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Recombinant production of eight human cytosolic aminotransferases and assessment of their potential involvement in glyoxylate metabolism.

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¹ Professor Stefania Petrucco prematurely passed away on February 23, 2009. This paper is dedicated to her memory.

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Abbreviations: PH1, Primary hyperoxaluria type 1; PLP, pyridoxal 5'-phosphate; GO, glycine oxidase. AGXT alanine-glyoxylate aminotransferase isoform 1 (normally peroxisomal); AGXT2 alanine-glyoxylate aminotransferase isoform 2 (mitochondrial). GRHPR, glyoxylate reductase/hydroxypyruvate reductase. LDH, lactate dehydrogenase. Other enzyme acronyms are given in the legend of Table 1. Italicized acronyms refer to the genes; non-italicized acronyms refer to the corresponding proteins.

SYNOPSIS

Primary hyperoxaluria type 1 (PH1) is a severe inborn disorder of glyoxylate metabolism, caused by a functional deficiency of the peroxisomal enzyme alanine:glyoxylate aminotransferase (AGXT), which converts glyoxylate to glycine using L-alanine as the amino-group donor. Even though pre-genomic studies indicate that other human transaminases can convert glyoxylate to glycine, in PH1 patients these enzymes are apparently unable to compensate for the lack of AGXT - perhaps due to their limited levels of expression, or to their localization in an inappropriate cell compartment, or to the scarcity of the required amino-group donor. Herein, we describe the cloning of eight human cytosolic aminotransferases, their recombinant expression as His6-tagged proteins and a comparative study on their ability to transaminate glyoxylate, using any standard amino acid as an amino-group donor. To selectively quantitate the glycine formed, we have developed and validated an assay based on bacterial glycine oxidase (GO); this assay allows the detection of enzymes that produce glycine by transamination, in the presence of mixtures of potential amino-group donors and without separation of the product from the substrates. We show that among the eight enzymes tested only alanine transaminase (GPT) and phosphoserine aminotransferase (PSAT1) can transaminate glyoxylate with good efficiency, using L-glutamate (and, for GPT, also L-alanine) as the best amino-group donor. These findings confirm that glyoxylate transamination can occur in the cytosol, in direct competition with the conversion of glyoxylate to oxalate. The potential implications for the treatment of primary hyperoxaluria are discussed.

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INTRODUCTION

Primary hyperoxaluria type 1 (PH1) is an autosomal-recessive inborn disease occurring in 1 per 120,000 live births [1]. It is caused by a deficiency of the enzyme alanine:glyoxylate aminotransferase (AGXT), which normally localizes to the peroxisomes in hepatocytes [1-3]. AGXT uses pyridoxal phosphate (PLP) as a cofactor [4] and catalyzes the nearly-irreversible transfer of the amino group from alanine to glyoxylate, to yield glycine and pyruvate [5, 6] (Figure 1).

In PH1 patients, glyoxylate cannot be efficiently converted to glycine and is instead oxidized to oxalate, leading to hyperoxalemia and hyperoxaluria. In turn, this causes the deposition of insoluble calcium oxalate in the kidney and in other tissues, leading to nephrolithiasis, nephrocalcinosis, systemic tissue damage and eventually kidney failure [1, 3, 7].

Since the human organism produces a variety of aminotransferases, and since these enzymes often show a broad substrate specificity [8], it is somewhat surprising that other transaminases may not functionally compensate for the deficiency of AGXT. As a matter of fact, another alanine:glyoxylate aminotransferase (known as AGXT2; [9]) is found in mammalian liver, but this enzyme localizes solely into the mitochondria [10] and from this subcellular compartment it cannot surrogate the function of AGXT. Furthermore, several pre-genomic studies suggest that other enzymes occurring in the liver of mammals can convert glyoxylate to glycine employing amino acids other than alanine as cosubstrates. For example, an aminotransferase that apparently prefers glutamate as amino-group donor has been purified from human liver [11]. Another glyoxylate transaminase that uses preferably aromatic amino acids as cosubstrates has been obtained from the liver of rodents and monkeys [12] while an enzyme with glutamine-glyoxylate transaminase activity was isolated from rat liver [13]. All these activities are attributable to PLP-dependent enzymes, but none of these catalysts has ever been characterized at a molecular level, and their subcellular localization is not known.

Today, the availability of the complete human genome sequence offers an opportunity to clarify the role of different aminotransferases in the metabolism of glyoxylate. The genes coding for aminotransferases can be identified based on homology criteria and their overall number is relatively modest [14]. Understanding which of these gene products can use glyoxylate as an amino-group acceptor would integrate the earlier biochemical literature and perhaps suggest new routes to the treatment of primary hyperoxaluria type 1. In fact, should some aminotransferase prove capable of efficiently converting glyoxylate to glycine, then stimulation of this enzyme (by means of drugs, or simply by providing a dietary supplement of the preferred amino-group donor) could represent a viable therapy to AGXT deficiency.

Herein, we report the cloning and recombinant expression of eight cytosolic aminotransferases and a preliminary assessment of their efficiencies and cosubstrate preferences in the transamination of glyoxylate.

EXPERIMENTAL

Enzymes and chemicals

Recombinant glycine oxidase (GO, EC 1.4.2.19) from *Bacillus subtilis* was expressed in *Escherichia coli* and purified as described [15]. The final enzyme preparation showed a specific activity of ~0.7 U/mg protein on glycine as substrate. Rabbit muscle lactate dehydrogenase (LDH) was purchased from Fluka; malate dehydrogenase from pig heart, glutamate dehydrogenase from bovine liver and horseradish peroxidase were from Sigma. All the chemicals were of the highest commercial purity available.

Molecular cloning

A plasmid carrying the AGXT coding sequence (NCBI accession n. X53414) was kindly provided by Prof. C.J. Danpure (University College London). Clones of the complete cDNAs for the other aminotransferases were obtained from ImaGenes (Berlin, Germany). The full-length coding sequences were PCR-amplified from these clones, using the PfuUltra High Fidelity Taq polymerase (Stratagene) or the Deep Vent DNA polymerase (New England Biolabs). The ID of the clones and the gene-specific PCR primers (purchased from MWG Biotech, Ebersberg, Germany) are listed in Table 1. All the primers bore 5'-tails such that the amplification products contained *Cpol* target sequences (uppercase letters) near to both ends.

A schematic description of the molecular cloning strategy is outlined in Figure 2. Amplicons were blunt-end cloned into an intermediate vector (usually pBlueScript II KS+ [Stratagene] treated with *Sma*I) using published protocols [16]. Ligated plasmids were then transformed into XL1B *E. coli* cells (Stratagene) and the inserts were sequence-verified. The plasmids were subsequently treated with *Cpol*, to extract the fragments corresponding to the amplified full-length cDNAs, ready for subcloning into the expression vector pET28-*Cpol*.

This plasmid is a derivative of pET28 (Novagen) modified to present a single *Cpol* restriction site in the cloning region, downstream to a sequence encoding a hexahistidine tag (Angelo Bolchi, unpublished; Supplementary Figure 1). *Cpol* cleaves its target sequence (CGGTCCG/CGGACCG) after the first CG, generating two nonidentical 3' overhangs (GTC on one strand and GAC on the other). Exploiting these features, the *Cpol*-cleaved fragments were cloned in-frame and directionally into pET28-*Cpol*.

Heterologous gene expression and protein purification

The expression vectors carrying the cloned inserts were transformed into BL21-CodonPlus®-RIL (Stratagene) or BL21 Star® (Invitrogen) *E. coli* cells.

The general expression protocol was as follows. A subculture was used to inoculate a flask of broth containing 50 µg/ml kanamycin (for BL21-CodonPlus®-RIL cells, also 170 µg/ml chloramphenicol). Bacteria were grown at 37 °C in vigorous aeration and agitation, until the cell density reached the appropriate value (usually, OD_{600 nm} ~0.6), at which point isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 1 mM. After further growth of the induced culture, cells were harvested by centrifugation and sonicated. Subsequently, the recombinant proteins present in the soluble lysate fraction were purified by immobilized metal affinity chromatography. The protein fractions were analyzed by gel electrophoresis and

those fractions with a purity > 90% were pooled, dialyzed against a buffer containing 4 μ M PLP and 1 mM dithiothreitol, and stored at -80 °C.

Details on (i) the culture conditions adopted for the overexpression of individual proteins, (ii) the specific purification procedures used for each protein and (iii) the overall protein yields are provided in the Supplementary Data.

The concentration of each purified recombinant protein was estimated based on the extinction coefficient at 280 nm calculated by ProtParam (www.expasy.ch/tools/protparam.html). Activity of the recombinant enzymes (when their specific reactions were known) was assessed through established methods (See Supplementary Table 1).

Glycine oxidase (GO) assay

The kinetics of glycine production were determined by a discontinuous assay based on the use of GO. The principle of the assay is illustrated in Supplementary Figure 2. Briefly, glyoxylate (1-10 mM final concentration) and one or more potential amino-group donors were equilibrated at the desired reaction temperature in 25 mM potassium phosphate, pH 7.4 before starting the reaction with addition of the aminotransferase (0.05-3 μ M final concentration).

Aliquots (20 μ l each) were removed at appropriate intervals, and further reaction was stopped by mixing with 2 μ l of 1 M phosphoric acid to reach pH ~2. These samples were then heated at 95 °C for 5 min to completely inactivate the aminotransferase. Each aliquot was subsequently mixed with 178 μ l of a solution containing Tris-HCl pH 8.0 (100 mM final concentration), GO (4 μ M final, corresponding to about 0.03 units), 3 units of horseradish peroxidase and 1 mM *o*-dianisidine, and incubated at 37 °C for 30 min (the time required to reach an endpoint). Finally, the mixtures were supplemented with 50 μ l of sulfuric acid (7 N final concentration) to dissolve the occasional precipitates of oxidized *o*-dianisidine and augment the sensitivity of the assay [17], before measuring absorption spectra in the 530 nm region to quantitate the extent of *o*-dianisidine oxidation.

In parallel, a calibration line was prepared. 2-40 nmoles of glycine (from a 10 mM standard solution) were added to a reaction mixture containing 1 mM *o*-dianisidine, 4 μ M GO, 15 units/ml horseradish peroxidase, 10 mM phosphoric acid and 100 mM Tris-HCl pH 8.0 in a final volume of 200 μ l. The reaction mixtures were incubated and treated as above, and the absorbance at 530 nm vs. the initial amount of glycine was used to generate a calibration line. Control reactions were performed in which glyoxylate and the potential amino-group donors were incubated together, in the absence of transaminases; under these conditions, the amount of spontaneous glyoxylate transamination was negligible.

GO-based assay in a 96-well format

To screen for the ability of recombinant aminotransferases to produce glycine, using a variety of potential amino-group donors, the GO-based assay was also adapted to a 96-well microtiter format.

In each well, a single aminotransferase (1 μ M) was incubated with glyoxylate (4 mM) and one or more potential amino-group donors (5-10 mM each) in a total volume of 40 μ l. After 2.5 hours of incubation at 25 °C, the enzymes were inactivated by adding phosphoric acid to a final concentration of 90 mM (pH~2). Then, the reaction mixtures were supplemented with 180 μ l of Tris-HCl 200 mM (pH 8)

containing glycine oxidase (1 μ M final), peroxidase (8 units/ml) and dianisidine (1 mM) and stored at 37°C for one hour. Finally 50 μ l of sulfuric acid were added to each well and the plate was scanned with an automated plate reader.

Continuous spectrophotometric assays for alanine-glyoxylate, serine-glyoxylate and glutamate-glyoxylate transaminase activities

Alanine-glyoxylate, serine-glyoxylate and glutamate-glyoxylate transamination reactions were measured using a continuous spectrophotometric assay at 220 nm [18, 19]. At this wavelength, most α -keto acids possess an extinction coefficient about tenfold higher than glyoxylate (Supplementary Figure 3) so that their formation can be easily quantified [18].

Typically the reaction mixture (0.9 ml, final volume) contained 1-150 mM amino acid (L-Glu, L-Ala or L-Ser) and 1-10 mM glyoxylate in 25 mM potassium phosphate at pH 7.5. Reactions were started by adding the recombinant enzyme, and the increase in absorbance was monitored continuously using a Cary 400 double-beam spectrophotometer (Varian). Initial velocities were calculated graphically and expressed as μ moles of keto acid product (e.g., pyruvate) formed per minute, per mg of protein. The kinetic data were analyzed using Sigma Plot (Systat Software Inc.).

The kinetics of the glutamate-glyoxylate transamination (catalyzed by, e.g., the enzyme PSAT1) could also be monitored spectrophotometrically by a coupled assay, in which the recombinant enzyme (0.1-0.5 μ M), glutamate and glyoxylate were incubated in the presence of glutamate dehydrogenase, NH_4^+ and NADH. The reaction mixture (200 μ l final volume) contained 25 mM potassium phosphate (pH 7.5), 32 mM ammonium acetate, 0.25 mM NADH and 15 units/ml glutamate dehydrogenase. The disappearance of NADH was monitored at 340 nm.

RESULTS

Identification, selection and cloning of the aminotransferase genes

An inventory of PLP-dependent enzymes encoded in the human genome [14] reveals 18 genes coding for aminotransferases - eight of which are predicted to localize in mitochondria. However, two arguments suggest that mitochondrial enzymes cannot compensate for the deficiency of peroxisomal AGXT. First, the existence of a mitochondrial AGXT isoenzyme (AGXT2) does not prevent the development of primary hyperoxaluria. Second, it is well known that PH1 is often caused by mutations that mistarget AGXT from peroxisomes to mitochondria [20, 21]. Even though AGXT retains its catalytic function in the mitochondria, it is metabolically ineffective because it is prevented from acting on the peroxisomal glyoxylate pool [21]. Instead, the glyoxylate produced in the peroxisomes can presumably be exported to the cytosol [22]. Thus, we focused in this study on the nine genes predicted to encode cytosolic transaminases, plus AGXT that was used as a positive control. The list of genes is shown in Table 1.

For all these genes, complete cDNAs were available from the IMAGE consortium; when multiple splice variants of a gene product were documented, we chose to clone the variant encoding the longest polypeptide. The coding sequences for the enzymes were then PCR-amplified from the cDNAs, subcloned, sequence-verified and finally cloned into *E. coli* expression vectors (Figure 2).

The enzymes were overexpressed as His6-tagged proteins in *E. coli* cells, and purified by immobilized metal affinity chromatography (see Experimental). In most cases it was possible to obtain a good yield of soluble protein. However, production of the protein corresponding to the gene *GOT1L1* encountered several problems: upon induction, multiple overexpressed protein species were detected in the cell lysate, and none of them could be purified by metal affinity chromatography. We thus decided not to pursue further the purification and characterization of this gene product.

The recombinant transaminases were soluble, could bind the PLP cofactor (as shown by their characteristic spectra; Supplementary Figure 4), reacted with potential substrates and could catalyze their specific reactions (when known) at rates comparable to those reported by other researchers (Supplementary Table 1).

Development and validation of an AGXT assay using glycine oxidase and peroxidase as coupling enzymes

To assess the formation of glycine by the various transaminases, we used a GO/peroxidase coupling system. GO catalyzes the conversion of glycine to glyoxylate and H_2O_2 [15, 23]. The latter is then used by peroxidase to oxidize *o*-dianisidine, yielding an intensely colored product [17] (Supplementary Figure 2).

In principle, the use of GO and peroxidase as indicator enzymes could allow monitoring the on-line formation of Gly by means of a continuous assay. However, in continuous coupled assays, the reaction catalyzed by the indicator enzyme must not be rate-limiting the overall kinetics. This requisite is difficult to meet by GO, which is a rather slow enzyme ($k_{\text{cat}}/K_{\text{M}} \approx 10^3 \text{ M}^{-1}\text{s}^{-1}$, under optimal conditions [24]). Hence we resorted to a discontinuous method, which, despite being more cumbersome, is not limited by the rate of the coupled reaction, affords an improved reliability of the assay and provides a much greater flexibility in terms of kinetic conditions that can be explored.

The details of the GO-based assay are given in the Experimental section. For comparison, the rates for glycine formation by recombinant AGXT were determined through this assay and through a continuous assay based on the absorbance change at 220 nm (α -keto acids production) [18, 19], using the same buffer and substrate concentrations (Table 2). The rates obtained through the two methods were virtually identical, validating the use of the GO assay for measuring the kinetics of enzymatic glycine production.

Screening of the recombinant aminotransferases for their ability to use glyoxylate as an amino group acceptor

The GO-based assay was adapted for use in a 96-well microtiter format (see Experimental). In fact, we used this plate assay to screen for the ability of the recombinant enzymes to transaminate glyoxylate, using the standard amino acids as amino-group donors (Figure 3).

Besides AGXT (that was included as the positive control) only two aminotransferases appeared able to produce glycine from glyoxylate to an appreciable extent – namely alanine aminotransferase (GPT) and phosphoserine transaminase (PSAT1). The activity of GPT towards glyoxylate was consistent with the results of pre-genomic studies on the rat liver enzyme [25, 26] and, not unexpectedly, L-Ala and L-Glu were by far the best amino-group donors (Figure 3).

On the other hand PSAT1 could use several different amino acid co-substrates. These included acidic amino acids (L-Glu and L-Asp, but also L-homocysteate) and small neutral amino acids such as L-Ala and L-Ser (Figure 3 and data not shown). A series of control kinetic experiments showed that L-Glu was the preferred amino-group donor both at 25°C (Table 3 and data not shown) and at 37°C (Supplementary Table 2), consistent with the notion that phosphoserine transaminase uses L-Glu as its standard cosubstrate [27]. In spite of the enzyme's name, phosphoserine was not an efficient amino-group donor (not shown).

A surprising result of the plate assay was the apparent inability of CCBL1 (also known as glutamine transaminase K) to act on glyoxylate. This was in contrast with a previous study by Han et al. [28], where the same enzyme was reported to employ quite efficiently glyoxylate as an amino-group acceptor. Nevertheless, the reaction conditions in that earlier study were significantly different from those of our plate assay (45°C, higher ionic strength and the nonstandard amino acid L-kynurenine used as the amino-group donor). More importantly, the reaction rates estimated in that earlier study were based on a single time-point, collected 10 min after starting the reaction [28]. When we performed specific kinetic assays on the reaction between phenylalanine, glutamine or methionine and glyoxylate, catalyzed by CCBL1, we observed modest initial transamination rates that declined after a few minutes, coming to a near complete stop well before completion of the reaction (Table 3 and Supplementary Figure 5A). Control experiments showed that the enzyme was not undergoing irreversible inactivation, but rather that it was being strongly inhibited by the keto acid products of the reaction (Supplementary Figure 5B and data not shown).

Reactions of GPT and PSAT1 with glyoxylate

We investigated in more detail the activity of GPT and PSAT1 towards glyoxylate. To this end, we measured the reaction rates of the two enzymes as a function of glyoxylate concentration, either in the presence of L-Ala (for GPT only; Figure 4A) or L-Glu (for both enzymes; Figure 4B-C). In these experiments the reaction conditions were chosen to approximate the intracellular environment (potassium phosphate buffer, pH 7.4, 37°C) and even the concentrations of amino-group donors employed (1 to 5-10 mM) represented rough lower and upper limits for the cytosolic concentration of L-Ala and L-Glu [29, 30].

GPT transaminated glyoxylate with higher efficiency. Reaction rates changed little when the concentration of L-Ala or L-Glu was raised from 1 to 5 mM, implying that GPT was essentially saturated by the amino-group donors at 1 mM concentrations. The apparent specificity constant, $k_{\text{cat}}/K_{\text{M}}^{\text{Glyoxylate}}$, fell in all cases in the range 6000-10000 $\text{M}^{-1} \text{s}^{-1}$ (as it can be calculated from the data in Figure 4A-B, based on a molecular mass of 57 kDa for recombinant GPT).

PSAT1 was comparatively less efficient, even though its maximum activity (3.5 $\mu\text{moles min}^{-1} \text{mg}^{-1}$, corresponding to a turnover number of about 2.5 s^{-1}) was just fivefold less than the activity of PSAT1 toward its 'physiological' substrate couple, glutamate-phosphohydroxypyruvate. The apparent specificity constant doubled (from 900 to 1800 $\text{M}^{-1} \text{s}^{-1}$) when L-Glu was raised from 1 to 10 mM, suggesting that PSAT1 was not fully saturated at the lowest L-Glu concentration tested. However, a further increase in glutamate concentration (to 20 mM) slightly depressed the apparent $k_{\text{cat}}/K_{\text{M}}^{\text{Glyoxylate}}$, possibly due to substrate inhibition (not shown).

DISCUSSION

We have expressed in *E. coli* eight cytosolic aminotransferases, plus the peroxisomal alanine:glyoxylate aminotransferase. For some of these human enzymes, overproduction by recombinant means had already been described [28, 32-35]. For others (GOT1, AGXT2L1 and AGXT2L2) this is the first report of cloning and recombinant expression in bacteria. In the long term, the recombinant expression of all human transaminases will allow the production of enzyme arrays useful for a synoptic analysis of the respective contributions to metabolic pathways, for quick comparisons of substrate specificities and for the screening of selective inhibitors. Furthermore, aminotransferases are also important tools in biocatalysis, e.g. for the deracemization of natural and non-proteinogenic α -amino acids [36].

An assay for glycine-forming transaminases

The specific goal of this study was the identification of aminotransferases that use glyoxylate as a substrate. To this end, we have devised a method for detecting the formation of glycine – a method whereby multiple potential amino-group donors can be tested simultaneously and where separation of the products from the substrates is not required. In fact, while GO is active on glycine and on a few D-amino acids, as well as on some primary and secondary amines (such as sarcosine), L-amino acids are neither good substrates nor inhibitors of this flavoenzyme [15].

In the past, several assays have been used to monitor the reactions catalyzed by transaminases that use glyoxylate [37]. Early methods were based (like the GO assay we developed) on the quantitation of the glycine produced [38]. However, these techniques were laborious and often unreliable, so that most researchers turned instead to assays for the quantitation of the keto acid co-product (e.g., pyruvate for the AGXT reaction) [37].

The most accurate and sensitive of these methods entail the derivatization of the keto acid product with some phenylhydrazine dye, followed by HPLC separation and quantitation (e.g., see [6]). These assays however require specific equipment and instrumental setup, and are difficult to apply to large-scale screenings.

The continuous assays based on the differential absorbance of glyoxylate versus other keto acids at ~ 220 nm are simple and reasonably accurate, provided that the extinction coefficient of the specific keto acid product is known [18]. A major limit of the method is the need to work at a wavelength where most substances absorb, thus affecting the sensitivity of the assay.

Finally, continuous coupled assays based on dehydrogenases (e.g., malate dehydrogenase to monitor the formation of oxaloacetate) are very convenient to study the kinetics of specific transamination reactions and can be easily employed in large-scale screenings. However, these assays are not general, since detection of different keto acid products requires the use of different coupling enzymes. Additionally, some dehydrogenases can use glyoxylate as a substrate, complicating the kinetic analysis [39].

Cytosolic transaminases that act on glyoxylate

The glycine oxidase assay could be adapted to a 96-well format, and was used to screen our set of recombinant aminotransferases (as well as libraries of potential amino-group donors). Notably, only PSAT1 and GPT, out of the eight enzymes tested, could transaminate glyoxylate to glycine with substantial efficiency.

The two homologs of the mitochondrial AGXT (AGXT2L1 and AGXT2L2) would not produce glycine using any standard amino acid as a co-substrate. Preliminary results suggest that these proteins are indeed transaminases, but they appear to strongly prefer pyruvate as the amino group acceptor. Studies are underway to elucidate the reaction preferences of these enzymes.

To our knowledge, the activity of PSAT1 on glyoxylate has never been described before. The observation that the most efficient amino-group donor for this enzyme was L-Glu, led us to consider the identification of PSAT1 with a glutamate-glyoxylate transaminase partially purified from human liver by Thompson and Richardson, over 40 years ago [11]. The enzyme studied by those authors reportedly showed a $K_M^{\text{Glyoxylate}} = 2 \text{ mM}$ [11], similar to the one observed by us with PSAT1 (Figure 4C). However, the enzyme from human liver was unable to use L-Ser as an amino-group donor, while it was very active with L-Ala [11]. Based on these considerations, it is most likely that the enzyme partially purified by Thompson and Richardson was GPT.

Implications for glyoxylate metabolism

The results of this paper help integrate the current view of the cytosolic metabolism of glyoxylate. It is well appreciated that, in the hepatocyte cytosol, glyoxylate can be oxidized to oxalate by lactic dehydrogenase (LDH) or reduced to glycolate by glyoxylate reductase/hydroxypyruvate reductase (GRHPR) [40, 41]. Oxalate is a dead-end product of the metabolism, as it can only be excreted and eliminated, mainly through the kidney. Glycolate can also be excreted; otherwise, it may enter the peroxisomes and be reconverted to glyoxylate [22], which would establish a sort of futile cycle.

We have confirmed a third fate for cytosolic glyoxylate, namely its conversion to glycine, carried out by GPT and PSAT1 (and, to a much lesser extent, by CCBL1) (Figure 5). This transamination reaction is virtually irreversible [6, 11], and has the advantage of producing a substance that the cell can readily incorporate into proteins or utilize in many biosynthetic pathways.

The actual importance of glyoxylate transamination, relative to the first two metabolic routes, remains to be established. The apparent $k_{\text{cat}}/K_M^{\text{Glyoxylate}}$ values (mirroring catalytic performance at low glyoxylate concentrations) for GPT and PSAT1 fall in the 10^3 - $10^4 \text{ M}^{-1} \text{ s}^{-1}$ range (Figure 4), which is lower than the values reported for LDH (3×10^4 to $10^5 \text{ M}^{-1} \text{ s}^{-1}$) and GRHPR (6×10^4 to $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) [40]. *In vivo*, however, the partitioning of cytosolic glyoxylate between oxidation, reduction and transamination will depend on several factors that may outweigh the intrinsic efficiencies of the individual catalysts. Such factors include the availability of specific coenzymes and cosubstrates (e.g., NADPH, PLP, L-Glu), the presence of inhibitors (e.g., keto acids that compete with glyoxylate) and above all the cytosolic concentration of the different enzymes, which in turn will depend on their relative expression levels.

Potential implications for the treatment of hyperoxaluria

It is believed that the excess oxalate produced in PH1 is formed mostly in the hepatocyte cytosol, *via* the LDH-catalyzed reaction [2, 42]. This implies that cytosolic transaminases are inefficient at competing with LDH for the common substrate, glyoxylate, released by the peroxisomes. However if expression of the genes *GPT*

and *PSAT1* were increased substantially above normal, this should steer more and more glyoxylate toward transamination – perhaps enough to significantly diminish oxalate production and the symptoms that ensue.

Are there practical ways to stimulate the expression of *GPT* and *PSAT1*, so as to test the above hypothesis? It is known that *GPT* expression is induced by corticosteroids – indeed, the gene promoter includes a glucocorticoid response element – and by prescription drugs such as the hypolipidemic compound fenofibrate [43]. As for *PSAT1*, its expression in hepatocytes seems highly regulated and may be strongly influenced by nutritional factors. For example, a survey of the data available at GEO profiles (<http://www.ncbi.nlm.nih.gov>) suggests that *PSAT1* in the mouse liver is highly induced by a ketogenic regimen (i.e., a diet based on fats and proteins, but low in carbohydrates) [44].

The possibility that off-the-shelf drugs and dietary changes may help manage PH1, while admittedly speculative, seems worth being explored. Since transgenic mice exist which recapitulate the metabolic defect of PH1 [45], it could be relatively straightforward to test whether treatments that augment the expression of *GPT* and *PSAT1* can decrease oxalate excretion in these animal models. Such studies would provide a better understanding of oxalate production *in vivo* and, in the best scenario, offer concrete suggestions for the therapy of genetic hyperoxaluria.

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FIGURE LEGENDS

Fig. 1. The reaction catalyzed by aminotransferases that use glyoxylate as an amino-group acceptor, producing glycine. For AGXT, the second substrate (amino-group donor) is L-alanine and the second product is pyruvate.

Fig. 2. Overview of the general procedure adopted for the molecular cloning and recombinant expression of human aminotransferase genes.

Fig. 3: A plate assay for the ability of recombinant aminotransferases to produce glycine from glyoxylate. Each well contained the indicated amino acids (10 mM each, except for aromatic amino acids that were 5 mM), glyoxylate (4 mM) and the specified transaminase (1 μ M). The transamination reactions were allowed to proceed for 2.5 hours before testing for the formation of glycine (see Experimental).

Fig. 4: Rates of the glyoxylate transaminations catalyzed by GPT and PSAT1, as a function of substrate concentration. Reactions were carried out at 37°C, in 25 mM potassium phosphate, and monitored by following the change in absorbance at 220 nm. Data were fitted to Michaelis-Menten hyperbolas. (A) Reaction of GPT with L-alanine as the amino-group donor. The titrations collected at 1 mM and 5 mM L-Ala yielded similar V_{\max} values (5.7 and 7.2 μ moles $\text{min}^{-1} \text{mg}^{-1}$, respectively) and similar apparent $K_M^{\text{Glyoxylate}}$ values (1 and 0.8 mM, respectively). (B) Reaction of GPT with L-glutamate as the donor. The titrations collected at 1 mM and 5 mM L-Glu were practically undistinguishable. $V_{\max} = 6.7 \mu\text{moles min}^{-1} \text{mg}^{-1}$ and $K_M^{\text{Glyoxylate}} = 0.75 \text{ mM}$. (C) Reaction of PSAT1 with L-glutamate as the amino-group donor. The catalytic parameters were: $V_{\max} = 3.5 \mu\text{moles min}^{-1} \text{mg}^{-1}$ and $K_M^{\text{Glyoxylate}} = 5.5 \text{ mM}$ (1 mM L-Glu); $V_{\max} = 3.6 \mu\text{moles min}^{-1} \text{mg}^{-1}$ and $K_M^{\text{Glyoxylate}} = 2.7 \text{ mM}$ (10 mM L-Glu). The relatively high $K_M^{\text{Glyoxylate}}$ values may mirror the fact that the carbonyl group of glyoxylate is >95% hydrated to the *gem diol* form in neutral aqueous solution [31].

Fig. 5: Metabolic fates available to glyoxylate in the cytosol of hepatocytes. GRHPR indicates glyoxylate reductase/hydroxypyruvate reductase. LDH is lactate dehydrogenase. Examination of the NCBI EST profile database (<http://www.ncbi.nlm.nih.gov/unigene>) suggests that LDH isozymes are normally expressed in the liver at higher levels than GRHPR, GPT or PSAT1.

Table 1: cDNAs corresponding to the aminotransferases cloned in this study

Gene*	EST Clone ID	PCR primers†	Protein size (aa)‡
<i>AGXT</i> §	-	FW: atCGGTCCG <u>at</u> ggcctctcacaagctgctg RE: taCGGACCG <u>tc</u> acagcttcttctggggc	415
<i>AGXT2L1</i>	IMAGE 4797767	FW: atCGGTCCG <u>at</u> gtgagctgtacagtaagc RE: atCGGACCG <u>at</u> cttgctttaaataatgcaaatacag	522
<i>AGXT2L2</i>	IMAGE 5220434	FW: atCGGTCCG <u>at</u> ggccgcagaccagcgc RE: atCGGACCG <u>at</u> ggcagagcagggctggc	473
<i>BCAT1</i>	DKFZp686E12175Q	FW: atCGGTCCG <u>at</u> ggattgcagtaacggatgc RE: taCGGACCG <u>tt</u> cattgtatcctctattttcc	408
<i>CCBL1</i>	IMAGE 4137942	FW: atCGGTCCG <u>at</u> ggccaacagctgcagggc RE: taCGGACCG <u>cca</u> aggcgtgactcaggg	445
<i>GOT1</i>	IMAGE 2820849	FW: atCGGTCCG <u>at</u> ggcaactcogtcagctcttg RE: taCGGACCG <u>tact</u> ggacgggtggtgtttc	436
<i>GOT1L1</i>	IMAGE 5273324	FW: atCGGTCCG <u>at</u> ggccaccctttcagtggtc RE: atCGGACCG <u>ttg</u> caaagactaaagttttattcca	444
<i>GPT</i>	IMAGE 4179699	FW: atCGGTCCG <u>at</u> ggcctcgagcacaggtg RE: taCGGACCG <u>cc</u> agcctggccccagctg	519
<i>PSAT1</i>	IMAGE 3883843	FW: atCGGTCCG <u>at</u> ggacgccccagggcag RE: taCGGACCG <u>ag</u> tatatcctggttaggatgtg	393
<i>TAT</i>	IMAGE 40146565	FW: atCGGTCCG <u>at</u> ggaccatacatgattcag RE: taCGGACCG <u>tc</u> caggagaatggatgcaggc	477

* *AGXT* encodes the peroxisomal alanine-glyoxylate aminotransferase. *AGXT2L1* and *AGXT2L2* encode two homologs (presumably cytosolic) of *AGXT2* (the mitochondrial alanine-glyoxylate aminotransferase). *BCAT1* encodes the cytosolic branched-chain amino acid transaminase. *CCBL1* (official HUGO Gene Nomenclature Committee symbol) encodes a cytosolic, promiscuous enzyme known by different names: glutamine transaminase K, kynurenine aminotransferase I and cysteine-conjugate β -lyase I. *GOT1* encodes the cytosolic aspartate aminotransferase. *GOT1L1* encodes a *GOT1* homolog. *GPT* encodes the cytosolic alanine transaminase. *PSAT1* encodes phosphoserine aminotransferase. *TAT* encodes the cytosolic tyrosine aminotransferase.

† Forward primers were designed to introduce a *CpoI* restriction site (uppercase letters) just before the start codon of the coding sequence (underlined). Reverse primers were usually designed to pair with the 5'-UTR of the cDNAs; however, when such sequence was unknown or too short, the primers were designed to pair with the 5'-end of the coding sequence, including the stop codon (doubly-underlined).

‡ The length reported here refers to the recombinant protein, which includes a 23-aa N-terminal tail encompassing a hexahistidine tag.

§ A vector containing the complete coding sequence of *AGXT* was kindly provided by Dr. C. J. Danpure (University College London).

|| Before subcloning in the expression vector, the cDNA contained in this EST clone was manipulated to remove a small intervening sequence and an internal *CpoI* restriction site.

Table 2: Comparing the reaction rates of AGXT measured through the GO assay or through a continuous spectrophotometric assay (α -keto acids formation)

Glyoxylate (mM)	L-Ala (mM)	L-Ser (mM)	Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	
			GO assay	Abs 220 nm assay
10	2		1.3 \pm 0.2	1.5 \pm 0.3
10	10		7.9 \pm 2.3	6.9 \pm 1.6
10	150		15.9 \pm 1.5	17.8 \pm 0.5
2	10		6.4 \pm 0.2	5.5 \pm 0.4
10		10	0.7 \pm 0.2	0.6 \pm 0.3

Reactions were measured in 25 mM potassium phosphate buffer (pH 7.4) at 25 °C. The reported activity values represent the average of at least two independent determinations.

Table 3: Glycine-forming activities of the recombinant aminotransferases, using selected amino-group donors

Enzyme	Amino-group donor	Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	
		1 mM donor	10 mM donor
AGXT	L-Ala	1	6.8
AGXT2L1	L-Ala	<0.006	<0.006
	β -alanine*	<0.006	<0.006
AGXT2L2	L-Ala	<0.006	<0.006
	β -alanine*	<0.006	<0.006
BCAT1	L-Glu	<0.006	<0.006
	L-Ile	<0.006	<0.006
CCBL1 [†]	L-Gln	(0.13)	(0.17)
	L-Phe	(0.10)	(0.13) [‡]
	L-Met	(0.26)	(0.3)
GOT1	L-Glu	<0.006	<0.006
	L-Asp	<0.006	<0.006
GPT	L-Glu	2.2	2.5
	L-Gln	-	<0.006
	L-Ala	1.7	2.1
PSAT1	L-Glu	1.5	1.1
	L-Gln	-	0.01
	L-Asp	0.02	0.15
	L-Ala	0.02	0.16
	L-Ser	0.03	0.13
TAT	L-Glu	<0.006	<0.006
	L-Tyr	<0.006	<0.006

The concentration of glyoxylate was 4 mM in all cases. Activities were measured in 25 mM potassium phosphate buffer (pH 7.4) at 25°C, using either the continuous assay at 220 nm or the GO-based assay. Upper limits are given for several enzymes that, at a 1 μM concentration, failed to yield a detectable amount of glycine in six hours (i.e., they formed less than 100 μM glycine, the minimum amount measurable in our GO assay setup).

* β -alanine was tested as a potential substrate for AGXT2L1 and AGXT2L2 since the homologous enzyme AGXT2 is known to transaminate this amino acid.

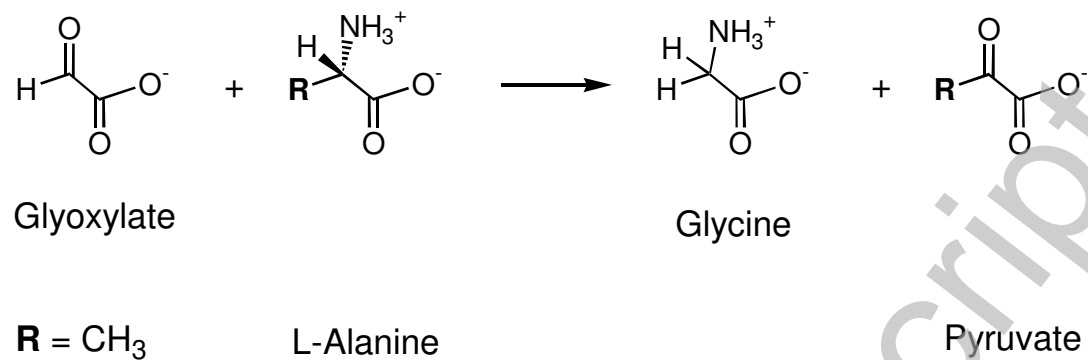
†The activities of CCBL1 (glutamine transaminase K) with L-Phe, L-Gln and L-Met were calculated based on the first few minutes of the kinetics, after which time the reaction rates slowed down substantially. See Supplementary Figure 5.

‡ This reaction rate was measured at 5 mM L-Phe.

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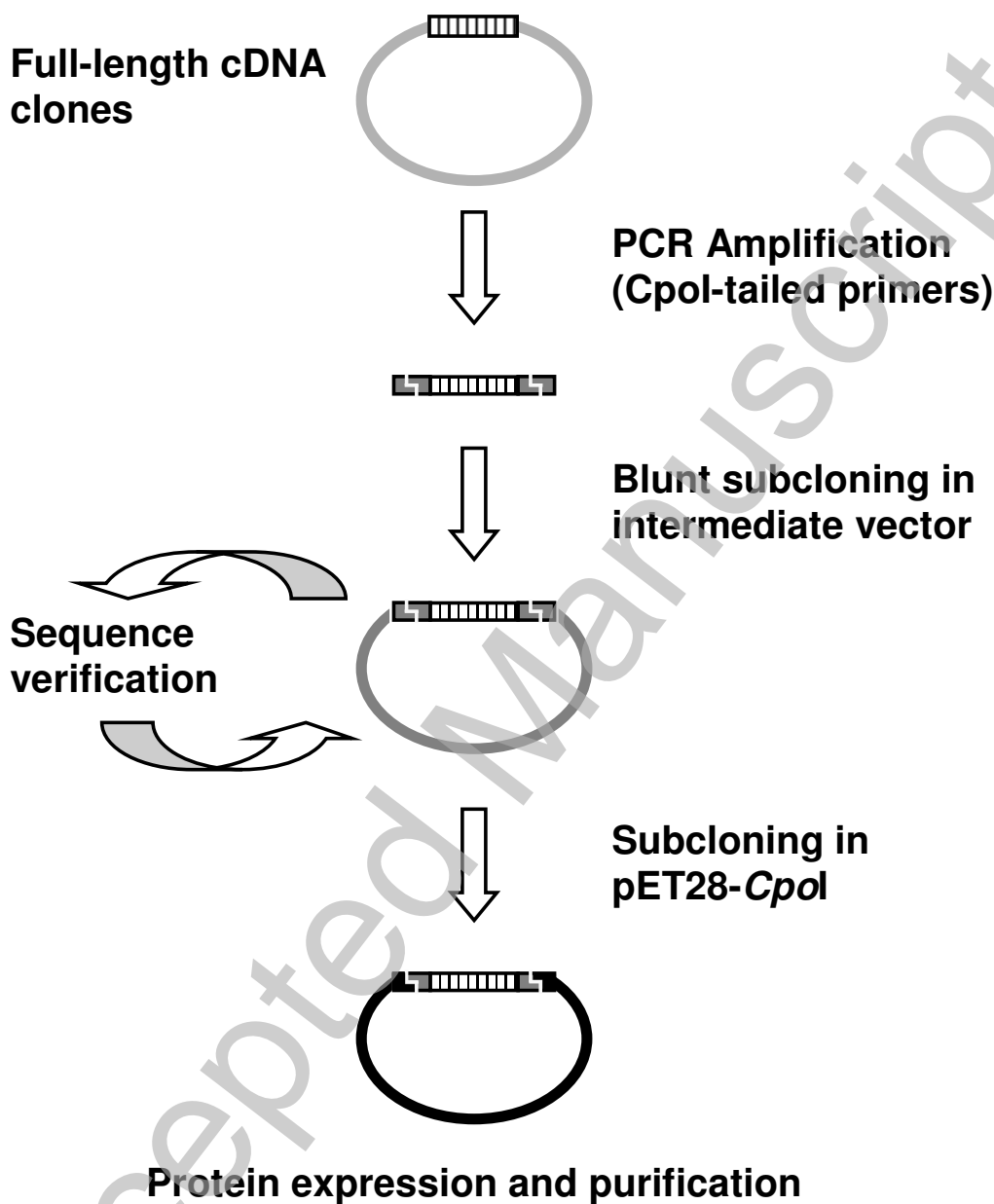
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Figure 1



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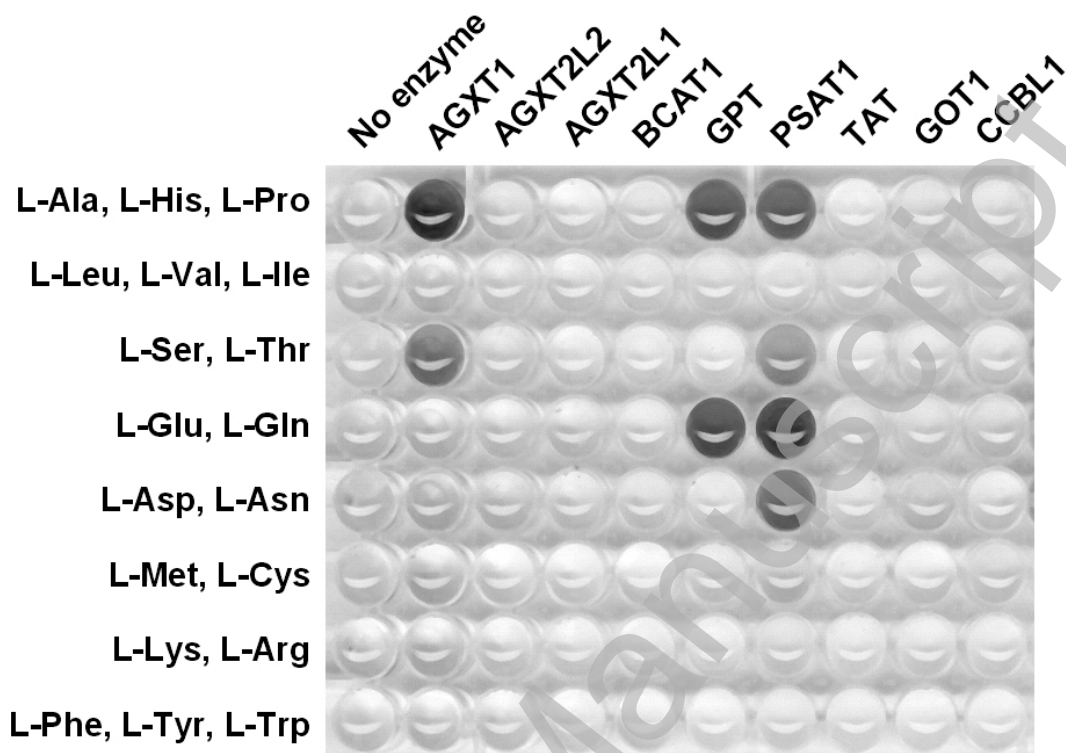
Figure 2



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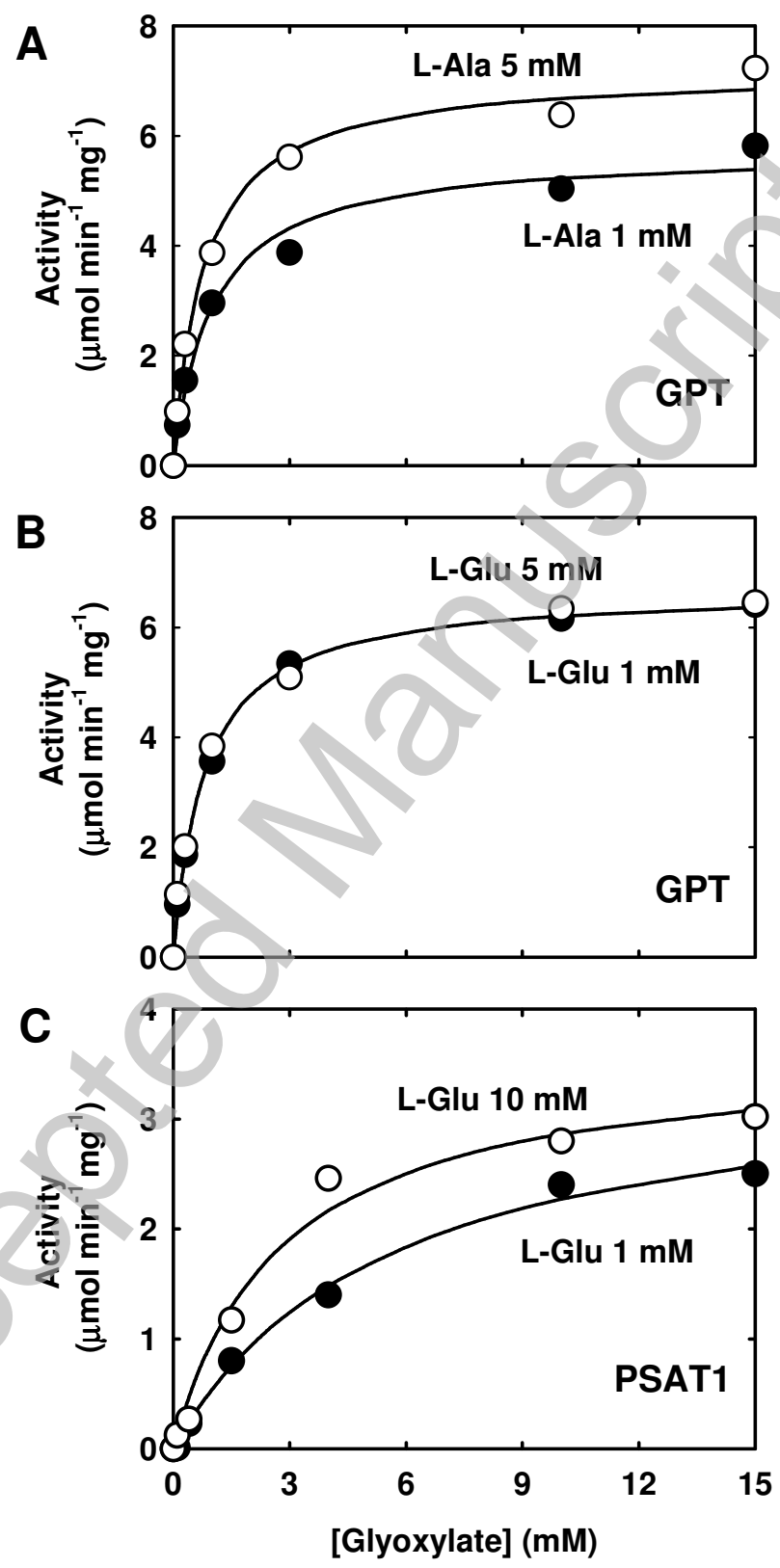
Figure 3



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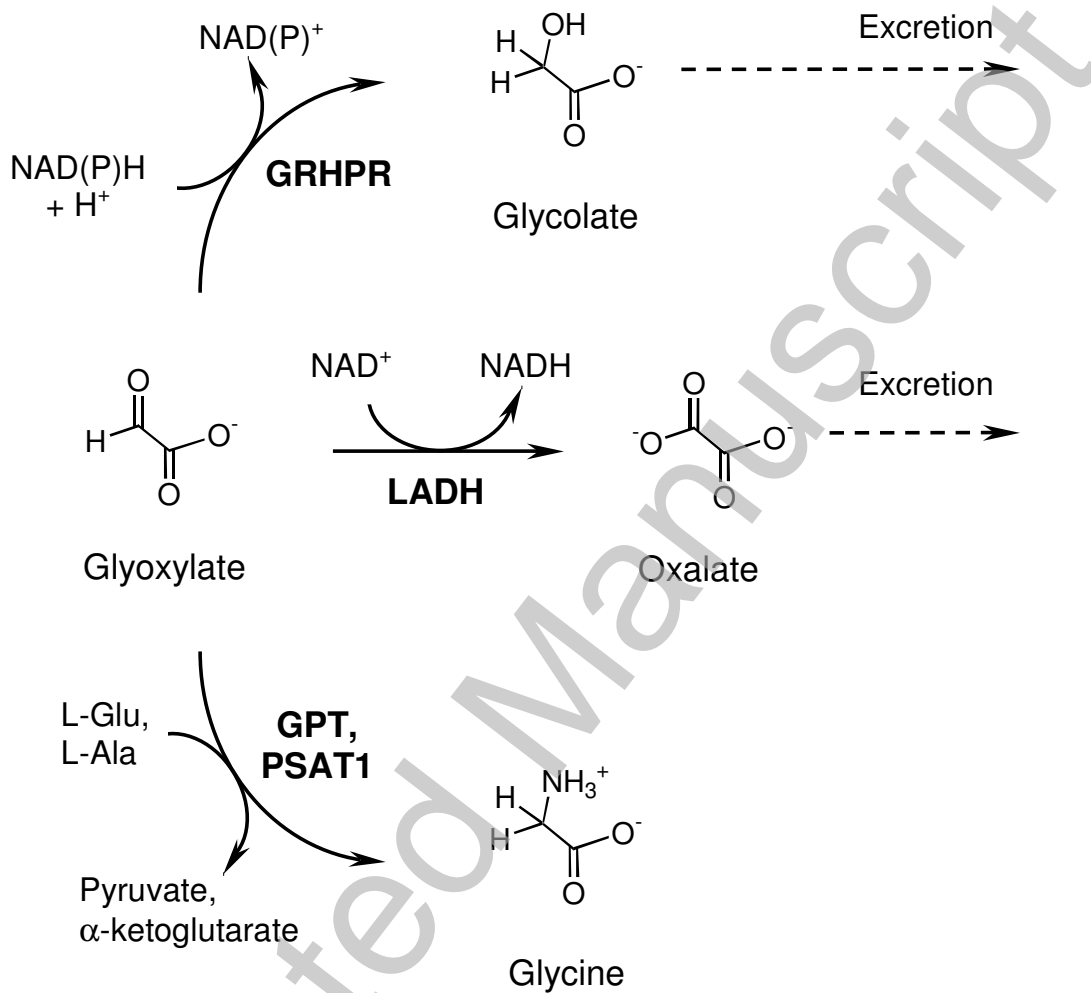
Figure 4



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Figure 5



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