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Assay and purification of ω -amidase/Nit2, a ubiquitously expressed putative tumor suppressor that catalyzes the deamidation of the α -keto acid analogues of glutamine and asparagine

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Abstract

ω -Amidase [ω -amidodicarboxylate amidohydrolase, E.C. 3.5.1.3] isolated from rat liver cytosol is a versatile enzyme that catalyzes a large number of amidase, transamidase and ester hydrolysis reactions. ω -Amidase activity toward α -ketoglutaramate and α -ketosuccinamate (the α -keto acid analogues of glutamine and asparagine, respectively) is present in mammalian tissues, tumors, plants, bacteria and fungi. Despite its versatility, widespread occurrence and high specific activity, the enzyme has been little studied, possibly because the assay procedure previously required a substrate (α -ketoglutaramate) that is not commercially available. Here we report a simplified method for preparing α -ketoglutaramate and an assay procedure that measures α -ketoglutarate formation from α -ketoglutaramate in a 96-well-plate format. We also describe a 96-well plate assay procedure that measures ω -amidase-catalyzed hydroxaminolysis of commercially available succinamic acid. The product, succinyl hydroxamate, yields a stable brown color in the presence of acidic ferric chloride that can be quantitated spectrophotometrically with negligible background interference. The two assay procedures (i.e. hydrolysis of α -ketoglutaramate and hydroxaminolysis of succinamate) were employed in purifying ω -amidase about ~3,600-fold from rat liver cytosol. The ratio of α -ketoglutaramate hydrolysis to succinamate hydroxaminolysis remained constant during the purification. ω -Amidase has recently been shown to be identical to Nit2, a putative tumor suppressor protein. It is anticipated that these new assay procedures will help characterize the function of ω -amidase/Nit2 in tumor suppression, will provide the basis of high-throughput procedures to search for potent inhibitors and enhancers of ω -amidase, and will assist in identifying biological interactions between nitrogen metabolism and tumor biology.

An apparent pyruvate-activated glutaminase and an apparent pyruvate-activated asparaginase were discovered more than 60 years ago [1]. Subsequently, Meister and co-workers [2-4] showed that both activities were actually due to a composite of two enzymes, namely a pyridoxal 5'-phosphate-dependent glutamine transaminase (Eq. 1) plus ω -amidase (Eq. 2), and a pyridoxal 5'-phosphate-dependent asparagine transaminase (Eq. 4) plus ω -amidase (Eq. 5). The net reactions are shown in Eqs 3 and 6. Transamination of glutamine and asparagine yield α -ketoglutaramate (α KGM)¹ and α -ketosuccinamate (α KSM), respectively, both of which are substrates of ω -amidase, yielding α -ketoglutarate and oxaloacetate, respectively [2].

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Intriguingly, neither asparagine nor glutamine is a substrate of rat liver ω -amidase [3]. On the other hand, both glutaramate and succinamate, where the α -keto group of α KGM and α KSM, respectively, is reduced to a $-\text{CH}_2-$ group are also substrates of ω -amidase. The transamination and associated deamination reactions involving glutamine and asparagine are shown in more detail in Fig. 1.

Mammalian tissues contain at least two glutamine transaminases [5], one of which (glutamine transaminase K; GTK) has been independently studied as kynurenine aminotransferase isozyme 1 [6]. [Note that in most cases the older term ‘transaminase’ has largely been replaced by ‘aminotransferase’, but the older term has been retained for enzymes that catalyze transamination of glutamine.] The glutamine transaminases play a role in the salvage of α -keto acids generated from essential amino acids by non-specific transamination reactions [5,7]. A glutamine transaminase (L-glutamine:keto-*scyllo*-inositol aminotransferase) is also important in the synthesis of aminocyclitol antibiotics [8]. Glutamine transamination and ω -amidase activities are thought to be part of a glutamine cycle that plays a role in nitrogen homeostasis in various microorganisms [9].

Transamination of asparagine with glyoxylate is involved in photorespiration ([10] and references cited therein). The plant enzyme is homologous to mammalian alanine-glyoxylate aminotransferase isozyme 1, which can utilize asparagine in place of alanine as a substrate. Asparagine transamination reactions occur in mammals as evidenced by the detection of α KSM in rat tissues [11]. The asparagine transamination/ ω -amidase pathway has been suggested to be important for the metabolism of asparagine in rat liver mitochondria [12].

Perhaps the most fascinating involvement of glutamine in transamination reactions relates to the methionine salvage pathway. During polyamine biosynthesis, the C1 of methionine is lost as CO_2 , the C2-C4 carbons are incorporated into the polyamine backbone, and the sulfur and

¹Abbreviations used: α KGM, α -ketoglutarate; α KSM, α -ketosuccinamate; DTT, dithiothreitol; GST, glutathione S-transferase; GTK, glutamine transaminase K; MTA, 5'-methylthioadenosine; Nit2, nitrilase-like protein 2.

methyl are incorporated into 5'-methylthioadenosine (MTA). In the methionine salvage pathway, which is present in mammals, plants and bacteria, MTA is converted to α KMB by a complex and unique series of reactions. Closure of the methionine salvage pathway in mammals is brought about by transamination of α KMB with glutamine. Thus, during the salvage pathway the original sulfur and methyl group of methionine are retained, the C1-C4 carbons lost during polyamine biosynthesis are obtained anew from the ribose moiety of MTA, and the amine nitrogen is obtained from glutamine. It is readily apparent that ω -amidase plays a crucial role in the salvage pathway by removing α KGM formed by transamination of glutamine and converting it to energetically useful α -ketoglutarate. The salvage pathway is reviewed in ref. 7.

Despite the biological importance of glutamine and asparagine transamination reactions, generation of α KGM and α KSM presents a problem. α KSM is relatively unstable and readily dimerizes [13]. The dimer can further react to form a plethora of aromatic compounds [11, 14] some of which may be toxic. α KGM has been detected in rat tissues and in human cerebrospinal fluid in μ M amounts [15]. Interestingly, the concentration of α KGM is greatly elevated in the cerebrospinal fluid of patients with hepatic encephalopathy – in some cases by as much as fifty fold [16]. Some evidence has been presented that α KGM may be neurotoxic [17].

Evidently, ω -amidase is important in converting two potentially toxic molecules to energetically useful compounds, namely α -ketoglutarate (Eq. 2) and oxaloacetate (Eq. 5). Perhaps not surprisingly given the extensive occurrence of glutamine and asparagine transamination reactions, ω -amidase activity is also widespread in nature. For example, ω -amidase activity (with α KGM and α KSM as substrates) has been detected in rat tissues, fungi, plants and tumors [13].

Recently, we showed that ω -amidase purified from rat liver cytosol is identical to Nit2 (nitrilase-like protein 2) (manuscript under review). Human Nit2 protein has been identified as a putative tumor suppressor protein, arresting cancer cells in the G2 cell cycle phase [18]. Interestingly, the Nit2 message is expressed in every human tissue investigated (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocytes) with highest expression in liver and kidney [18]. This finding correlates remarkably well with the widespread occurrence of ω -amidase activity previously reported in rat tissues. Thus, Meister found ω -amidase activity in every rat tissue investigated (liver, kidney, spleen, pancreas, skeletal muscle, cardiac muscle, testis, brain) with highest specific activity in liver and kidney [13].

A recent search for ω -amidase in PubMed generated 25 citations, all from more than a decade ago. A possible explanation for the scientific neglect of ω -amidase, despite its obvious importance in nitrogen and sulfur metabolism, is that neither α KGM nor α KSM is available commercially. In most previous studies of the enzyme, α KGM was used as the substrate. This compound is made from glutamine by oxidation with L-amino acid oxidase in the presence of catalase [13]. We reasoned that facile analytical methods would greatly advance the study of ω -amidase/Nit2. To that end, we have adapted our previous end-point assay of ω -amidase with α KGM [19] to a 96-well plate format. In addition, we have devised a relatively easy procedure to generate α KGM in solution in amounts suitable for a large number of assays. Lastly, we describe a spectrophotometric assay suitable for 96-well plate assays based on the hydroxaminolysis of commercially available succinamate. This assay has the advantage of negligible background and generation of a relatively stable colored product. The ratio of the hydroxaminolysis reaction to α KGM hydrolysis reaction remained constant throughout a 3,600-fold purification of ω -amidase from rat liver cytosol.

Materials and methods

Reagents

All reagents were of the highest quality available. Bovine serum albumin (fraction V), dithiothreitol (DTT), HEPES, EGTA, glutamine, sodium α -ketoglutarate, hydroxylamine hydrochloride, ferric chloride, trichloroacetic acid, lyophilized *Crotalus adamanteus* venom (containing 0.46 U of L-amino acid oxidase activity per mg of solid), and catalase (from horse liver, 36,000 Sigma units/mg), were obtained from Sigma Aldrich Chemical Company (St. Louis, Mo). Hydroxylapatite was from BioRad (Philadelphia, PA). Succinamic acid was obtained from Aldrich Chemical Company (Milwaukee, WI). 2,4-Dinitrophenylhydrazine was obtained from Eastman Kodak (Rochester, NY).

Purification of ω -amidase from rat liver cytosol

The rat liver cytosol was obtained by the procedure of Krasnikov et al. originally described for the isolation of highly purified rat liver mitochondria [20]. The isolation was carried out at 0 – 4°C. Briefly, a single liver was removed from an adult male Sprague Dawley rat and placed in a small beaker with 40 ml of ice-cold isolation buffer containing 300 mM sucrose, 5 mM HEPES, 500 mM EDTA, 100 mM EGTA and 0.5% (w/v) bovine serum albumin. The pH was adjusted with Tris base to 7.4. Minced and washed liver tissue was homogenized in a loose-fitting Dounce homogenizer (100 ml volume) at a tissue/buffer ratio of 1 g/8–10 ml. The homogenate was centrifuged for 10 min at 1000 $\times$ g. The pellet, which contained blood, nuclei and cell membrane fragments, was discarded. The supernatant fraction, which contained cytosol together with mitochondria, was carefully poured into a clean tube and centrifuged for 10 min at 10,000 $\times$ g. The supernatant, which contains mostly cytosol, was used for the purification of ω -amidase. Theoretically, the added bovine serum could lower the specific activity of the ω -amidase in the supernatant by as much as 18%. However, the type of bovine serum albumin added (essentially fatty acids free, fraction V) binds fatty acids and most will be removed in the fatty acid layer adhering to the tube in the last centrifugation step. The cytosolic fraction was stored at -20°C. ω -Amidase is stable in the frozen cytosol for at least a year.

The purification procedure was a modification of the methods of Cooper et al. [19] and Hersh [21]. The modified procedure requires fewer steps, but generates a preparation of higher specific activity (see below). A unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the formation of 1 μ mol of α -ketoglutarate per min from a reaction mixture containing 5 mM α KGM, 5 mM DTT and 100 mM Tris-HCl buffer (pH 8.5) at 37°C. Protein concentrations greater than 1 mg/ml were measured with the Total Protein Reagent, Biuret Method (Sigma Chemical Company, St. Louis, Mo). Protein concentrations less than 1 mg/ml were measured with the Quick Start™ Bradford reagent (BioRad, Philadelphia, PA). Bovine serum albumin was used as a standard.

Spectrophotometry

All spectrophotometric measurements were carried out with a SpectraMax M5 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Results and Discussion

Preparation of solutions of α -ketoglutaramate (α KGM) suitable for ω -amidase assays

Meister [13] showed that L-glutamine is a substrate of *C. adamanteus* L-amino acid oxidase and that this enzyme could be used to prepare α KGM suitable for ω -amidase assays. After oxidation of glutamine to α KGM in the presence of dialyzed *C. adamanteus* snake venom (which contains an appreciable amount of L-amino acid oxidase) and catalase (to remove

H₂O₂) at 37°C, protein was removed by dialysis and the solution was passed through a Dowex 50 (H⁺) column. The effluent was decolorized with activated charcoal, taken to pH ~4.5 with barium hydroxide, concentrated by flash evaporation, and the barium salt of αKGM was precipitated with 4 volumes of ethanol. The sodium salt was then prepared from the barium salt by passage through another Dowex 50 (H⁺) column followed by neutralization with a solution of concentrated NaOH [13].

We have simplified the Meister procedure. Glutamine (5 g; 34.2 mmols) was incubated with 2 g of lyophilized *C. adamanteus* venom previously dialyzed against distilled water (insoluble material was removed by centrifugation) and 10,000 U catalase at 37°C in 200 ml of distilled water (pH adjusted to 7.4 with 1M NaOH). [Note that the dried venom is a toxicant and should be carefully weighed so as to avoid inhalation. The dried venom should be constituted without shaking or stirring.] The incubation was carried out in a 12-liter flask with gentle agitation to ensure a large surface area for sufficient oxygen delivery to the enzyme. After incubation for 24 h, protein was removed by passage through an Amicon filter (*M_r* 10,000 cutoff), and the deproteinized solution was added to a Dowex 50 (H⁺) column (2.5 × 15 cm). The column binds sodium ions, unreacted glutamine and ammonium. The column was eluted with distilled water and the effluent, which contained α-ketoglutaric acid, was decolorized with ~250 mg of activated charcoal and taken to pH ~6.0 with 1 M NaOH, followed by filtration to remove the charcoal. This solution is directly suitable for enzymatic assays. The yield of sodium αKGM in solution was about 20 mmols (~58% yield) in a final volume of ~300 ml. This amount of substrate is sufficient for more than four thousand 96-well plate assays.

Glutamine has a tendency to slowly cyclize non-enzymatically in solution to 5-oxoproline (2-pyrrolidone-5-carboxylate), which does not bind to Dowex 50 (H⁺). There is also some slow hydrolysis of αKGM to α-ketoglutarate. Thus, the oxidation of glutamine must be carried out on a time scale that avoids major contamination with 5-oxoproline and α-ketoglutarate. Typically, solutions of αKGM prepared by our modification of the Meister procedure contain less than 5% of 5-oxoproline (by GC analysis [22]) and less than 1% of α-ketoglutarate relative to αKGM. The amount of αKGM in this solution may be standardized by quantitative conversion to α-ketoglutarate with purified rat liver ω-amidase. The α-ketoglutarate is then quantitated by conversion to glutamate with glutamate dehydrogenase [19] or with 2,4-dinitrophenylhydrazine (see below). Stock solutions of sodium αKGM (~15 – 20 mM) are stable for at least 5 years at -20°C. However, traces of α-ketoglutarate may be formed on repeated freeze-thawing.

96-Well plate assay for ω-amidase using αKGM as substrate

ω-Amidase was purified about 50-fold from rat liver by Meister [13] and later about 150-fold from rat liver cytosol by Hersh [21]. In routine end-point assays of this enzyme, Meister and colleagues measured ammonia production with Nessler's reagent after trapping in sulfuric acid [2,13]. Since this procedure uses mercury salts it should be avoided. Moreover, ammonia is a ubiquitous contaminant, which adds to the background. Hersh [21] devised a kinetic assay in which α-ketoglutarate generated from αKGM is continuously measured by reductive amination in the presence of NADH, ammonium, and glutamate dehydrogenase. The decrease in absorbance of NADH at 340 nm ($\epsilon = 6,220 \times \text{M}^{-1}\text{cm}^{-1}$) is continuously monitored.

In later work, we devised an end-point assay that measures α-ketoglutarate as its 2,4-dinitrophenylhydrazone. This assay is about 2.5 more sensitive than the Hersh assay and measures appearance of product rather than disappearance of product [19]. The basis of the 2,4-dinitrophenylhydrazone assay is as follows: When αKSM is employed as substrate, rat liver ω-amidase exhibits a very broad pH optimum – from pH 4 to 9.5 [13]. This wide pH range is consistent with the canonical CYS-GLU-LYS triad deduced to be present in the putative active site of Nit2, in which the glutamate acts as a general base catalyst [23]. However, the

pH profile with α KGM as substrate is markedly different from that exhibited with α KSM [13]. The enzyme is active at pH 8.0 – 9.5, but activity with α KGM is low at pH 7.0 [13]. This fact is explained by the finding of Meister [13] and Hersh [21] that α KGM is in equilibrium with a cyclic (lactam) form, which predominates (~99.7%) at pH close to neutral (Fig. 1). Hersh showed that at pH values above 8.0, the rate of interconversion is rapid, so that the concentration of open-chain substrate for the enzyme is not limiting for enzyme turnover. However, at lower pH values the rate of ring opening is slow, so that the amount of open-chain substrate becomes limited [21]. Thus, in the coupled assay with excess glutamate dehydrogenase (indicator enzyme), the rate of reductive amination at 30°C of product α -ketoglutarate (as assessed by disappearance of absorbance at 340 nm due to oxidation of NADH) was rapid and monophasic at pH 8.3. However, at pH values below 8.0 the rate of disappearance of NADH was biphasic – an initial “burst” in the rate of reductive amination could be discerned, followed by a slower linear rate of loss of absorbance. As the pH of the buffer was decreased below 8.0 the magnitude of the burst was largely unaltered, but the steady state rate of loss NADH absorbance following the burst was slowed. From the magnitude of the change in absorbance at 340 nm during the burst phase, Hersh was able to calculate that the amount of open-chain α KGM was ~0.3% in the pH range 6.5 to 7.8 [21]. Hersh also noted that the interconversion between cyclic and open-chain forms is specific base (i.e. OH⁻) catalyzed [21]. Formation of α -keto acid 2,4-dinitrophenylhydrazones are typically generated under highly acidic conditions. Under these conditions, ring opening of the lactam form of α KGM is predicted to be extremely slow. Thus, α -ketoglutarate may be measured as its 2,4-dinitrophenylhydrazone even in the presence of a large excess of α KGM.

With these considerations in mind, we adapted our previous end point assay [19] for 96-well plate analysis. The reaction mixture contains 5 mM α KGM, 5 mM DTT, 100 mM Tris-HCl buffer (pH 8.5) and enzyme in a final volume of 0.05 ml. After incubation at 37°C the reaction is terminated by addition of 0.02 ml of 5 mM 2,4-dinitrophenylhydrazine in 2 M HCl. After a further incubation for 5 min at 37°C, 0.13 ml of NaOH is added and the absorbance is read at 430 nm within 5 min. The $\epsilon_{430\text{nm}}$ of α -ketoglutarate 2,4-dinitrophenylhydrazone under these conditions is 16,000 M⁻¹cm⁻¹. The incubation with 2,4-dinitrophenylhydrazine reagent should not be longer than 5 min because there is slow acid-catalyzed deamidation of α KGM to α -ketoglutarate. To lower the background absorption, the volumes of the reaction mixture and 2,4-dinitrophenylhydrazine reagent may be reduced to 0.02 and 0.01 ml, respectively, if necessary.

When assays were conducted with purified ω -amidase, the blank contained complete reaction mixture but no added enzyme. For assays of crude tissue homogenates, the blank contained complete reaction mixture plus homogenate plus 200 mM glycylglycine. Glycylglycine was previously reported to be an inhibitor of ω -amidase at pH 8.5 [24]. This blank corrects for small carbonyl-containing molecules in crude homogenates that may react with the 2,4-dinitrophenylhydrazine reagent. These molecules are removed after ammonium sulfate fractionation of tissue homogenates followed by dialysis (see below).

When the α KGM assay mixture contains a relatively high amount of ω -amidase activity, incubation is generally carried out for 5 or 10 min at 37°C before addition of 2,4-dinitrophenylhydrazine reagent. However, we have noted that for dilute enzyme preparations, the formation of α -ketoglutarate is linear up to at least 90 min.

96-Well plate assay for ω -amidase – hydroxaminolysis of succinamate

In addition to catalyzing hydrolysis of α KGM and α KSM, purified rat liver ω -amidase also catalyzes a wide variety of amidation, transamidation, esterification, transesterification and hydroxaminolysis reactions [3,13,21,25]. Meister et al. [3] showed that semi-purified rat liver ω -amidase exhibits a pH optimum of 6.5 – 7.5 for the hydroxaminolysis of glutaramate and

succinamate. The hydroxaminolysis reaction with succinamate was noted to be especially favorable [3,13]. Other workers have shown that *Bacillus subtilis* [26,27] and *Thermus aquaticus* [27] possess an enzyme activity that catalyzes the hydroxaminolysis of succinamate. The enzyme was assumed to be an ω -amidase [26,27]. Hersh presented evidence that the hydroxaminolysis reaction with glutaramate catalyzed by the purified rat liver ω -amidase proceeds by a two step (ping-pong) process involving a covalent acyl intermediate formed with an active site cysteine residue [25]. Using glutaramate and methyl glutarate Hersh showed that both the first step of the ping-pong reaction (i.e. acylation of the active site cysteine sulfhydryl) and the second step (i.e. deacylation of the acyl-enzyme intermediate) are partially rate determining. Water is the “normal” attacking nucleophile in the regeneration of the active site cysteine. However, when hydroxylamine is present in the reaction mixture it begins to compete with water as the concentration of hydroxylamine is increased. Eventually, at very high hydroxamate concentrations hydroxaminolysis outcompetes water and the primary product is glutaryl hydroxamate rather than glutarate [25]. Hersh reported K_m values for glutaramate (hydrolysis reaction, pH 7.0) and hydroxylamine (hydroxaminolysis reaction, pH 8.5) of 2 mM and 3.7 mM, respectively, with ping-pong kinetics for rate of glutaramate disappearance in the presence of increasing hydroxylamine [25].

We used the findings of Meister et al. [3] and Hersh [25] to devise a convenient 96-well plate colorimetric assay for ω -amidase based on hydroxaminolysis reactions. Since succinamate is available commercially whereas glutaramate is not, and succinamate is more active than glutaramate in the hydroxaminolysis reaction [3], we used succinamate rather than glutaramate. The proposed ping-pong mechanism with succinamate and hydroxylamine is shown in Fig. 2. Our hydroxaminolysis assay procedure is as follows: The reaction mixture (0.05 ml) contains 20 mM succinamate, 5 mM DTT, 100 mM potassium phosphate buffer (pH 7.4), 100 mM hydroxylamine-HCl and enzyme. [Stock solutions of succinamic acid and hydroxylamine-HCl were previously neutralized with NaOH and stored at -20°C] After incubation at 37°C, 0.15 ml of a reagent containing 0.37 M FeCl₃, 0.67 M HCl, and 0.2 M trichloroacetic acid is added. The absorbance of the succinyl hydroxamate ferric complex is determined at 535 nm. The blank lacks either hydroxylamine or enzyme. When assays are conducted with purified protein no protein precipitate is discernible. However, when relatively crude homogenates are used as a source of enzyme, it is necessary to remove denatured aggregated protein by centrifugation prior to absorbance measurement.

The ferric chloride reagent used in the present study is identical to that used previously by Meister et al. [3] and by Ronzio et al. to measure γ -glutamyl hydroxamate formation catalyzed by sheep brain glutamine synthetase [28]. The extinction coefficient at 535 nm of γ -glutamyl hydroxamate with the ferric chloride reagent was reported to be 850 M⁻¹cm⁻¹ [28]. Meister et al. [3] stated that the color yield of succinyl hydroxamate relative to that observed with γ -glutamyl hydroxamate is 1.15. Therefore, we used a value of 920 M⁻¹cm⁻¹ as the extinction coefficient for the ferric complex of succinyl hydroxamate at 535 nm. A disadvantage of the hydroxamate assay is that the sensitivity is considerably less than that of the α KGM 2,4-dinitrophenylhydrazine assay. However, ω -amidase catalyzes hydroxaminolysis reactions very rapidly [3,25]. Indeed, we show here that, under the conditions of our assay procedures, rat liver cytosolic ω -amidase catalyzes hydroxaminolysis of succinamate about five times more rapidly than the hydrolysis of α KGM. Moreover, blank absorbance values are negligible in the hydroxamate assay and the color yield is stable for at least three hours.

When the α KSM assay mixture contains a relatively high amount of ω -amidase activity, incubation is generally carried out for 30 min at 37°C before addition of ferric chloride reagent. However, we have noted that for dilute enzyme preparations, the formation of hydroxamate is linear up to at least 90 min.

In a recent study, his₆-tagged mouse Nit2 was overexpressed, purified, crystallized and the X-ray crystallographic structure determined to 1.4 Å resolution [29]. The protein was recognized to be an amidase although the *in vivo* substrates were not known to the authors. Interestingly, despite the lack of knowledge concerning the actual *in vivo* substrates, a detailed picture of the active site, including residues postulated to be critical to catalysis, was obtained [29]. The hydroxaminolysis reaction should be especially amenable to high throughput screening for potential inhibitors and enhancers of rodent and human ω-amidases. Now that the substrates of Nit2 are actually known, inhibitors and enhancers identified by high throughput screening should be useful for comparative studies of the active site topology.

Purification of ω-amidase from rat liver cytosol

All steps were carried out at 0–4°C, unless otherwise stated. Centrifugation steps were carried out at 12,000 *g* for 15 min. ω-Amidase is sensitive to oxidative inactivation [19,21]. Therefore, all isolation buffers contained 1 or 2 mM DTT. All potassium phosphate buffers were pH 7.4. Frozen (–20°C) rat liver cytosol was thawed and centrifuged, and the pellet was discarded. The supernatant was treated with 313 g/L solid ammonium sulfate. Insoluble protein was removed by centrifugation and the supernatant was treated with additional ammonium sulfate (137 g/L). The active pellet was dissolved in the minimal amount of 10 mM potassium phosphate buffer and thoroughly dialyzed against 5 mM potassium phosphate buffer. An inactive precipitate that formed during dialysis was removed by centrifugation. The dialyzed sample was then added to a column of DE-52 (2.5 × 55 cm) equilibrated with 10 mM potassium phosphate buffer and eluted with the same buffer. ω-Amidase was present in the reddish colored eluate. The eluate was added to a second DE-52 column (2.5 × 55 cm) equilibrated with 5 mM potassium phosphate buffer. The column was then eluted with 500 ml of the same buffer. Active ω-amidase was eluted with 600 ml of 10 mM buffer and the eluate was allowed to “drip” into collection tubes containing 0.5 ml of 100 mM potassium phosphate buffer such that the final phosphate concentration was about 20 mM. [Addition of more concentrated potassium phosphate buffer was a precaution, because we previously noted that alkaline pH shifts occurred in the eluate during the purification step on DE-52 columns.] The active fractions were combined, dialyzed against 2.5 mM potassium phosphate buffer and then added to a hydroxylapatite column (1.7 × 5 cm) equilibrated with 5 mM potassium phosphate buffer. The column was successively eluted with 100 ml of 5 mM phosphate buffer and 100 ml of 10 mM potassium phosphate buffer. Finally, active enzyme was eluted with 60 ml of 50 mM potassium phosphate buffer. Tubes containing the most active fractions were combined. A summary of the purification is presented in Table 1.

Previous workers obtained a specific activity of 11.7 μmol·mg^{–1}·min^{–1} (30°C) [21] and 42.4 μmol·min^{–1}·min^{–1} (37°C) [19] for ω-amidase purified from rat liver cytosol using αKGM as substrate. In our previous preparation we noted that SDS-PAGE analysis revealed four major protein bands and several smaller bands [19]. The present purification procedure requires less steps than the purification procedures reported previously and yields the most active preparation to date for cytosolic rat liver ω-amidase (126 μmol·mg^{–1}·min^{–1} with αKGM as substrate; ~570 μmol·mg^{–1}·min^{–1} for the hydroxaminolysis of succinamate; 37°C). Our findings serve to emphasize the remarkably high inherent catalytic activity of ω-amidase. SDS-PAGE analysis of our purified enzyme revealed the two major bands (data not shown). These bands have now been identified as Nit2 (*M_r* ~ 31,000) and inactive GSTMu (~26,000) (manuscript under review).

Conclusions

The present work provides a convenient method for the preparation of the natural ω-amidase substrate, αKGM. In addition, we have provided a new assay based on the hydroxaminolysis

of succinamate. Although this assay is less sensitive than the assay that measures hydrolysis of α KGM, this is offset by the much higher (five-fold) rate of ω -amidase-catalyzed hydroxaminolysis of succinamate versus ω -amidase-catalyzed hydrolysis of α KGM. Additionally, the hydroxaminolysis assay utilizes a commercially available substrate (succinamate). Moreover, the negligible background is an advantage of the hydroxaminolysis reaction, especially in relatively crude homogenates where small- M_r carbonyl-containing molecules add to the background absorbance in the α KGM assay. The finding that the ratio of succinamate hydroxaminolysis/ α KGM hydrolysis remains relatively constant throughout the purification establishes ω -amidase as the sole catalyst in rat liver cytosol for the hydroxaminolysis of succinamate. This is important, because with the expected heightened interest in ω -amidase (as a result of its identity with the putative tumor suppressor Nit2), the hydroxaminolysis reaction will provide a convenient assay for high throughput searches for suitable inhibitors and enhancers of the purified enzyme and of the enzyme in tissue homogenates. Identification of such molecules will be important in modeling the role of ω -amidase/Nit2 in cancer suppression. It remains to be established, however, whether the tumor suppressor properties of ω -amidase are related to the amidase activity or are independent of the amidase activity. Finally, the enzyme may be a useful tool for the analysis of α KGM in body fluids as a marker for liver disease.

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References

1. Greenstein JP, Carter CE. The influence of α -keto acids on the desamidation of amino acid amides. *J Nat Canc Inst* 1947;7:57–60.
2. Meister A, Sober HA, Tice SV, Fraser PE. Transamination and associated deamidation of asparagine and glutamine. *J Biol Chem* 1952;197:319–330. [PubMed: 12981062]
3. Meister A, Levintow L, Greenfield RE, Abendschein PA. Hydrolysis and transfer reactions catalyzed by ω -amidase. *J Biol Chem* 1955;215:441–460. [PubMed: 14392177]
4. Otani TT, Meister A. ω -Amide and ω -amino acid derivatives of α -ketoglutaric and oxalacetic acids. *J Biol Chem* 1957;224:137–148. [PubMed: 13398393]
5. Cooper AJL, Meister A. Comparative studies of glutamine transaminases from rat tissues. *Comp Biochem Physiol* 1981;69B:137–145.
6. Mosca M, Croci C, Mostardini M, Breton J, Malyszko J, Avanzi N, Toma S, Benatti L, Gatti S. Tissue expression and translational control of rat kynurenine aminotransferase/glutamine transaminase K mRNAs. *Biochim Biophys Acta* 2003;1628:1–10. [PubMed: 12850267]
7. Cooper AJL. The role of glutamine transaminase K (GTK) in sulfur and α -keto acid metabolism in the brain, and possible bioactivation of neurotoxicants. *Neurochem Int* 2004;44:557–577. [PubMed: 15016471]
8. Lucher LA, Chen YM, Walker JB. Reactions catalyzed by purified L-glutamine keto-*scyllo*-inositol aminotransferase, an enzyme required for biosynthesis of aminocyclitol antibiotics. *Antimicrob Agents Chemother* 1989;33:452–459. [PubMed: 2729940]
9. Mora J. Glutamine metabolism and cycling in *Neurospora crassa*. *Microbiol Rev* 1990;54:293–304. [PubMed: 2145504]
10. Ta TC, Joy KW, Ireland RJ. Role of asparagine in the photorespiratory nitrogen metabolism of pea leaves. *Plant Physiol* 1985;78:334–337. [PubMed: 16664240]
11. Cooper AJL, Raps SP, Meister A. Fluorometric determination of α -ketosuccinamic acid in rat tissues. *Anal Biochem* 1987;167:312–320. [PubMed: 3442326]
12. Moraga-Amador DA, MacPhee-Quiggley KM, Keefer JF, Schuster SM. Asparagine catabolism in rat liver mitochondria. *Arch Biochem Biophys* 1989;258:314–326. [PubMed: 2912380]

13. Meister A. Preparation and enzymatic reactions of the keto analogues of glutamine and asparagine. *J Biol Chem* 1953;200:571–589. [PubMed: 13034816]
14. Stephani RA, Meister A. Structure of the dimeric α -keto acid analogue of asparagine. *J Biol Chem* 1971;246:7115–7118. [PubMed: 5129723]
15. Duffy TE, Cooper AJL, Meister A. Identification of α -ketoglutaramate in rat liver, kidney and brain. Relationship to glutamine transaminase and ω -amidase activities. *J Biol Chem* 1974;249:7603–7606. [PubMed: 4436326]
16. Vergara F, Plum F, Duffy TE. α -Ketoglutaramate: increased concentrations in the cerebrospinal fluid of patients in hepatic coma. *Science* 1974;183:81–83. [PubMed: 4808789]
17. Duffy TE, Vergara F, Plum F. α -Ketoglutaramate in hepatic encephalopathy. *Res Publ Assoc Res Nerv Ment Dis* 1974;53:39–52. [PubMed: 4438817]
18. Lin CH, Chung MY, Chen WB, Chien CH. Growth inhibitory effect of the human *Nit2* gene and its allelic imbalance in cancers. *FEBS J* 2007;274:2964–2956.
19. Cooper AJL, Duffy TE, Meister A. α -Keto acid ω -amidase from rat liver. *Methods Enzymol* 1985;113:350–358. [PubMed: 3003502]
20. Krasnikov BF, Zorov DB, Antonenko YN, Zaspaa AA, Kulikov IV, Kristal BS, Cooper AJL, Brown AM. Comparative kinetic analysis reveals that inducer-specific ion release precedes the mitochondrial transition. *Biochim Biophys Acta (Bioenergetics)* 2005;1708:375–392.
21. Hersh LB. Rat liver ω -amidase: Purification and properties. *Biochemistry* 1971;10:2884–2891. [PubMed: 5114531]
22. Cooper AJL, Dhar AK, Kutt H, Duffy TE. Determination of 2-pyrrolidone-5-carboxylic acid and α -ketoglutaramic acids in human cerebrospinal fluid. *Anal Biochem* 1980;103:118–126. [PubMed: 7377537]
23. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganoski P, Pekarsky Y, Croce CM, Brenner C. Crystal structure of the worm NitFhit Rosetta Stone protein reveals a Nit tetramer binding two Fhit dimers. *Current Biol* 2000;10:907–917.
24. Cooper AJL. Asparagine transaminase from rat liver. *J Biol Chem* 1977;252:2032–2038. [PubMed: 14957]
25. Hersh LB. Rat liver ω -amidase. Kinetic evidence for an acyl-enzyme intermediate. *Biochemistry* 1972;11:2251–2256. [PubMed: 5028495]
26. Ramaley RF, Fernald NJ, DeVries T. Dicarboxylate ω -amidase of *Bacillus subtilis* 168: Evidence for a membrane-associated form. *Arch Biochem Biophys* 1972;153:88–94. [PubMed: 4631108]
27. Fernald NJ, Ramaley RF. Purification and properties of dicarboxylate ω -amidase from *Bacillus subtilis* 168 and *Thermus aquaticus* YT-1. *Arch Biochem Biophys* 1972;153:95–104. [PubMed: 4631109]
28. Ronzio RA, Rowe WB, Wilk S, Meister A. Preparation and studies on the characterization of sheep brain glutamine synthetase. *Biochemistry* 1969;8:2670–2674. [PubMed: 5799143]
29. Barglow KT, Saikatendu KS, Bracey MH, Huey R, Morris GM, Olson AJ, Stevens RC, Cravatt BF. Functional proteomics and structural insights into the molecular recognition in the nitrilase family enzymes. *Biochemistry* 2008;47:13514–13523. [PubMed: 19053248]

Fig. 1.
Associated transamination – deamidation of glutamine and asparagine.

Fig. 2. Ping-pong mechanism for the hydroxaminolysis of succinamate. In step 1, the succinamate reacts covalently with an active site cysteine (E-SH) to generate an acyl-enzyme intermediate (thioester) and ammonia. In step 2, nucleophilic attack of hydroxylamine generates succinyl hydroxamate and regenerates the active site cysteine.

Table 1

Purification of ω-Amidase from Rat Liver Cytosol ^a

Step	Volume (ml)	Total protein (mg)	Units – αKGM assay (μmol·min ⁻¹)	Specific activity – αKGM assay (μmol·mg ⁻¹ ·min ⁻¹) A	Recovery (%)	Purification (fold)	Specific activity – hydroxamate assay (μmol·mg ⁻¹ ·min ⁻¹) B	Ratio B/A
1. Cytosol	107	2620	91.9	0.035	100	1.0	0.178	5.1
2. Ammonium Sulfate fractionation and dialysis	30	420	60.9	0.145	66.2	4.1	0.664	4.6
3. DE-52 chromatography (1)	150	113	49.2	0.436	53.6	12.4	2.44	5.6
4. DE-52 chromatography (2) and dialysis	65	3.5	17.4	4.96	18.9	141	31.3	6.3
5. Hydroxylapatite chromatography	8.8	0.11	13.9	126	15.1	3600	570	4.5

^aFrom approximately 25 g of liver.