzed by GTK/KAT1 and GTL/KAT3 *in vivo* in human/mammalian tissues is *quantitatively* likely to be orders of magnitude greater than that of L-kynurenine [13].

In addition to catalyzing transamination reactions, both GTK and GTL catalyze competing β-elimination reactions with many cysteine *S*-conjugates [Eq. (10)], where -SR is a good leaving group [e.g., [13, 21, 29-32]. Because GTK was the first enzyme recognized to have strong cysteine *S*-conjugate β-lyase activity ([32] and references cited therein) the alternative abbreviation CCBL1 (cysteine *S*-conjugate beta-lyase 1) is given in UniProt for GTK/KAT1. This name is also used by some commercial vendors. Inasmuch as GTL/KAT3 also has strong cysteine *S*-conjugate β-lyase activity [30]. This enzyme has been given the alternative name CCBL2 in the UniProt and by some commercial vendors.

In addition, Commandeur et al. [29] have shown that selenocysteine *Se*-conjugates are excellent transaminase and β-lyase substrates of highly purified rat liver GTK [Eq. (11)].

$$RSCH_2CH(CO_2^-)(NH_3^+) + H_2O \leftrightarrows CH_3C(O)CO_2^- + {}^+NH_4 + RSH \quad (10)$$

$$RSeCH_2CH(CO_2^-)(NH_3^+) + H_2O \leftrightarrows CH_3C(O)CO_2^- + {}^+NH_4 + RSeH \quad (11)$$

Substrate specificity of GTK and GTL. The wide L-amino acid and α-keto acid specificity noted by Cooper and Meister for rat kidney GTK and rat liver GTL [14, 15] was also noted for human KAT1 (GTK) [19] and mouse KAT3 (GTL) [20]. In general, amino acid substrates have the general structure $RCH(CO_2^-)NH_3^+$ and α-keto acid substrates have the structure $RC(O)(CO_2^-)$ where R is a relatively hydrophobic, non-charged group. Thus, for example, methionine and leucine (and their respective α-keto acids) in addition to glutamine, are good substrates of both enzymes [14, 15]. Valine and isoleucine and their corresponding α-keto acids, however, are poor substrates [14, 15, 19, 20], presumably due to steric hindrance at the active site as a result of branching at the β position. Interestingly, glyoxylate [$HC(O)(CO_2^-)$] (but not glycine [$CH_2(CO_2^-)NH_3^+$]) is a good substrate of both GTK and GTL, presumably because its small size allows ready entry at the active site.

METABOLIC IMPORTANCE OF THE GTωA PATHWAY

Here we summarize the suggested metabolic role of this pathway as recently reviewed in [13].

Closure of the methionine salvage pathway [Eq. (12)]. Strong evidence suggests that the GTωA pathway is largely responsible for closure of the methionine salvage pathway in mammals ([13] and references cited therein). Moreover, glutamine transaminase and ω-amidase have been shown to act in tandem to close the methionine salvage pathway in plants and bacteria [33].

$$\text{L-Glutamine} + \text{α-keto-γ-methiolbutyrate (KMB)} + H_2O \rightarrow \text{α-ketoglutarate} + \text{L-methionine} + {}^+NH_4 \quad (12)$$

Salvage of α-keto acids. We have proposed that the glutamine transaminases may act as repair enzymes

to correct the "mistakes" of other transaminases. Such mistakes may generate α-keto acids that are potentially toxic/neurotoxic at elevated levels (e.g., phenylpyruvate, *p*-hydroxyphenylpyruvate). Owing to the irreversibility of the GTωA pathway, the α-keto acid will be favorably converted back to the less toxic amino acid, while at the same time providing anaplerotic α-ketoglutarate ([13] and references cited therein). Caligiore et al. suggest that GTK may be especially important in salvaging tryptophan from its corresponding α-keto acid [34].

Detoxification of α-keto acids. Excess glyoxylate may be toxic due to its potential conversion to oxalate. Highly insoluble calcium oxalate is a major contributor to kidney stones. It has been suggested that glutamine transaminases/KATs may divert glyoxylate to glycine in the kidney thereby lowering the possibility of the conversion of glyoxylate to oxalate [35].

Possible antioxidant role of the GTωA pathway. Many α-keto acids are oxidatively decarboxylated by H_2O_2 which, in the process, is converted to H_2O ([36] and references quoted therein). For example, α-ketoglutarate is oxidized by H_2O_2 to succinate and CO_2 [Eq. (13)]. We have suggested that the GTωA pathway, as a source of α-ketoglutarate, may play a role in antioxidant defenses and upregulation of defense systems under hypoxic conditions [13]. Moreover, it has been recently shown that oxidation of specific cysteine residues in ωA by H_2O_2 inhibit ωA's catalytic activity which, in turn, would decrease the production of the α-ketoglutarate, making it less available to neutralize H_2O_2 [37]. In light of these new findings, although α-ketoglutarate itself does react with H_2O_2, it now seems less likely that the α-ketoglutarate, which has come directly from the GTωA pathway, would be the α-ketoglutarate acting as an intracellular antioxidant.

$$^{-}O_2CCH_2CH_2C(O)CO_2^{-} + H_2O_2 \rightarrow {}^{-}O_2CCH_2CH_2CO_2^{-} + CO_2 + H_2O \quad (13)$$

Possible role of the GTωA pathway in the transport of α-keto acids/L-amino acids. A large number of cellular and subcellular transporters have been described for L-amino acids and α-keto acids. We have suggested that the GTωA pathway, coupled to an α-ketoglutarate-linked aminotransferase, may assist in the transport of L-amino acids [13]. For example, consider transamination of an L-amino acid with α-ketoglutarate to the corresponding α-keto acid in compartment 1 and uptake of that α-keto acid into compartment 2. The GTωA pathway will ensure stoichiometric appearance of the corresponding L-amino acid in compartment 2 [Eqs. (14), (15)]. Removal of the α-keto acid in compartment 2 via the GTωA pathway will ensure unidirectional movement of the α-keto acid from compartment 1 into compartment 2. The compartments may be at the cellular or subcellular level.

$$\text{Compartment 1: L-Amino acid + α-ketoglutarate} \leftrightarrows \text{α-keto acid + L-glutamate} \quad (14)$$

$$\text{Compartment 2: L-Glutamine + α-keto acid + } H_2O \rightarrow \text{α-ketoglutarate + L-amino acid + } {}^{+}NH_4 \quad (15)$$

SUBSTRATE SPECIFICITY, DISTRIBUTION AND STRUCTURE OF ω-AMIDASE

Substrate specificity. Meister and colleagues showed that partially purified rat liver ω-amidase hydrolyzes the ω-monoamides of 4- and 5-C dicarboxylates such as KGM, α-ketosuccinamate (KSM; the α-keto acid analogue of asparagine), glutaramate, succinamate [9, 10]. However, neither glutamine nor asparagine was found to be a substrate of this preparation of ω-amidase [9, 10]. Thus, the -C(O)- in the α position may be replaced by a $-CH_2-$ but not by a $-CH(NH_3^{+})-$. Subsequently, Hersh [16, 17] showed that rat liver ω-amidase catalyzes hydroxaminolysis reactions with a number of carboxamide substrates, in which water is replaced by hydroxylamine as the attacking nucleophile, generating the corresponding hydroxamate [Eq. (16)]. In addition, rat liver ω-amidase was shown to catalyze the hydrolysis of the terminal carboxylate esters of a number of 5C- and 4C-dicarboxylates and some transamidation reactions at the ω-carboxamide [16, 17]. For a detailed discussion of these reactions see [38].

$$RC(O)NH_2 + NH_2OH \rightarrow RC(O)NHOH + {}^{+}NH_4 \quad (16)$$

Assay procedures. Meister showed that partially purified rat liver ω-amidase exhibits a relatively sharp pH optimum at ~9.0 for KGM, yet a much broader pH optimum (~5.0-9.0) with KSM as substrate, despite the apparent close similarity in structure between these two α-keto acids [11]. An explanation was provided by Hersh using a continuous coupled enzyme assay procedure in which the formation of α-ketoglutarate from KGM, catalyzed by ω-amidase [Eq. (8)], is reductively aminated by the action of GDH in the presence of ammonia and NADH – the reverse direction of Eq. (5) [13]. The disappearance of NADH is continuously monitored spectrophotometrically at 340 nm (ε = 6220 $M^{-1}cm^{-1}$). Hersh showed that, at the enzyme concentration used in the assay, the rate of change of NAD^{+} production at pH values below 8.0 is biphasic [16]. From this, Hersh was able to calculate the ratio of open-chain form (0.3%; substrate) to the lactam form (99.7%; non substrate). [Nevertheless, throughout this review, unless stated otherwise, it is understood that the term KGM refers to the sum of open-chain form plus lactam.]

Hersh concluded that the rate of interconversion between open-chain and cyclized forms of KGM is $^{-}$OH-catalyzed. As a result, assays of ω-amidase with KGM as substrate are typically carried out in buffers at pH 8.5-9-0 where rate of ring opening is unlikely to be rate limiting for amounts of enzyme used in a typical assay [39].

An alternative assay procedure uses succinamate [R = $^{-}O_2CH_2CH_2$- in Eq. (16)] as substrate. ω-Amidase catalyzes the conversion of succinamate to the corresponding hydroxamate in the presence of hydroxylamine. The hydroxamate exhibits a brown color (ε = 920 $M^{-1}cm^{-1}$ at 535 nm) when acidic ferric chloride is added to the reaction mixture [39]. The advantages of this assay are that succinamate, unlike KGM, is commercially available and the assay can be carried out at physiological pH values (i.e., ~7.0-7.4). A third assay procedure involves the use of KGM as a substrate dissolved in buffers at pH 8.5-9.0. The reaction is quenched by addition of acidic 5 mM 2,4-dinitrophenylhydrazine. After incubation for a few minutes, the pH of the reaction is adjusted by the addition of 1 M KOH and the absorbance of the 2,4-dinitrophenylhydrazone is measured at 430 nm (ε ~ 16,000 $M^{-1}cm^{-1}$) [39].

Distribution of ω-amidase. The enzyme activity is ubiquitously expressed in nature, attesting to its central importance in nitrogen metabolism in the biosphere. For example, Meister showed that ω-amidase activity (KGM and KSM as substrates) is present in all eight rat tissues investigated [11]. It was also shown to be present in a Novikoff tumor, lettuce leaves, spinach leaves, *Escherichia coli* and *Streptococcus faecalis* [11]. In another study, ω-amidase activity was shown to be present in all ten rat organs investigated, with highest specific activity in liver and kidney [40]. Prostate was not included in these studies, but later work showed that glutamine transaminase and ω-amidase specific activities are exceptionally high in that organ [41]. Lin et al noted the presence of Nit2 (nitrilase-like protein 2) protein in all sixteen human tissues/cells investigated with highest levels in liver and kidney [42]. [Nit2 is identical to ω-amidase (see below)]. GeneCards indicates that the message for ω-amidase/Nit2 is present in all 37 human tissues investigated [43].

ω-Amidase is well represented in *Neuropora crassa* where it is thought to be an important intermediate in the cycling of glutamine nitrogen [44, 45]. In plants, transamination of asparagine to KSM with glyoxylate as co-substrate [Eq. (17)] followed by hydrolysis of KSM, catalyzed by ω-amidase [Eq. (18)], is thought to play a key role in photorespiration – the net reaction is shown in Eq. (19) [46, 47].

$$\text{L-Asparagine + glyoxylate} \rightarrow \text{α-ketosuccinamate (KSM) + glycine} \quad (17)$$

$$\text{KSM} + H_2O \rightarrow \text{oxaloacetate} + {}^{+}NH_4 \quad (18)$$

$$\text{Net: L-Asparagine + glyoxylate} + H_2O \rightarrow \text{oxaloacetate + glycine} + {}^{+}NH_4 \quad (19)$$

Special role of an ω-amidase in microbial nicotine metabolism. Gram-positive soil bacteria *Arthrobacter nicotinovorans*, *Nocardioides* sp. JS614 and *Rhodococcus opacus* were shown to contain a similarly organized gene cluster for the catabolism of nicotine [48]. An intermediate in the catabolism of nicotine by *A. nicotinovorans* was shown to be KGM and one of the genes in the gene cluster was found to code for an enzyme that hydrolyzes KGM to α-ketoglutarate and ammonia [48]. The enzyme was found to have a monomer mass of 32.3 kDa – only slightly larger than that of the mammalian ω-amidase monomer (30.6 kDa; see below) – and possess a characteristic EKC triad at the active site [48]. Interestingly, the mammalian ω-amidase is a homodimer (see below), whereas preliminary data suggested that the *A. nicotinovorans* enzyme is a homotetramer [48].

Structure and location of mammalian ω-amidase/Nit2. The enzyme is annotated as Nit2 in human and mammalian gene data banks – see [49, 50]. The human enzyme (Q9NQR4 (UniProt) [51]) is a homodimer; each monomer consists of 276 amino acids with a molecular mass of 30,608 Da. Predicted three dimensional structures for the human enzyme are reported in [52] and [53] – the AlphaFold predicted structure [AF-Q9NQR4] is classified as “very high, with a pLDDT>90. The enzyme active site on each monomer possesses a reactive cysteine residue [16, 17], part of a glutamate, lysine, cysteine triad (E43; K112; C153 in the human enzyme) [52, 53].

Cellular and extracellular location of ω-Amidase/Nit2. The enzyme is present in rat liver cytosol [10, 40] and mitochondria [40, 54]. The mechanism for the import of ω-amidase into mitochondria requires a detailed study. Interestingly, UniProt lists a protein – epididymis secretory sperm binding protein Li 8a (Q9NQR4-1) that has a sequence identical to that of ω-amidase and is present in the centrosome [51]. However, we are unable to find any additional information on the apparent identity of ω-amidase/Nit2 with this binding protein. Finally, it has been shown that Nit2 (i.e., ω-amidase) is one of 1160 proteins identified in urinary exosomes by the NHLBI Epithelial Systems Biology Laboratory [55].

Possible post translational modification sites. PhosphSitePlus® indicates about thirty potential sites for post translational modifications of Nit2/ω-amidase of which Y49 (phosphorylation), Y145 (phosphorylation) and K249 (acetylation) are the most prominent [56]. In this context, it was previously noted that folate deficiency in human colonocytes in culture resulted

in a remarkable, ~98% loss of Nit2 (i.e., ω-amidase) protein, compared to that of control cultures as assessed by staining intensity of the protein spot on a 2D gel [57]. Possibly, Nit2 is phosphorylated under low folate conditions and moves to a different position on the 2D gel. Moreover, in a proteomic study of MCF7 breast cancer cells, a shift in isoelectric point of Nit2 (i.e., ω-amidase) compared to normal breast cells was noted, but only in those cancer cells overexpressing ERBB2 (Erb-B2 receptor tyrosine kinase) [58]. Whether phosphorylation of the enzyme under normal physiological conditions occurs *in vivo*, and whether this plays a role in regulating enzyme activity and location requires a detailed study.

ω-AMIDASE/Nit2 IS A MEMBER OF THE NITRILASE SUPERFAMILLY

The nitrilase superfamily of enzymes, which includes nitrilases, amidases and amidotransferases, has been classified into 13 families [59, 60]. ω-Amidase/Nit2 [along with Nit 1 (deaminated glutathione (dGSH) amidase [61])] are the sole mammalian members of family 10 of the nitrilase superfamily. All members of the superfamily, including ω-amidase, contain a canonical glutamate (E), lysine (K), cysteine (C) triad at the active site [60, 62]. Interestingly, this superfamily does not include the two mammalian glutaminase isozymes. The architecture of the active site in the two mammalian glutaminase isozymes is different from those of the nitrilase superfamily and the active site contains a reactive serine residue rather than a reactive cysteine residue – see below. The importance of the EKC triad (i.e., E43, K112, C153) for the catalytic activity in human ω-amidase is shown by mutation studies. Separate mutations of each of these residues to an alanine residue results in an inactive enzyme [52]. Moreover, removal of the loop that forms a lid over the active site (residues 116-128) also results in loss of activity [52]. In addition to the EKC triad, according to Weber et al. “All known nitrilase superfamily amidase and carbamoylase structures have an additional glutamate that is hydrogen bonded to the catalytic lysine” [62]. *Geobacillus pallidus* amidase catalyzes the hydrolysis of simple aliphatic amides [62]. Both an E(142)L mutation and an E(142)D mutation result in an inactive enzyme [62]; see also [63]. Interestingly, one of the substrates studied in the latter study was glutaramate, which is also a substrate of ω-amidase [11, 17].

Yeast Nit2 (yNit2) is an ortholog of mammalian Nit1 but NOT of mammalian Nit2. The crystal structure of yeast Nit2 (yNit2) has been reported by Liu et al. [64]. Confusingly, yNit2 is more related to mammalian Nit1 than to mammalian Nit2 ([61, 64] and references cited therein). A compound that is present in the active site of yNit2 was tentatively identified as GSH-like by Liu et al. [64]. Subsequently, Peracchi et al. [61] showed that dGSH is a major substrate of both yNit2 and mouse Nit1 (mNit1) [61]. KGM is a relatively poor substrate of both yNit2 [61, 64] and mNit1 [64]. As noted by Perrachi et al. for mouse Nit2 (mNit2) [61] “Sequence comparisons and structural data indicate that the residues of the Nit2 catalytic pocket that surround the substrate α-KGM are conserved in Nit1.” A major difference is, however, found in the subsite where the amido group of α-KGM is predicted to bind, which is a quite small niche in the structure of Nit2, but a much larger cavity in Nit1 – see also [65, 66]. These considerations are elaborated further in the next section which discusses the mechanism of human ω-amidase/Nit2.

Catalytic mechanism of ω-amidase/Nit2. Based on a QM/MM study of hNit2 (i.e., human ω-amidase) a theoretical four-step process that includes two transition states (TSs) at the active site during turnover of KSM to oxaloacetate and ammonia has been suggested [53]. This mechanism, depicted using KGM as the substrate and the numbering of the human EKC triad (i.e., Glu_{43}, Lys_{112}, Cys_{153}), is depicted in Fig. 2. In the first step, the amide group of the substrate is activated by coordination with Lys_{112} of the catalytic triad, and the supporting Glu_{128} residue, followed by nucleophilic attack by Cys_{153}. The stabilized tetrahedral intermediate collapses to form the thioester intermediate with concomitant loss of ammonia/ammonium. Subsequently, a water molecule, activated by coordination to Glu_{43}, attacks the thioester intermediate releasing KG followed by redistribution of active site residues for a subsequent round of catalysis. Curiously, the authors mention that the enzyme has 276 residues (as also noted above for human ω-amidase and in UniProt [51]) yet show the active site residues to be Glu81, Lys150, and Cys191, which is shifted by 38 amino acids. We assume that the authors recapitulate the residues mentioned in the work of Barglow et al. [66] and also assume that the 38 additional residues are a product of the expression system used for production of the enzyme (N-terminal hexahistidine fusion with the gene 10 leader sequence).

ω-AMIDASE/Nit2 – CLINICAL ASPECTS

Differentiation of Crohn’s disease (CD) from ulcerative colitis (UC). Burakoff et al. investigated whether blood-based biomarkers can differentiate UC from CD and noninflammatory diarrhea [67]. These authors generated whole blood gene expression profiles for 21 patients with UC, 24 patients with CD, and 10 control patients with diarrhea, but without colonic pathology. According to the authors: “A supervised

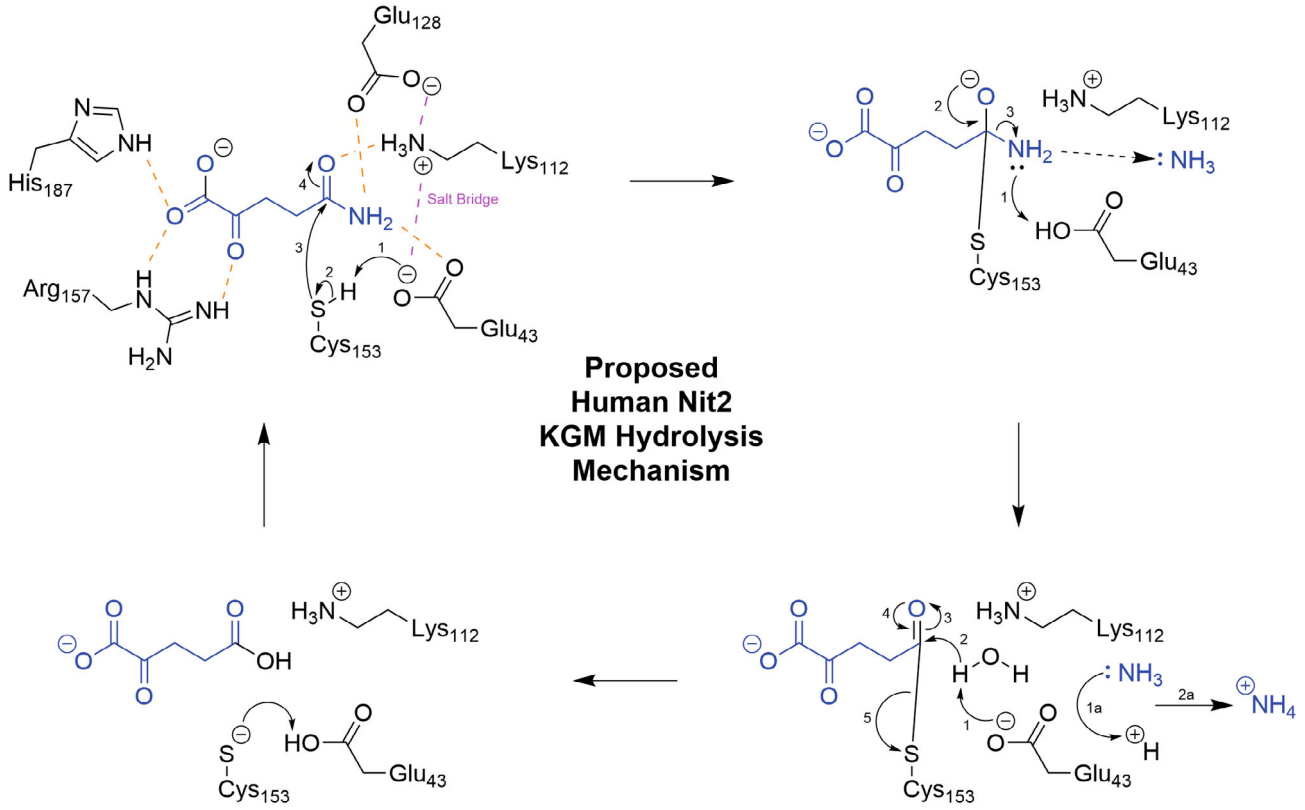

Fig. 2. Proposed reaction mechanism for the hydrolysis of KGM by human ω-amidase. The catalytic triad residue numbers reflect that on the human enzyme correlating to that of Chien et al. [52]. Molecular modeling of the mechanism was performed by Silva Teixeira et al. [53] and are the basis of the mechanism proposed.

learning method (logistic regression) was used to identify specific panels of probe sets which were able to discriminate between UC and CD and from controls. The UC panel consisted of the four genes, CD300A, KPNA4, IL1R2, and ELAVL1; the CD panel comprised the four genes CAP1, BID, NIT2, and NPL. These panels clearly differentiated between CD and UC" [67]. Note the inclusion of Nit2 (i.e., ω-amidase) in the CD panel. ω-Amidase/Nit2 as a possible diagnostic biomarker in CD requires further study.

Nit2 and Down syndrome. Myung et al. carried out MALDI-TOF mass-spectrometric identification and quantification of spots on 2D gel electrophoresis after in-gel digestion of proteins using cortical brain samples from 7 controls and 7 samples from fetal Down syndrome at the early second trimester [68]. Three proteins tentatively identified as reduced by 50% in Down syndrome fetal brain versus normal fetal brain were identified as Rik protein, mitochondrial inner membrane protein and Nit2 [68]. The significance of this finding also requires further study.

Role of ω-amidase/Nit2 in tumor progression. Nit1, as noted above, is now known to be a dGSH deaminase. The enzyme catalyzes the hydrolysis of dGSH to α-ketoglutarate and cysteinylglycine [61]. Considerable evidence suggests that Nit1 is a tumor suppressor (e.g., [69-71]). Because Nit2 has moderate sequence similarity to Nit1, Lin et al. considered the possibility that Nit2 (i.e., ω-amidase) is also a tumor suppressor [42]. Ectopic expression of Nit2 in HeLa cells was found to inhibit cell growth through G(2) arrest rather than by apoptosis [42]. The authors also showed, using proteomic and RT-PCR analysis, that Nit2 up-regulated the protein and mRNA levels of 14-3-3sigma, an inhibitor of both G(2)/M progression and protein kinase B (Akt)-activated cell growth, and down-regulated 14-3-3beta, a potential oncogenic protein [42]. The authors concluded that Nit2 may be a tumor suppressor candidate [42]. However, other workers have concluded that Nit2 may be a cancer promoter in colorectal cancer [72]. Moreover, the authors suggested that Nit2 may be a promising target for the treatment of colon cancer [72]. In another study, it was shown that the expression of Nit2 in tongue squamous cell carcinoma is significantly higher than that in normal tongue tissues [73]. The authors also suggested that Nit2 may be a therapeutic target [73]. As noted above, GTK and ω-amidase are well represented in rat prostate tissue [41]. Furthermore, it was shown that protein levels of these enzymes increased in human prostate cells in culture in tandem with the aggressiveness of the cancer [41]. *We suggest that, on balance, ω-amidase*

is a tumor promoter by providing anaplerotic α-ketoglutarate to rapidly dividing cancer cells (see the next section).

Glutamine addiction in tumors – the canonical pathway. It is well known that many cancers utilize glutamine as a major source of anaplerotic α-ketoglutarate and nitrogen for DNA synthesis. This has been termed "glutamine addiction" ([13] and references cited therein). Most researchers in the field only consider one pathway for the conversion of glutamine to α-ketoglutarate. This pathway (the canonical pathway) consists of the conversion of glutamine to glutamate via the action of a glutaminase, followed by conversion of glutamate to α-ketoglutarate by a transamination reaction [Eqs. (20)-(22)]. Note that Eq. (21) depicts the reverse direction of Eq. (4). Alternatively, glutamate may be converted to α-ketoglutarate by the action of GDH [Eq. (5)].

$$\text{L-Glutamine} + H_2O \rightarrow \text{L-glutamate} + {}^{+}NH_4 \quad (20)$$

$$\text{L-Glutamate} + \text{α-keto acid} \leftrightarrows \text{α-ketoglutarate} + \text{L-amino acid} \quad (21)$$

$$\text{Net: L-Glutamine} + H_2O + \text{α-keto acid} \rightarrow \text{α-ketoglutarate} + \text{L-amino acid} + {}^{+}NH_4 \quad (22)$$

Human cells contain two glutaminase isozymes encoded by *GLS* and *GLS2*. *GLS* encodes a kidney-type glutaminase (KGA) – also known as GLS1 (or simply GLS). *GLS* also encodes its active, shorter splice variant (glutaminase C, GAC). GAC is present in many cancers ([74] and references quoted therein) and is thought to be a major enzyme contributing to their glutamine addiction. *GLS2* encodes an enzyme (GLS2) that is normally predominant in the liver and also exists as splice variants – LGA and GAB (glutaminase B) [75-77]. In addition, LGA exists as two isoforms, one of which is more active than the other [76]. There appears to be some confusion in the literature on the naming of the more active isoform of GLS2 [75-77]. In their recent article, Feng et al., use the term LGA to describe the more active GLS2 isoform [74]. Unlike GLS1, which is a tumor promoter in many cancers, GSL2 may be either a tumor promoter or tumor suppressor [74, 78].

An Aside – Glutaminases involved in glutamine addiction – can their catalytic properties reveal insights into ω-amidase catalysis and regulation? Many enzymes are activated by forming polymers [79, 80]. Perhaps the most well studied such enzyme is acetyl-CoA carboxylase ([80] and references cited therein). The enzyme is active as a homodimer, but its activity is greatly increased upon polymerization. In addition, the activity is regulated in a complex fashion by phosphate binding and by allosteric regulators [80]. It is now becoming apparent that the mammalian glutaminases are activated in a similar fashion. GLS1 can exist as an inactive dimer as well as an active tetramer [80-82]. Moreover, both glutaminase isozymes are activated by phosphate and contain allosteric binding sites ([74] and references cited therein). In addition, it is known that GLS1 (i.e., KGA), GAC (i.e., active shortened form of KGA) and GLS2 can exist in active polymeric forms [74, 81-83]. Recently, Feng et al. demonstrated that the ability of GAC and GLS2 to form filaments is directly coupled to their catalytic activity [74]. The authors further noted that "Filament formation guides an 'activation loop' to assume a specific conformation that works together with a 'lid' to close over the active site and position glutamine for nucleophilic attack by an essential serine." The active site of GLS1 is different from that of ω-amidase. For example, as noted above, the active site of GLS/GAC contains a reactive serine residue [74], whereas that of ω-amidase contains a reactive cysteine residue [16, 52, 53]. However, a point of similarity is that both enzymes require a lid to cover the active site for catalysis to proceed [52, 53, 74]. Nothing is currently known about possible regulation of ω-amidase activity. Nevertheless, based on the fact that KGM and glutamine are almost identical in size (differing in molecular mass by just 1 Da) and are monoamides of 5-C dicarboxylic acids, it is possible that ω-amidase shares some regulatory features with GLS1 and GLS2. This is another area that requires detailed study.

Glutamine addiction in tumors – the alternative GTωA pathway for the formation of anaplerotic α-ketoglutarate. Several clinical studies have been completed or are ongoing for the treatment of various cancers with the allosteric glutaminase inhibitor CB-839 monotherapy or in combination therapy with other drugs (e.g., [84-87]). Whereas some of these treatments seem to be well tolerated, they have only been moderately successful. We have suggested that this may be due, in part, to an alternative pathway for the conversion of glutamine to α-ketoglutarate – i.e., the GTωA pathway (originally referred to as the glutaminase II pathway), that can provide anaplerotic α-ketoglutarate to cancer cells when GLS1 is inhibited [13, 88]. Although this pathway has mostly been ignored (or unrecognized) by cancer researchers, strong evidence has recently been presented that the GTωA pathway is a major source of anaplerotic α-ketoglutarate in pancreatic cancer. Thus, Udupa et al. showed that genetic suppression of GTK in pancreatic tumors (P198 shGTK-KD cancer cells injected in the back of nude mice) led to *complete* suppression of pancreatic tumorigenesis [89]. The authors suggested that a GTK inhibitor may be useful, either alone or in combination with a GLS1 inhibitor, for the treatment of cancer [89]. In another study, Pham et al., investigated the isotopomer patterns in L-glutamate generated from L-[U-^{13}C]glutamine, in orthotopic human Myc-amplified

D425MED medulloblastoma tumor and showed that this tumor preferentially uses the GTωA pathway over the canonical GLS1 pathway to convert L-glutamine to L-glutamate (in this case, via α-ketoglutarate) [90]. The authors noted that *KYAT1* (i.e., the gene for GTK) and its mRNA are upregulated in medulloblastoma compared to other pediatric brain tumors in the Children's Brain Tumor Network/KidsFirst Pediatric Brain Tumor Atlas RNAseq dataset [90]. In another study, as noted above, not only does GLS1 protein increase in prostate cancer cells in culture as the aggressiveness of the tumor derived therefrom increases, but GTK and ω-amidase proteins also increase concomitantly [41]. It has been suggested that inhibitors of glutamine transaminases may be useful anti-cancer agents, perhaps in combination with GLS1 inhibitors [41, 89, 90]. Glutamine transaminase inhibitors are discussed in the next section.

Glutamine transaminase inhibitors. Transition state (TS) mimetics that inhibit KMB transamination in cells in culture have been developed, including L-methionine ethyl ester pyridoxal (MEEP) [91, 92]. Inasmuch as KMB is a substrate of both GTL and GTK, it is reasonable to assume that these TS mimetic inhibitors will block L-glutamine transaminases. Interestingly, MEEP was shown to induce DNA strand breaks in HeLa cells typical of apoptotic cell death [92]. Some inhibitors of KAT1 (i.e., GTK) have been synthesized with K_i values ranging from ~20 μM to ~1 mM [93] but, to our knowledge, have not been tested as anti-cancer agents. The anti-cancer agent cisplatin is known to be nephrotoxic at least in part due to formation of the corresponding cysteine *S*-conjugate [94]. Mitochondrial AspAT is known to catalyze cysteine *S*-conjugate β-lyase reactions [95]. Thus, it was reasoned that this enzyme might catalyze a β-lyase reaction with the cysteine *S*-conjugate of cisplatin. Overexpression of mitAspAT in LLC-PK_1 cells led to increased toxicity of cisplatin, increased platination in mitochondria and increased inhibition of α-ketoglutarate dehydrogenase complex, possibly due to close juxtapositioning of mitAspAT to the enzyme complex [96].

In that study (i.e., [96]), GTK was not studied as a possible β-lyase contributing to the nephrotoxicity. Nevertheless, in a recent study, Sukeda et al. purified human recombinant CCBL1 (i.e., GTK/KAT1) and used a high-throughput screening assay (transamination between kynurenine and pyruvate) to screen chemical libraries (not specified) for potential inhibitors [97]. 2′,4′,6′-Trihydroxyacetophenone (THA; a naturally occurring metabolite in *Curcuma comosa*) was identified as a dose-dependent inhibitor of the human recombinant GTK/KAT1 with an IC_{50} of 13.2 μM [97]. The authors stated that THA inhibited the β-lyase activity, but inspection of their data shows that the inhibition was actually of the transaminase activity [97]. Nevertheless, the authors noted some protective effects of THA against cisplatin-induced toxicity toward mouse kidney *in vivo* and LL-C-PK1 cells in culture. However, the compound did not prevent the proliferation of cancer cell lines – LLC and MDA-MB-231 [97]. Sukeda et al. also recognized other potential caveats. For example, THA may have been protective by acting as an antioxidant. Nevertheless, THA could be the lead compound in the search for compounds that will result in more powerful glutamine transaminase inhibitors.

In conclusion, much work remains to be carried out in the search for a selective and potent inhibitor of GTK (and GTL). Such an inhibitor will be useful in studies of the enzyme mechanism, but it may have limitations as an anti-cancer agent due to the possible involvement of the glutamine transaminases in so many physiologically relevant processes (see the above discussion). For this reason, compounds designed to disrupt the GTωA pathway may be better directed toward inhibition of ω-amidase rather than toward inhibition of glutamine transaminases. We will return to this point later.

ENDOGENOUS CONCENTRATIONS OF α-KETOGLUTARAMATE (KGM) IN NORMAL RAT TISSUES AND HUMAN BODY FLUIDS

Despite the fact that ω-amidase is inherently of high specific activity in tissues, it is now well established that KGM is an endogenous, natural metabolite. This is presumably due to the cyclization of open-chain (substrate) form to a lactam (inactive as a substrate), and that the rate of ring opening is relatively slow at neutral pH [16]. Because the rate of ring opening is hydroxide ion ($^-$OH)-dependent, any pathological condition that will result in lowering of the cellular pH (e.g., hypercarbia [98]) will impede the ω-amidase reaction with KGM even further.

The occurrence of KGM as a natural metabolite was first observed in human cerebrospinal fluid (CSF). In the procedure used by the investigators, endogenous KGM was converted to α-ketoglutarate with ω-amidase purified from rat liver. The generated α-ketoglutarate was then measured fluorometrically with GDH in the presence of NADH and ammonia [99-101]. The presence of KGM in human CSF was later verified by using a gas chromatography-mass spectrometry (GC-MS) procedure [102]. KGM has also been measured in human urine by a GC-MS procedure [103, 104] – see also below. Both GC-MS procedures measure the cyclized lactam form of KGM. In another procedure, KGM was measured in human CSF rat liver, rat kidney and rat brain by an isotope dilution method [105]. Finally, a high-performance liquid chromatography (HPLC) procedure has been developed to measure underivatized

KGM in rat tissues and plasma [106]. Taken together, the data indicate that the concentration of KGM in normal human CSF is ~5 μM, whereas the concentration in rat liver, kidney, brain, and plasma is ~8-216, 5-13, 6-11, and 19 μM, respectively [99, 101, 105, 106]. The concentration in normal human urine is ~1-3 μmol KGM/mmol creatinine [103]. Halámková and colleagues showed that two separate spectrophotometric coupled enzyme assays could be used to determine the concentration of KGM estimated to be present in human serum [107]. The authors estimated that the concentration of KGM in normal human plasma is ~5-10 μM [107] – a concentration in the same order of magnitude as that noted by Shurubor et al. of 19 μM [103].

CLINICAL STUDIES IN WHICH KGM WAS MEASURED/DETECTED IN BODY FLUIDS

Increased concentrations of KGM in the CSF of patients with liver disease. Duffy and colleagues showed that KGM occurs in human CSF and that the concentration is increased in hyperammonemic patients with hepatic encephalopathy (HE) [99, 101, 105]. Moreover, the concentration was found to increase in proportion to the severity of the disease. In some severe cases of HE, CSF KGM concentrations exceeded 50 μM [99, 105]. A strong correlation was also noted between concentrations of KGM and glutamine in the CSF [99, 105]. Most likely, increased ammonia in the HE patients resulted in increased tissue glutamine concentrations, which in turn contributed to increased transamination of glutamine. Mild neurotoxic effects were noted upon infusion of KGM into rat CSF [99, 105]. This finding led Duffy et al. to suggest that KGM may be a neurotoxic agent contributing to the encephalopathy noted in HE patients [99, 101, 105]. However, the concentration of KGM in the infusate (10 mM) is >2000-fold greater than that expected in normal CSF and 100-fold greater than the highest concentration found in the CSF of an HE patient.

KGM exhibits a red color when spotted onto filter paper (or onto paper chromatograms) and then sprayed with an ethanolic solution of Ehrlich's reagent [(4-dimethylamino)benzaldehyde] [108]. The procedure is sensitive enough to detect KGM at a concentration of 20 μM in 20 μL of CSF, spotted directly onto paper without prior treatment [108]. The chemistry behind this reaction is unknown, but the spot test may possibly serve as a starting point for the development of a clinical test for KGM.

The KGM used in the studies of Duffy et al. was synthesized by the method of Meister [11] in which a solution of glutamine is incubated with snake venom L-amino acid oxidase in the presence of catalase, followed by purification of KGM by cation exchange chromatography. However, it has been known for more than 75 years that glutamine spontaneously cyclizes in solution to 5-oxoproline (5-OP) and formation of ammonia [109]. Thus, even the best preparations of KGM prepared by this enzymatic procedure contain a few percent 5-OP. This compound is known to be enzymatically converted to L-glutamate [110] – an amino acid in the CSF suggested to be an excitotoxin even at μM concentrations (e.g., [111, 112] and references cited therein).

Increased urinary KGM/creatinine ratios in primary and secondary hyperammonemia. Primary hyperammonemia is due to a defect that directly affects enzymes or transporters of the urea cycle [113]. Secondary hyperammonemia occurs when the function of the urea cycle is inhibited by toxic metabolites or by substrate deficiencies [113, 114]. The possibility that increased production of KGM also occurs in patients who do not have overt liver disease but have an inborn error of metabolism resulting in primary hyperammonemia was investigated by Kuhara and colleagues [103]. The KGM/creatinine ratio was shown to be markedly elevated in urine obtained from patients with primary hyperammonemia due to an inherited metabolic defect in any one of the five enzymes of the urea cycle [103]. Kuhara et al. also noted increased urinary KGM in three patients with a defect resulting in lysinuric protein intolerance and one of two patients with a defect in the ornithine transporter I [103]. It was suggested that the increase in urinary KGM concentrations in patients with primary hyperammonemia is related to concomitant increase of glutamine concentration [103]. On the other hand, urinary KGM levels were not well correlated with secondary hyperammonemia in patients with propionic acidemia or methylmalonic acidemia, possibly as a result, in part, of decreased glutamine levels [103].

In another study, Kuhara and colleagues noted an increase in urinary KGM in most patients with secondary hyperammonemia resulting from citrin deficiency, despite normal levels of urinary glutamine [104]. Citrin is a hepatic mitochondrial aspartate-glutamate carrier coded by the *SLC25A13* gene and a defect in this gene can lead to episodic hyperammonemia and disturbed consciousness ([115] and references cited therein).

Frainay et al. have devised a network-based recommendation system to interpret and enrich metabolomics results that they have named MetaboRank [116]. Interestingly, the authors state "...MetaboRank recommended the overlooked α-ketoglutaramate as a metabolite which should be added to the metabolic fingerprint of HE..." [116].

In summary, KGM may be a useful biomarker for many hyperammonemic diseases including hepatic encephalopathy, inborn errors of the urea cycle, citrin deficiency and lysinuric protein intolerance.

Strong association of urinary KGM with uromodulin in chronic kidney disease patients. In a recent study, it was shown that KGM is one of five metabolites (out of 607) that strongly (P 2.11e^{-440}) correlated with the protein uromodulin (UMOD) in a cohort of kidney disease patients (n = 462) in Germany [117]. The kidney-specific UMOD has been suggested to be a potential marker for tubular functional mass in population-based studies [117-121]. According to Pruijm et al. [118] the associations of UMOD excretion with markers of tubular functions and kidney dimensions may reflect tubule activity in the general population. It has also been suggested to be a marker for structural integrity of the distal nephron, and renal function [120, 121]. The reason for the association of KGM with uromodulin is not obvious and requires further study.

KGM as a potential biomarker in serum obtained from stroke victims. Sidorov et al. used metabolomic analysis to prospectively analyze potential serum biomarkers in acute ischemic (7 h) and chronic (3-6 months) stages of 60 stroke victims [122]. The authors noted the presence of four unknown metabolites at the acute stage that were significantly associated with infarct volume (all $p < 0.01$) [122]. Nine metabolites at the chronic stage were found to significantly associate with infarct volume, namely 3-indolepropionate, α-ketoglutaramate, picolinate, and six unknowns (all $p < 0.048$) [122].

OTHER STUDIES INVOLVING BIOLOGICALLY OCCURING KGM

Plasma and tissue KGM in rats treated with the hepatotoxin thioacetamide. Shurobor et al. used an HPLC method to show that the concentration of KGM in normal rat plasma is ~19 μM [106, 123]. The authors treated rats with various doses (200, 400, and 600 mg/kg) of hepatotoxic thioacetamide and, after six days of recovery, measured the concentration of several TCA cycle metabolites, and that of KGM, in the plasma [123]. Despite the apparent physiological recovery of the rats at this time, a metabolic imbalance was still apparent [123]. Notably, the concentration of plasma KGM was decreased by about 15-20% in rats treated with all three doses, relative to that of a control [123]. The authors noted that the concentration of KGM in liver and kidney, but not in brain, tended to decrease in proportion to the amount of thioacetamide administered [123]. The α-ketoglutarate/KGM ratio was significantly increased in brain and kidney as the concentration of administered thioacetamide increased, but the ratios in the kidney were not significantly different from that of the control [123].

Increased serum KGM in dogs with exocrine pancreatis insufficiency (EPI). EPI results from insufficient secretion of pancreatic digestive enzymes ([124] and references cited therein). Barko et al., used UPLC-MS/MS to carry out untargeted serum metabolomics to identify metabolic disturbances associated with EPI [124]. Fasted serum samples were collected from dogs with EPI (n = 20) and healthy controls (n = 10), all receiving pancreatic replacement therapy [122]. 759 serum metabolites were detected, of which the concentration of 114 varied significantly ($p < 0.05$, $q < 0.2$) between dogs with EPI and healthy controls. Of note was a marked increase in the concentration of serum KGM [124]. The reason for this increase requires further study.

ADDIONAL ASPECTS OF ω-AMIDASE/KGM BIOLOGY

Wing and eye development in *Drosophila melanogaster*. Knockdown of the gene *CG813* in *D. melanogaster*, that encodes a homologue of mammalian ω-amidase, results in severe eye- [125] and wing defects [126]. It is not clear whether the defects are due to a buildup of KGM that is specifically toxic to eye and wing development, decreased synthesis of anaplerotic α-ketoglutarate that specifically affects wings and eyes, or to an as yet unidentified moonlighting property of ω-amidase required for correct eye and wing development.

Role of KGM in stimulating nitrogen assimilation in plants. Unkefer et al. have recently shown that KGM (also known as 2-hydroxy-5-oxoproline) stimulates nitrate uptake by plants and is a “likely signal for ammonium assimilation” [127]. These authors suggest that application of KGM to agricultural plants “would potentially reduce the use of nitrate fertilizer per unit of yield, leading to a further reduction in agriculture’s carbon footprint” [127].

THE NEED FOR LARGE SCALE SYNTHESIS OF KGM CHEAPLY AND ON AN INDUSTRIAL SCALE

One of the reasons why ω-amidase has been so little studied is that the substrate KGM is not available commercially and, until recently, could only be made enzymatically by oxidation of L-glutamine with snake venom L-amino acid oxidase in the presence of catalase [11]. However, as noted above, such preparations are invariably contaminated with small amounts of 5-oxoproline and trace amounts of α-ketoglutarate. Recently, one of us (TTD) has devised a laboratory scale organic synthesis of KGM that is not contaminated with 5-OP and/or α-ketoglutarate [128]. Martinez and Unkefer have patented another organic synthesis procedure [127].

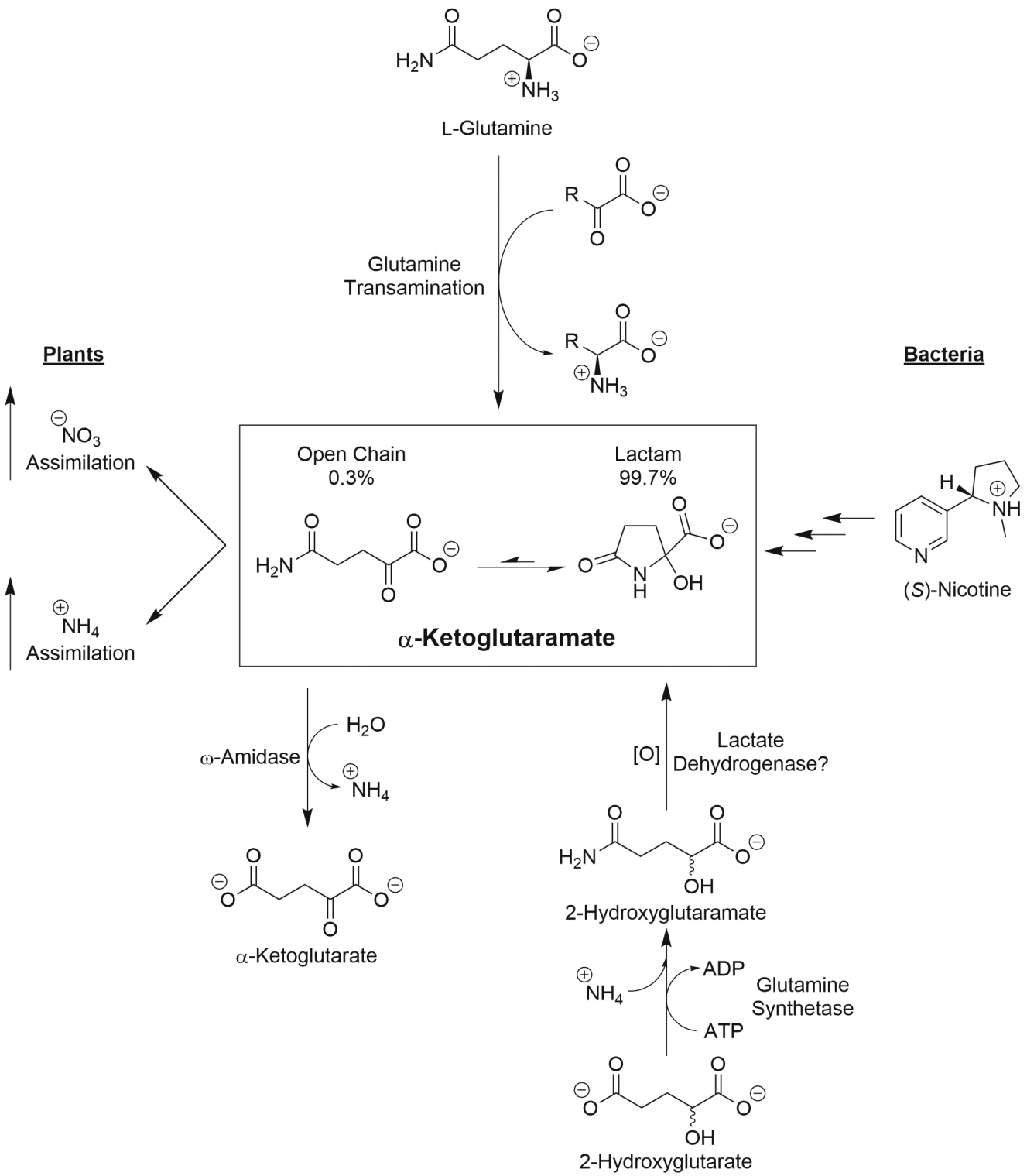

Fig. 3. The importance of ω-amidase as a clinical marker; the central role of KGM as a clinical marker and as a marker of nitrogen metabolism; the GTωA pathway as a source of anaplerotic α-ketoglutarate to cancer cells.

This procedure relies on the oxidation of glutamine or 5-oxoproline with Fremy's salt. The yield is reported to be high [129]. The possible advantages and disadvantages of the procedure are discussed in [130]. In conclusion, (i) given the potentially enormous agricultural benefit of using KGM to stimulate nitrate assimilation and (ii) the need to study the biological and clinical significance of ω-amidase/KGM, it is of utmost importance that methods be devised for the cheap, large-scale synthesis of KGM.

THE NEED FOR KGM LABLED WITH HEAVY ISOTOPES

Most of the metabolomic studies mentioned above that recognized KGM as an important intermediate were carried out by Metabolon Inc. The company does not currently have access to authentic KGM or a suitable internal standard. Nevertheless, Metabolon recognizes the KGM peak in the MS profile with a high degree of certainty based on fragmentation pattern. In other studies, Kuhara et al. measured KGM peak heights relative to creatinine by using authentic KGM but, in this case, γ-methyl KGM was used as an internal standard. Given the growing recognition of the metabolic and clinical importance of KGM, it is important that KGM, labeled with one or more heavy isotopes, be synthesized as an appropriate internal standard and for quantitation.

THE NEED FOR SELECTIVE INHIBITORS OF ω-AMIDASE

Given the many roles of the pathway in intermediary metabolism and especially in providing anaplerotic α-ketoglutarate to cancer cells, it will be very important to develop inhibitors of the enzymes of the GTωA pathway. As noted above, some researchers have suggested that inhibitors of GTK may be useful anticancer agents, perhaps in combination with a GLS1/GAC inhibitor. Some inhibitors of GTK have been described in the literature. These have not been tested in a clinical setting but can perhaps be used as lead compounds in the development of more potent inhibitors. However, as also noted, selective inhibitors of ω-amidase may be even more useful as anticancer agents, perhaps in combination with a GLS1/GAC inhibitor. Unfortunately, no selective and potent inhibitor of ω-amidase is currently available, although some strategies for the development of such inhibitors have been suggested [130].

CONCLUSIONS

ω-Amidase is ubiquitously expressed in nature, often in high levels, yet its biological roles remain largely unappreciated by biomedical scientists. *This review emphasizes (i) the central role of KGM in nitrogen metabolism, (ii) ω-amidase and its substrate KGM as clinical markers and (iii) the proposed importance of the GTωA pathway as a source of anaplerotic α-ketoglutarate to cancer cells.* This is schematically shown in Fig. 3. Finally, we emphasize the need for the development of selective inhibitors of GTK and especially of ω-amidase.

Abbreviations. $^{-}$OH, Hydroxide ion; 2D, Two dimensional; 5-OP, 5-Oxoproline (synonym: pyroglutamate); ω-Amidase, ω-Amidodicarboxylate amidohydrolase; AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; BID, BH3 interacting domain death agonist is a member of the BCL-2 family; CAP1, adenylyl cyclase-associated protein-1; CCBL1, cysteine *S*-conjugate β-lyase 1; CCBL2, cysteine *S*-conjugate β-lyase 2; CD, Crohn disease; CD300A, Cluster of differentiation 300A; CSF, cerebrospinal fluid; dGSH, deaminated glutathione; EKC, Glutamate (E), lysine (K), cysteine (C); ELAVL1, Embryonic lethal abnormal vision [synonym: or HuR (Drosophila-like Hu antigen R)]; EPI, exocrine pancreatic insufficiency; GABA, γ-aminobutyric acid; GAB, glutaminase B; GAC, glutaminase C; dGSH, deaminated glutathione; GC-MS, Gas chromatography-mass spectrometry; GDH, glutamate dehydrogenase; GLS or GLS1, glutaminase isozyme 1 (synonyms: kidney type glutaminase); GLS2, glutaminase isozyme 2 (synonyms: liver type glutaminase); GTK, glutamine transaminase K [synonyms: kynurenine aminotransferase 1; GTL, glutamine transaminase L [synonyms: kynurenine aminotransferase 3; GTωA, glutamine transaminase ω-amidase; GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; GSH, glutathione; HE, hepatic encephalopathy; HPLC, high performance liquid chromatography; IL1R2, interleukin 1 receptor, type II; KAT/KYAT, kynurenine aminotransferase; KGA, kidney-type glutaminase; KGM, α-Ketoglutaramate (synonyms: 2-oxoglutaramate, 5-amino-2,5 dioxopentanoate); KMB, α-Keto-γ-methiolbutyrate (synonyms: 4-methylthio-2-oxobutanoate, 4-methylthio2-oxobutyrate); KOH, potassium hydroxide; KPNA4, karyopherin alpha 4 (synonym: importin alpha 3); KSM, α-ketosuccinamate (synonyms: 2-oxosuccinamate, 4-amino-2,4-oxobutanoate); LGA, liver type glutaminase; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MEEP, L-methionine ethyl ester pyridoxal; NADH, nicotinamide adenine dinucleotide; mNit2, mouse nitrilase like protein 2; NPL, N-Acetylneuraminate pyruvate lyase; mRNA, messenger ribonucleic acid; Nit2, Nitrilase like protein 2 (synonyms: ω-amidase); PLP, pyridoxal 5′-phosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; TCA, tricarboxylic acid; THA, 2′,*A*′,*6*′-Trihydroxyacetophenone; TS, transition state; UC, ulcerative colitis; UMOD, uromodulin; UPLC-MS/MS, ultraperformance liquid chromatography-tandem mass spectrometry; yNit2, yeast nitrilase like protein 2.

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