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

Stefano DONINI*, Manuela FERRARI*, Chiara FEDELI*, Marco FAINI*,†, Ilaria LAMBERTO*, Ada Serena MARLETTA*, Lara MELLINI*, Michela PANINI*, Riccardo PERCUDANI*, Loredano POLLEGIONI†, Laura CALDINELLI†, Stefania PETRUCCO*‡ and Alessio PERACCHI*‡

*Department of Biochemistry and Molecular Biology, University of Parma, 43100 Parma, Italy, and †The Protein Factory – Department of Biotechnology and Molecular Sciences, University of Insbruck, 21100 Varese, Italy

INTRODUCTION

PH1 (primary hyperoxaluria type 1) is a severe inborn disorder of glyoxylate metabolism caused by a functional deficiency of the peroxisomal enzyme AGXT (alanine-glyoxylate aminotransferase), which converts glyoxylate into glycine using L-alanine as the amino-group donor. Even though pre-genomic studies indicate that other human transaminases can convert glyoxylate into glycine, in PH1 patients these enzymes are apparently unable to compensate for the lack of AGXT, perhaps due to their limited levels of expression, their localization in an inappropriate cell compartment or the scarcity of the required amino-group donor. In the present paper, we describe the cloning of eight human cytosolic aminotransferases, their recombinant expression as His6-tagged proteins and a comparative study on their ability to transamine glyoxylate, using any standard amino acid as an amino-group donor. To selectively quantify the glycine formed, we have developed and validated an assay based on bacterial GO (glycine oxidase); 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 GPT (alanine transaminase) and PSAT1 (phosphoserine aminotransferase 1) can transamine 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 into oxalate. The potential implications for the treatment of primary hyperoxaluria are discussed.

Key words: alanine-glyoxylate aminotransferase, glyoxylate, hyperoxaluria, pyridoxal 5'-phosphate (PLP), transaminase.

Abbreviations used: AGXT, alanine-glyoxylate aminotransferase; CCBL1, glutamine transaminase K; GO, glycine oxidase; GOT1, glutamic-oxaloacetic transaminase 1; GPT, alanine transaminase; GRHPR, glyoxylate reductase/hydroxyproline reductase; LDH, lactate dehydrogenase; PH1, primary hyperoxaluria type 1; PLP, pyridoxal 5'-phosphate; PSAT, phosphoserine aminotransferase.

1 Present address: European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany.
2 Professor Stefania Petrucco prematurely passed away on 23 February 2009. This paper is dedicated to her memory.
3 To whom correspondence should be addressed (email peracchi@unipr.it).
Figure 1: The reaction catalysed 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.

A supplement of the preferred amino-group donor) could represent a viable therapy to AGXT deficiency.

In the present paper, we report the cloning and recombinant expression of eight cytosolic aminotransferases and a preliminary assessment of their efficiencies and co-substrate preferences in the transamination of glyoxylate.

EXPERIMENTAL

Materials

Recombinant GO (glycine oxidase; EC 1.4.2.19) from Bacillus subtilis was expressed in Escherichia coli and purified as described in [15]. The final enzyme preparation showed a specific activity of ~0.7 unit/mg of protein on glycine as substrate. Rabbit muscle LDH (lactate dehydrogenase) 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 number X53414) was provided by Professor C.J. Danpure (Department of Genetics, Evolution and Environment, University College London, London, U.K.). 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) were subsequently treated with CpoI to extract the fragments corresponding to the amplified full-length cDNAs, ready for subcloning into the expression vector pET28-CpoI.

This plasmid is a derivative of pET28 (Novagen), modified to present a single CpoI restriction site in the cloning region downstream of a sequence encoding a His tag (A. Bolchi, personal communication; Supplementary Figure S1 at http://www.BiochemJ.org/bj/422/bj4220265add.htm). CpoI cleaves its target sequence (CGGTCCG/CGGACCG) after the first CG.

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

See the Experimental section for further details.

Generating two nonidentical 3′ overhangs (GTC on one strand and GAC on the other). Exploiting these features, the CpoI-cleaved fragments were cloned in-frame and directionally into pET28-CpoI.

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 (plus 170 μg/ml chloramphenicol) for BL21-CodonPlus®-RIL cells. Bacteria were grown at 37°C with vigorous aeration and agitation, until the cell density reached the appropriate value (usually D600 ~0.6), at which point isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM. After further growth of the induced culture, cells were harvested by centrifugation (10,000 g for 10 min) and sonicated (30×20 s bursts). Subsequently, the recombinant proteins present in the soluble lysate fraction were purified by immobilized metal-affinity chromatography. The protein fractions were analysed by gel electrophoresis and those fractions with a purity > 90% were pooled, dialysed against a buffer containing 4 μM PLP and 1 mM dithiothreitol, and stored at −80°C.

Details of (i) the culture conditions adopted for the over-expressing of individual proteins, (ii) the specific purification procedures used for each protein, and (iii) the overall protein yields are provided in the Supplementary Online Data (at http://www.BiochemJ.org/bj/422/bj4220265add.htm).

The concentration of each purified recombinant protein was estimated based on the absorption coefficient at 280 nm calculated by ProtParam (http://www.expasy.org/tools/protparam.html). Activity of the recombinant enzymes (when their specific reactions were known) was assessed through established methods (see Supplementary Table S1 at http://www.BiochemJ.org/bj/422/bj4220265add.htm).
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 S2 (at http://www.BiochemJ.org/bj/422/bj4220265add.htm). 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 μM final concentration). Aliquots (20 μl each) were removed at appropriate intervals, and further reaction was stopped by mixing with 2 μl 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 μl of a solution containing Tris/HCl, pH 8.0 (100 mM final concentration), GO (4 μM final concentration, corresponding to approx. 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 end-point). Finally, the mixtures were supplemented with 50 μl of sulfuric acid (3.5 M 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 quantify the extent of o-dianisidine oxidation.

In parallel, a calibration line was prepared. Glycine (2–40 nmol from a 10 mM standard solution) was added to a reaction mixture containing 1 mM o-dianisidine, 4 μM GO, 15 units/ml horseradish peroxidase, 10 mM phosphoric acid and 100 mM Tris/HCl, pH 8.0, in a final volume of 200 μl. The reaction mixtures were incubated and treated as above, and the absorbance at 530 nm was plotted against the initial amount of glycine 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 microtitre format. In each well, a single aminotransferase (1 μM) was incubated with glyoxylate (4 mM) and one or more potential amino-group donors (5–10 mM each) in a total volume of 90 mM (pH ∼2). Then, the reaction mixtures were supplemented with 180 μl of 200 mM Tris/HCl, pH 8, containing glycine oxidase (1 μM final concentration), peroxidase (8 units/ml) and dianisidine (1 mM) and stored at 37 °C for 1 h. Finally, 50 μl of sulfuric acid was 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 absorption coefficient approx. 10-fold higher than that of glyoxylate (Supplementary Figure S3 at http://www.BiochemJ.org/bj/422/bj4220265add.htm) so that their formation can be easily quantified [18]. Typically, the reaction...
mixture (final volume 0.9 ml) 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 μmol of keto acid product (e.g. pyruvate) formed per min per mg of protein. The kinetic data were analysed using Sigma Plot (Systat Software).

The kinetics of the glutamate-glyoxylate transamination [catalysed, for example, by e.g. the enzyme PSAT1 (phosphoserine aminotransferase 1)] could also be monitored spectrophotometrically by a coupled assay, in which the recombinant enzyme (0.1–0.5 μM), glutamate and glyoxylate were incubated in the presence of glutamate dehydrogenase, NH₄⁺ and NADH. The reaction mixture (200 μl final volume) contained 25 mM potassium phosphate, pH 7.5, 32 mM ammonium acetate, 0.25 mM NADH and 15 units/ml of 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 to mitochondria. However, two arguments suggest that the 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. Secondly, 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 the present study on the nine genes predicted to encode cytosolic transaminases, plus AGXT, which was used as a positive control. The list of genes is shown in Table 1. For all of 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 the Experimental section). In most cases, it was possible to obtain a good yield of soluble protein. However, the production of the protein corresponding to the gene GOT1LI [GOT1 (glutamic-oxaloacetic transaminase 1)-like 1] 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 other recombinant transaminases were soluble, could bind the PLP cofactor (as shown by their characteristic spectra; see Supplementary Figure S4 at http://www.BiochemJ.org/bj/422/bj4220265add.htm), reacted with potential substrates and could catalyse their specific reactions (when known) at rates comparable with those reported by other researchers (Supplementary Table S1).

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

<table>
<thead>
<tr>
<th>Glyoxylate (mM)</th>
<th>L-Ala (mM)</th>
<th>L-Ser (mM)</th>
<th>Activity (μmol·min⁻¹·mg⁻¹)</th>
<th>GO assay</th>
<th>A_220 assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td></td>
<td>1.3±0.2</td>
<td>1.5±0.3</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td></td>
<td>7.9±2.3</td>
<td>6.9±1.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>150</td>
<td></td>
<td>15.9±1.5</td>
<td>17.8±0.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td></td>
<td>6.4±0.2</td>
<td>5.5±0.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td></td>
<td>0.7±0.2</td>
<td>0.8±0.3</td>
<td></td>
</tr>
</tbody>
</table>

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

To assess the formation of glycine by the various transaminases, we used a GO–peroxidase coupling system. GO catalyses the conversion of glycine into glyoxylate and H₂O₂ [15,23]. The latter is then used by the peroxidase into oxidize o-dianisidine, yielding an intensely coloured product [17] (Supplementary Figure S2).

In principle, the use of GO and peroxidase as indicator enzymes could allow monitoring of glycine formation in real time by means of a continuous assay. However, in continuous coupled assays, the reaction catalysed by the indicator enzyme must not be rate-limiting to the overall kinetics. This requisite is difficult to meet with GO, which is a rather slow enzyme (kcat/Km ≈ 10³ M⁻¹·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 the 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-based 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 microtitre format (see the Experimental section). 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 (which was included as the positive control), only two aminotransferases appeared able to produce glycine from glyoxylate to an appreciable extent, namely GPT (alanine aminotransferase) and PSAT1 (phosphoserine transaminase). 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,
Reactions were allowed to proceed for 2.5 h before testing for the formation of glycine (see the and even the concentrations of amino-group donors employed not shown) and at 37°C in the cellular environment (potassium phosphate buffer, pH 7.4, 37°C). The reaction conditions were chosen to approximate the intra-cellular reaction conditions 6000–10000 M⁻¹·s⁻¹.

such as L-Ala and L-Ser (Figure 3 and results 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 results not shown) and at 37°C (Supplementary Table S2 at http://www.BiochemJ.org/bj/422/bj4220265add.htm), consistent with the notion that phosphoserine transaminase uses L-Glu as its standard co-substrate [27]. In spite of the enzyme’s name, phosphoserine was not an efficient amino-group donor (results not shown).

A surprising result of the plate assay was the apparent inability of CCBL1 (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 quite efficiently employ 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 non-standard amino acid L-kyurenine 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, catalysed 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 S5A at http://www.BiochemJ.org/bj/422/bj4220265add.htm). 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 S5B, and results 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; Figures 4B–4C). 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 (from 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, $k_{cat}/K_{m}^{glyoxylate}$, fell in all cases in the range 6000–10000 M⁻¹·s⁻¹ (as it can be calculated from the data in Figures 4A and 4B, based on a molecular mass of 57 kDa for recombinant GPT).

PSAT1 was comparatively less efficient, even though its maximum activity (3.5 μmol·min⁻¹·mg⁻¹, corresponding to a turnover number of approx. 2.5 s⁻¹) was just 5-fold less than the activity of PSAT1 towards its ‘physiological’ substrate couple, glutamate–phosphohydroxypyruvate. The apparent specificity constant doubled (from 900 to 1800 M⁻¹·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 $k_{cat}/K_{m}^{glyoxylate}$, possibly due to substrate inhibition (results not shown).

**DISCUSSION**
We have expressed in *E. coli* eight cytosolic aminotransferases, plus the peroxisomal AGXT. 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...
Reactions were carried out at 37°C, in 25 mM potassium phosphate, and monitored by following the change in absorbance at 220 nm. Results 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 $V_{\text{max}}$ values (5.7 and 7.2 μmol·min$^{-1}·$mg$^{-1}$ respectively) and similar apparent $K_{\text{Glyoxylate}}$ 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 indistinguishable. $V_{\text{max}} = 6.7 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_{\text{Glyoxylate}} = 0.75 \text{mM}$. (C) Reaction of PSAT1 with L-glutamate as the amino-group donor. The catalytic parameters were: $V_{\text{max}} = 3.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_{\text{Glyoxylate}} = 5.5 \text{mM}$ (1 mM L-Glu), $V_{\text{max}} = 3.6 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_{\text{Glyoxylate}} = 2.7 \text{mM}$ (10 mM L-Glu). The relatively high $K_{\text{Glyoxylate}}$ values may mirror the fact that the carbonyl group of glyoxylate is > 95% hydrated to the gem-diol form in neutral aqueous solution [31].

Figure 4 Rates of the glyoxylate transaminations catalysed by GPT and PSAT1, as a function of substrate concentration

Cytosolic transaminases that act on glyoxylate

The GO-based 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 homologues of the mitochondrial AGXT (AGXT2L1 and AGXT2L2) could 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 that was partially purified from human liver by Thompson and Richardson [11] over 40 years ago. The enzyme studied by those authors reportedly showed a $K_{m}$ of 2 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, whereas it was very active with L-Ala [11]. On the basis of these considerations, it is most likely that the enzyme partially purified by Thompson and Richardson [11] was GPT.

Implications for glyoxylate metabolism

The results of the present study help integrate the current view of the cytosolic metabolism of glyoxylate. It is well appreciated from the substrates is not required. In fact, although 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 catalysed by transaminases that use glyoxylate [37]. Early methods were based (similarly to the GO-based assay we developed) on the quantification of the glycine produced [38]. However, these techniques were laborious and often unreliable, so that most researchers turned instead to assays for the quantification of the keto acid co-products (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 phenylhydrazine dye, followed by HPLC separation and quantification (e.g. see [6]). These assays, however, require specific equipment and instrumental set up, and are difficult to apply to large-scale screenings.

The continuous assays based on the differential absorbance of glyoxylate compared with other keto acids at ~220 nm are simple and reasonably accurate, provided that the absorption coefficient of the specific keto acid product is known [18]. A major limitation 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].

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 the present 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
that, in the hepatic cytosol, glyoxylate can be oxidized to oxalate by LDH or reduced to glycolate by GRHPR (glyoxylate reductase/hydroxyxpyruvate reductase) [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 into glyoxylate [22], which would establish a futile cycle.

We have confirmed a third fate for cytosolic glyoxylate, namely its conversion into 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 $k_{cat}/K_m^{glyoxylate}$ values (mirroring catalytic performance at low glyoxylate concentrations) for GPT and PSAT1 fall in the $10^{-5}$–$10^3$ M$^{-1}$·s$^{-1}$ range (Figure 4), which is lower than the values reported for LDH ($3 \times 10^{-9}$ to $10^8$ M$^{-1}$·s$^{-1}$) and GRHPR ($6 \times 10^4$ to $1.1 \times 10^5$ M$^{-1}$·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 co-substrates (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-catalysed 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 towards 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 hypolipidaemic 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, although admittedly speculative, seems worth being explored. Since transgenic mice exist that 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.

**REFERENCES**

SUPPLEMENTARY ONLINE DATA

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

Stefano DONINI*, Manuela FERRARI*, Chiara FEDELI*, Marco FAINI*, Ilaria LAMBERTO*, Ada Serena MARLETTA*, Lara MELLINI*, Michela PANINI*, Riccardo PERCUDANI*, Loredano POLLEGIONI†, Laura CALDINELLI†, Stefania PETRUCCO* and Alessio PERACCHI*†

*Department of Biochemistry and Molecular Biology, University of Parma, 43100 Parma, Italy, and †The Protein Factory – Department of Biotechnology and Molecular Sciences, University of Insubria, 21100 Varese, Italy

EXPERIMENTAL

Expression and purification conditions for individual aminotransferases

Buffers used

Buffer A: 5% glycerol, 300 mM NaCl and 50 mM sodium phosphate, pH 7.0. Buffer B: 300 mM NaCl and 50 mM sodium phosphate, pH 8.0. Buffer C: 10% glycerol, 100 mM NaCl and 50 mM Tris/HCl, pH 8.0. Buffer D: 300 mM NaCl and 25 mM Tris/HCl, pH 8.0.

AGXT

AGXT was expressed in E. coli BL21-CodonPlus®-RIL cells (Stratagene). Bacteria were grown in 1 litre of M9-glucose medium (plus antibiotics) at 37°C, until the D600 reached ~0.6. At that point, the culture was induced with IPTG (isopropyl β-D-thiogalactoside; 1 mM) and grown for a further 3 h at 30°C. Cells were then collected by centrifugation and resuspended in 100 ml of Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors (1 μM leupeptin, 1 μM pepstatin, 0.5 mM benzamidine and 0.5 mM PMSF).

After sonication, the soluble lysate fraction was loaded on to a His-Select nickel-affinity resin (Sigma), equilibrated in the same buffer as above, and the recombinant His6-tagged protein was purified following the manufacturer’s instructions. The final yield of purified AGXT was ~2.5 mg per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 2 μM PLP and 1 mM dithiothreitol, concentrated by ultrafiltration and stored at ~80°C.

AGXT2L1

AGXT2L1 was expressed in E. coli BL21-CellStar cells (Invitrogen). The cells were grown at 37°C in 1 litre of LB (Luria–Bertani) broth, supplemented with 50 mg/l of kanamycin, until the D600 ~0.6. After induction with IPTG, the bacteria were cultured for 72 h at cold-room temperature (4–8°C). Cells were then centrifuged, resuspended in 100 ml of Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors and sonicated.

The soluble lysate fraction was loaded on to a Talon cobalt-affinity resin (Clontech) equilibrated in the same buffer as above, and the recombinant protein was purified following the manufacturer’s instructions. The final yield of purified AGXT2L1 was ~14 mg per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 4 μM PLP and 1 mM DTT (dithiothreitol), concentrated by ultrafiltration and stored at ~80°C.

AGXT2L2

AGXT2L2 was expressed in E. coli BL21-Star cells. These were grown in 1 litre of M9-glucose medium (plus kanamycin) at 37°C, until the D600 reached ~0.6. After induction with IPTG, the culture was continued for 3 days at cold-room temperature. Cells were then centrifuged and resuspended in 100 ml of Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors.

After sonication, the recombinant protein was purified from the soluble lysate fraction using a Talon metal-affinity resin (equilibrated in the same buffer as above). The final yield was ~3 mg of purified AGXT2L2 per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 4 mM PLP and 1 mM DTT, concentrated by ultrafiltration and stored at ~80°C.

BCAT1

BCAT1 was expressed in E. coli BL21-CodonPlus®-RIL cells. The cells were grown in 1 litre of LB medium (plus antibiotics) at 37°C, until the D600 reached ~0.6. After induction with IPTG, cells were grown for three more hours at 37°C, centrifuged and resuspended in Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors.

Bacteria were then sonicated and the soluble lysate fraction was loaded on to a Talon cobalt-affinity resin equilibrated in the same buffer as above, and the recombinant His6-tagged BCAT1 was purified following the manufacturer’s instructions. The final yield of purified protein was ~2 mg per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 4 μM PLP and 1 mM DTT, concentrated by ultrafiltration and stored at ~80°C.

CCBL1

CCBL1 was expressed in E. coli BL21-CodonPlus®-RIL cells. Bacteria were grown in 1 litre of LB broth at 37°C until the D600 reached ~0.6. The culture was then induced with IPTG and grown for 3 h at 30°C. Cells were centrifuged, resuspended

1 Current address: European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany
2 Professor Stefania Petrucco prematurely passed away on 23 February 2009. This paper is dedicated to her memory.
3 To whom correspondence should be addressed (email peracchi@unipr.it).
The activities of BCAT1 and TAT in the previous studies had been measured using different dehydrogenases (leucine dehydrogenase and α-2-hydroxyisocaproate dehydrogenase respectively) and this may explain in part the lower specific activities measured on our enzyme preparations.

The glutamine-phenylpyruvate activity of CCBL1 was assayed by exploiting the absorption of the enol-borate adduct of phenylpyruvate [10]; the reaction mixtures contained 60 mM L-glutamine, 0.4 mM sodium phenylpyruvate, 300 mM sodium borate buffer, pH 8.5, and enzyme in a final volume of 1 ml. GOT1 activity was assayed via a coupled assay with malate dehydrogenase [11]. Conditions were: 100 mM Pipes, pH 7.5, 25°C, 10 mM L-Asp, 1.5 mM α-ketoglutarate, 0.25 mM NADH and 25 units/ml malate dehydrogenase. GPT activity was probed using a continuous assay based on LDH [3]. Conditions were: 125 mM Tris/HCl buffer, pH 7.5, 25°C, 575 mM L-Ala and 16.3 mM α-ketoglutarate, 0.25 mM NADH and 25 units/ml LDH.

<table>
<thead>
<tr>
<th>Recombinant enzyme</th>
<th>Reaction</th>
<th>Specific activity (μmoles · min⁻¹ · mg⁻¹)</th>
<th>The present study</th>
<th>Previous study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCAT1</td>
<td>4-Methyl-2-oxovalerate + L-glutamate = L-leucine + α-ketoglutarate</td>
<td>5.3 ± 0.7</td>
<td>98 ± 15 (reverse direction)</td>
<td>[1]</td>
<td></td>
</tr>
<tr>
<td>CCBL1</td>
<td>Phenylpyruvate + L-glutamine = phenylalanine + α-ketoglutarate</td>
<td>4.8 ± 0.7</td>
<td>3.7 (rat enzyme)</td>
<td>[2]</td>
<td></td>
</tr>
<tr>
<td>GPT</td>
<td>α-Ketoglutarate + L-alanine = L-glutamate + pyruvate</td>
<td>87 ± 20</td>
<td>∼70</td>
<td>[3]</td>
<td></td>
</tr>
<tr>
<td>GOT1</td>
<td>α-Ketoglutarate + L-aspartate = L-glutamate + oxaloacetate</td>
<td>30 ± 5</td>
<td>48.5 (rat enzyme)</td>
<td>[4]</td>
<td></td>
</tr>
<tr>
<td>PSAT1</td>
<td>Phosphohydroxypropionate + L-glutamate = L-phosphoserine + α-ketoglutarate</td>
<td>12 ± 0.6</td>
<td>∼15 (bovine enzyme)</td>
<td>[5]</td>
<td></td>
</tr>
<tr>
<td>TAT</td>
<td>p-Hydroxyphenylpyruvate + L-glutamate = L-tyrosine + α-ketoglutarate</td>
<td>17 ± 2</td>
<td>83 ± 6</td>
<td>[6]</td>
<td></td>
</tr>
</tbody>
</table>

**Figure S1** Schematic map of the expression vector pET28-CpoI used for the cloning of the complete aminotransferase coding sequences

Image reproduced with permission from Angelo Bolchi (Universita degli studi di Parma, Parma, Italy).

in 200 ml of Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors, and sonicated. The soluble lysate fraction was loaded on to a Talon cobalt-affinity resin equilibrated in the same buffer as above, and the recombinant protein was purified following the manufacturer’s instructions (Clontech). The final yield of purified CCBL1 was ∼0.75 mg per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 2 μM PLP and 1 mM DTT, concentrated by ultrafiltration and stored at −80°C.

**GOT1**

GOT1 was expressed in *E. coli* BL21-Star cells. After induction, cells were grown for 3 h at 30°C, centrifuged and
resuspended in Buffer B supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors.

Cells were then sonicated, and the recombinant protein was purified from the soluble lysate fraction using a Talon metal-affinity resin (equilibrated in the same buffer as above). The final yield was ~5 mg of purified GOT1 per litre of bacterial culture. The protein was dialysed against buffer B supplemented with 4 μM PLP and 1 mM DTT, and then concentrated by ultrafiltration. Glycerol (5%) was added to the enzyme solution before storing at −80°C.

GPT

GPT was expressed in E. coli BL21-CodonPlus®-RIL cells. Bacteria were grown in 400 ml of LB broth, supplemented with antibiotics, glucose (0.5%) and L-Ala (40 mg/l) at 37°C until \( D_{600} \) reached ~1. The culture was then induced with IPTG and grown further for ~18 h at 20°C. Cells were then centrifuged and resuspended in 200 ml Buffer C supplemented with 40 μM PLP, 2 mM Tris[2-carboxyethyl] phosphine, 10 mM L-Ala, 0.05% Triton X-100 and protease inhibitors.

After sonication, the soluble lysate fraction was loaded on to a His-Select nickel-affinity resin equilibrated in the same buffer as above, and the recombinant protein was purified following the manufacturer’s instructions (Sigma). The final yield was ~1 mg of purified GPT per 400 ml of bacterial culture (~2.5 mg per litre). The protein was dialysed against buffer C supplemented with 2 μM PLP and 2 mM Tris[2-carboxyethyl] phosphine, concentrated by ultrafiltration and stored at −80°C.

PSAT1

PSAT1 was expressed in E. coli BL21-CodonPlus®-RIL cells. Bacteria were grown in 1 litre of M9-glucose medium (plus antibiotics) at 37°C, until the \( D_{600} \) reached ~0.6. After induction with IPTG, the bacteria were grown for 3 days at cold-room temperature. The culture was then centrifuged, resuspended in 100 ml of Buffer D supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol, 0.2% Tween 20 and protease inhibitors and sonicated.

The soluble lysate fraction was loaded on to a Talon cobalt-affinity resin equilibrated in the same buffer as above, and the recombinant protein was purified following the manufacturer’s instructions. The final yield was ~29 mg of purified PSAT1 per litre of bacterial culture. The protein was dialysed against buffer D supplemented with 4 μM PLP and 1 mM DTT and concentrated by ultrafiltration. Glycerol (5%) was added to the enzyme solution before storing at −80°C.

TAT

TAT was expressed in E. coli BL21-CodonPlus®-RIL cells. Bacteria were grown in 1 litre of LB broth at 37°C, until the \( D_{600} \) reached ~0.6. Induced cells were grown for 3 h at 30°C, centrifuged and resuspended in Buffer A supplemented with 40 μM PLP, 5 mM 2-mercaptoethanol and protease inhibitors.

After sonication, the soluble lysate fraction was loaded on to a Talon cobalt-affinity resin equilibrated in the same buffer as above, and the recombinant His-tagged protein was purified following the manufacturer’s instructions (Clontech). The final yield was ~3.5 mg of purified TAT per litre of bacterial culture. The protein was dialysed against buffer A supplemented with 4 mM PLP and 1 mM DTT, and then concentrated by ultrafiltration and stored at −80°C.
Figure S4 Native absorption spectra of the eight cytosolic enzymes purified in the present study

The spectrum of most enzymes shows a prominent peak at 410–420 nm, typical of bound PLP. For GPT, GOT and PSAT, the highest peak is centered in the 320–350 nm region and is indicative of the presence of bound pyridoxamine phosphate.

Table S2 Activity of PSAT towards glyoxylate measured by different assays

Reactions were measured in 25 mM potassium phosphate buffer, pH 7.4, at 37°C. When errors are indicated, the reported activity values represent the average of at least two independent determinations.

<table>
<thead>
<tr>
<th>Glyoxylate (mM)</th>
<th>Amino group donor (mM)</th>
<th>Activity (μmol·min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GO assay</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.16 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2.20 ± 0.23</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>3.1 ± 0.15</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>1.7</td>
</tr>
<tr>
<td>L-Ala</td>
<td></td>
<td>0.048</td>
</tr>
<tr>
<td>L-Homocysteine</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Figure S5 Product inhibition of CCBL1

Transamination between glyoxylate (4 mM) and L-Phe (5 mM) catalysed by CCBL1 (0.22 μM) was measured in 25 mM potassium phosphate, pH 7.4, 25°C. The reaction progress was monitored based on the absorbance change in the 250–300 nm region (compare with Supplementary Figure S3). (A) The observed reaction rate begun to decrease after ~10 min (and the formation of ~20 μM products). (B) When the same reaction was performed in the presence of 120 μM 3-phenylpyruvate, the observed initial rate was 6-fold lower.

REFERENCES

Cytosolic aminotransferases involved in glyoxylate metabolism


Received 15 May 2009/18 June 2009; accepted 23 June 2009
Published as BJ Immediate Publication 23 June 2009, doi:10.1042/BJ20090748