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# Synthesis of pyridoxamine 5′-phosphate using an MBA:pyruvate transaminase as biocatalyst

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ABSTRACT

Transaminases (TAs) have useful applications as biocatalysts because of their capability of introducing amino groups into ketones and keto acids with high enantioselectivity, regioselectivity and broad substrate specificity. In this study we have shown that purified His-tagged omega-TA CV2025 from *Chromobacterium violaceum* is capable of complete conversion of pyridoxal 5′-phosphate (PLP) to pyridoxamine 5′-phosphate (PMP) in the presence of (*S*)-α-methylbenzylamine (MBA) as the amine donor. Conversions of 5 mM PLP with at least 0.8 mg/ml CV2025 TA (5.8 U/ml) were complete within 24 h. The fastest completion was achieved with an enzyme concentration of 3 mg/ml (22 U/ml): Within 4 h 5 mM PLP/MBA were converted to 100% and 10 mM PLP/MBA to 70%. PLP amination was only partially inhibited in the presence of 0.5 mM gabaculine, whereas the MBA:pyruvate transamination was shown to be inhibited completely. PMP formation of comparable efficiency could not be achieved with equivalent units of porcine α-TA. This represents the first example of a PLP-converting TA with an attributed gene and the first demonstration of quantitative biocatalytic PMP synthesis.



## 1. Introduction

### 1.1. Chemical transamination of aldehydes and ketones

The formal conversion of the carbonyl functional group in aldehydes and ketones to the corresponding amines is an important chemical transformation in many synthetic routes in organic chemistry and can be achieved by reductive amination or transamination. The Schiff base formation of ketones with amines and subsequent reduction of the C=N bond by catalytic hydrogenation is of interest for the asymmetric synthesis of α-amino acids from α-keto acids. Chemical transaminations are part of numerous routes in total synthesis, often following aldol condensation reactions. Dialkylaluminium amides, prepared *in situ* from the appropriate trialkylaluminium and the amine or amine hydrochloride, have been used as amine transfer reagents compatible with many functional groups. The use of trimethylaluminium and N,O-dimethylhydroxylamine hydrochloride allows chemical transaminations to the N-methoxy-N-methylamides, also known as the Weinreb amides. Tris(dimethylamino)borane undergoes facile transamination reactions with primary and secondary amines leading to tris(alkylamine)borane higher homologs as products. As many of these stoichiometric transamination reactions utilize unstable, air- and moisture-sensitive reagents which lead to waste in stoichiometric quantities, the development of biocatalytic processes for transamination reactions is desirable.

### 1.2. Biocatalytic transamination of aldehydes and ketones

Aminotransferases or transaminases (TAs) have broad potential for the synthesis of a wide variety of enantiomerically pure (*R*)- and (*S*)-compounds with amine functional groups [1]. Various examples of the use of TAs for the production of D- and L-amino acids, both naturally occurring and non-natural ones, have been described [2–9]. ω-TAs can be employed in two different ways for the production of enantiomerically pure amines. A racemic mixture can be separated by kinetic resolution into the corresponding ketone and the remaining amine enantiomer, which is typically obtained in high enantiomeric excess, while the ketone can be recycled to the starting racemic mixture. The same ω-TA can be utilized on the other hand to synthesize the amine of the

*Abbreviations:* TA, transaminase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; MBA, (*S*)-α-methylbenzylamine; TFA, trifluoroacetic acid; TBAS, tetra-*n*-butyl ammonium hydrogen sulphate; α-KG, α-ketoglutarate.

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opposite configuration straight from the ketone. In addition to enantioselective transaminations, the chemo-selective transamination of sensitive non-chiral aldehydes and ketones represents a synthetically useful connection. Against this background we have chosen pyridoxal 5′-phosphate (PLP) as a starting point for the exploration of a path to pyridoxamine 5′-phosphate (PMP).

#### 1.2.1. The role of PLP in enzyme catalysis

PLP, a derivative of vitamin B6, plays a crucial role as cofactor of many amino acid metabolising enzymes [10–14]. PLP is also involved in the transamination of some amines [15] as well as aminosugars [16] and is essential for biotin biosynthesis being a cofactor of δ-aminolevulinic acid synthase. PLP is also the cofactor in many other enzymes such as the decarboxylases and is an essential vitamin to higher organisms. It is an additive to foods and animal feeds because of this. PLP dependent enzymes bind the cofactor through an imine linkage between the aldehyde group of PLP and the ε-amino group of a conserved active site lysine residue. During catalysis the amino group of the substrate displaces the ε-amino group of the lysine residue resulting in an internal aldimine. The pyridoxine ring acts as electron sink and – depending on the possibility of abstraction of the α-proton – allows for a wide range of reaction specificities such as transaminations, racemisations, α-decarboxylations, aldol cleavage and β- and γ-elimination and replacement reactions.

The aminated form of PLP, pyridoxamine 5′-phosphate (PMP), appears in transamination reactions only (Fig. 1). Transamination consists of two half reactions. First the aldimine becomes deprotonated to become a quinoid intermediate, which in turn accepts a proton at a different position to become a ketimine. The resulting ketimine is hydrolysed leaving behind PMP which then acts as amine donor in the second half-reaction. PLP is subsequently recycled. Whereas PLP is covalently bound to the active site lysine, PMP interacts with the apoenzyme via non-covalent interactions only. The dissociation constant of E-PMP is much higher than that of E-PLP [17]. Apotransaminases bind PMP about 100-fold less tightly than PLP. PMP can be displaced from the enzyme with high concentrations of sulphate or phosphate ions [17].

#### 1.2.2. Pyridoxal- and PLP-converting TAs

If PMP is rather displaceable, the question arises – would any TA catalyze the amination of its own cofactor without recycling it? The answer is yes, with PLP acting as substrate rather than cofactor. In the 1960s, Snell discovered a pyridoxamine:pyruvate TA in a *Pseudomonas* strain [18]. This enzyme accepts pyridoxal as substrate but contains no PLP as cofactor. Just one half reaction of the typical TA two-step sequence is performed and PMP and PLP are only poor substrates [19]. Recently a pyridoxamine:pyruvate TA from *Mesorhizobium loti* (EC 2.6.1.30) was cloned, expressed and characterized by Yoshikane et al. [20]; it was not active with PLP or PMP either. It was found to belong to the class V aminotransferases of fold type 1 [21] or α family, AT IV [11]. The recently solved structure shows that an active site glutamate prevents binding of the phosphate moiety of PLP [22]. But PMP-specific TAs have been found as well. A PMP:α-KG TA (EC 2.6.1.54) from *Clostridium kainantoi* was first purified by Tani et al. [23] and its mode of action is supposed to be similar to that of pyridoxamine:pyruvate TA. An *E. coli* PMP-specific TA which catalyzes the reversible interconversion of PMP to PLP [24] was also suggested by Beechey and Happold [25]. No gene has yet been allocated to any of these PMP-specific TAs.

### 1.3. PLP bioconversion to PMP

Mechanistic and structural studies on TAs and other PLP-dependent enzymes as well as inhibitor design require the commercial availability of PLP and PMP. We investigated the possibility to use ω-TAs as biocatalysts for PMP production (Fig. 2). Other PLP-dependent enzymes such as decarboxylases and racemases might be able to use PLP as substrate, too. However, previous reports on transaminases capable of pyridoxamine and pyridoxamine phosphate conversion suggested ω-TAs were the most promising PLP-dependent enzymes for investigation of PLP amination.

We recently recruited several novel amine:pyruvate TAs using an enzyme from *Vibrio fluvialis* JS17 [15] as search model. These enzymes belong to class III aminotransferases of fold type 1 [21] or α family, AT II [11]. One of these enzymes which has been recently described in literature [26] proved to convert PLP efficiently to PMP in presence of the aromatic amine methyl-benzylamine. We report here for the first time on the applicability of an overexpressed ω-TA for PMP biosynthesis.

Pyridoxamine-5′-phosphate (PMP) is both scientifically and industrially important as co-factor of a variety of enzymes, central metabolite, potent antioxidant, vitamin $B_6$ vitamer and enzyme substrate. Since the worldwide availability of this important metabolite broke down suddenly, new direct and flexible routes to PMP were evaluated. Because chemical phosphorylation of pyridoxamine requires lengthy chromatographic purification, a direct reductive amination of pyridoxal-5-phosphate seemed to be an attractive route, if the challenge of the common equilibrium in the transamination reaction could be overcome.

## 2. Experimental

### 2.1. Materials

The majority of chemicals were obtained from Sigma–Aldrich, including porcine L-Asp:α-KG TA (#G2751). The latter comes as a powder containing sodium citrate salt. 1 mg (124 U) was dissolved in 100 μl water and used immediately. *Vibrio fluvialis* TA was purchased from Juelich Fine Chemicals (Juelich, Germany; now Codexis). *Escherichia coli* strain BL21 Star (pLysS) was obtained from Invitrogen (Paisley, UK).

### 2.2. Methods

#### 2.2.1. His-tagging of CV2025 transaminase and enzyme purification

The two oligonucleotides 5′-CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGGCCATCATCATCATCATCA-3′ and 5′-TATGATGATGATGATGGCCCATGGTATATCTCCTTCTTAAAGTTAAACAAAATTATTT-3′ were annealed to create a double-stranded poly-His encoding DNA fragment with a 5′ *Xba*I cut end, a 3′ *Nde*I cut end and an internal unique *Nco*I site [27]. The DNA fragment was inserted into *Xba*I *Nde*I digested expression plasmid pQR800 [26] at the 5′ terminus of the TA gene. *E. coli* BL21 Star (pLysS) was transformed with this construct and the preparation of cell-free extract was performed as described previously [26] except for the lysis buffer being 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8, supplemented with 0.5 mM pyridoxal 5′-phosphate (PLP). Extracts were kept as 5 ml aliquots in the freezer for months without detectable loss of activity. For His-tag purification, 5 ml extract were mixed with 5 ml of lysis buffer and with 5 ml Ni-NTA agarose (Qiagen, Dorking, UK). After agitation for 1 h at 4 °C, the beads were pelleted by 6 min centrifugation at 1700 g at 4 °C. After two wash steps with 50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, pH 8, four elution steps with 2.5 ml elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 250 mM imidazole, pH 8) containing 0.25 mM PLP were performed and residual agarose

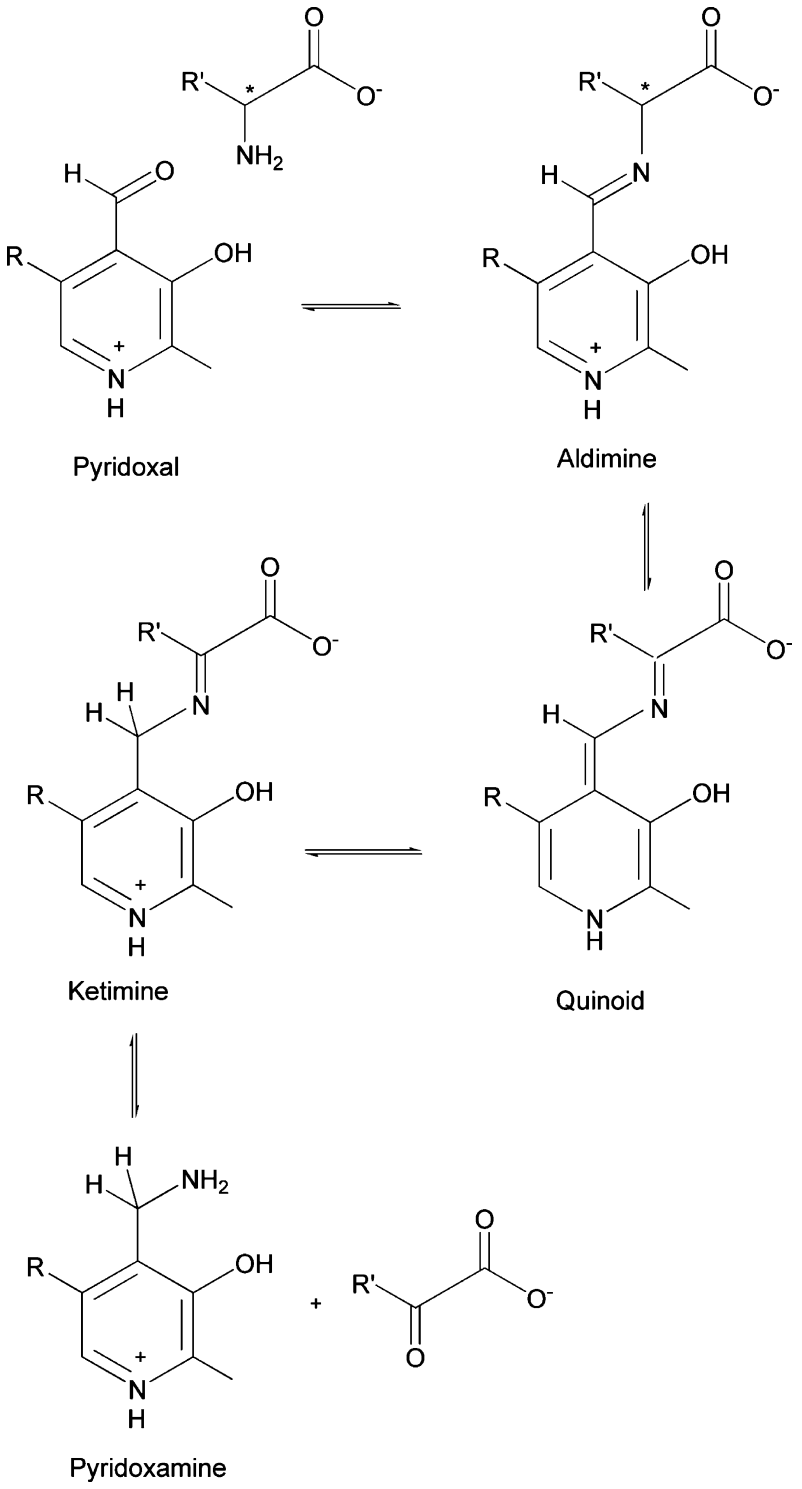


**Fig. 1.** Schematic half-reaction of the catalytic transamination cycle (modified slightly from [35]).

spun down for 5 min at 2900 × *g*. 10 ml eluate were transferred into dialysis tubing (molecular weight cutoff: 10000, SnakeSkin™, Pierce, Rockford, USA) and dialysed against 100 mM HEPES pH 7.5, 150 mM NaCl, 0.25 mM PLP. 19 ml dialysate were obtained and concentrated using Amicon Ultra-15 devices (Millipore, Watford, UK). 0.6 ml final concentrate was mixed with 0.2 Vol 80% glycerol and frozen at −20 °C. The purity of the enzyme was checked by SDS PAGE and its concentration determined by the Bradford method [28].

*2.2.2. Enzyme activity assays*

*2.2.2.1. ω-TA and MBA/pyruvate as substrates.* Activities for His-tagged CV2025 and *V. fluvialis* TA, both being (*S*)-specific amine:pyruvate TAs, were determined from initial rate measurements with (*S*)-α-methylbenzylamine (MBA) and pyruvate as the substrates [26,29,30]. Duplicate samples containing 100 mM HEPES pH 7.5, 10 mM MBA, 10 mM pyruvate and enzyme were incubated at 37 °C and stopped after 3 min by quenching with 4 vol of 0.1% trifluoroacetic acid (TFA). 1 unit (U) of enzyme is defined as the amount

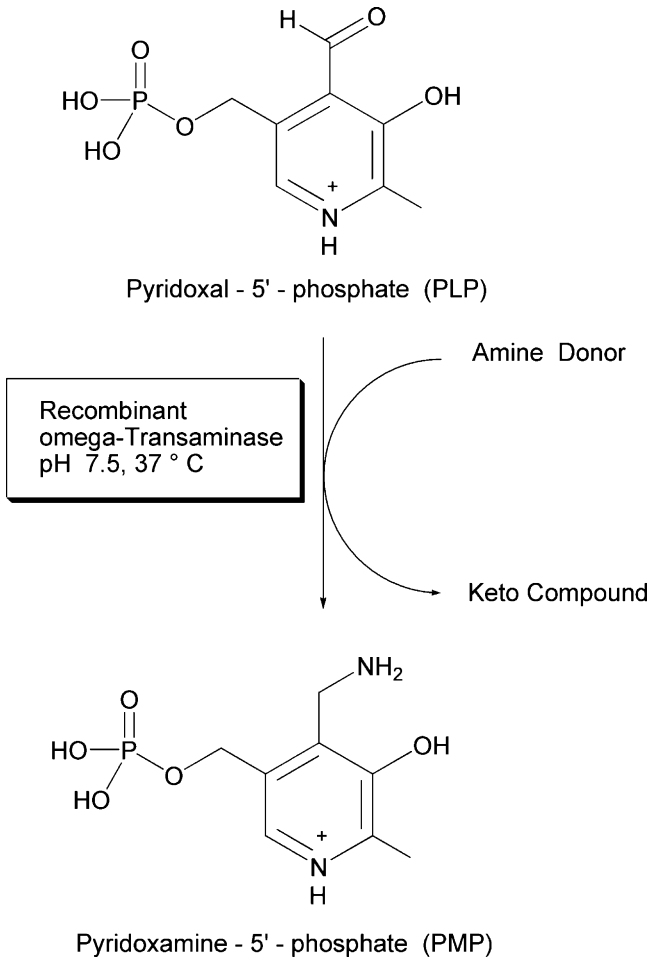


**Fig. 2.** Scheme for biocatalytic PMP formation using recombinant ω-TA.

that catalyses the formation of 1 μmol of acetophenone in 1 min at 10 mM MBA, 10 mM of pyruvate, pH 7.5 and 37 °C. All assays were performed and measured in HPLC glass vials since it was found that acetophenone adsorbs significantly to polypropylene. For the initial rate measurements, the volume of added enzyme was adjusted such that the AP production within 3 min was 1–2 mM (with the exception of pH and temperature studies where the enzyme concentration was kept constant). For practical reasons the initial rate measurements were performed after 3 min and not less. Measurements after 10 min resulted in rates of approximately 60% of those determined after 3 min. With the conditions applied the assay over 3 min might not be completely linear and activities might be underestimated.

*2.2.2.2. α-TA and L-Asp/α-KG as substrates.* Porcine L-Asp:α-KG TA was tested for its activity with HEPES as buffer. 1U is described as the amount of enzyme which converts 1 μmol of α-KG to L-Glu at pH 7.5 and 37 °C in 1 min. Hence, in a 1 ml reaction, 5 mM α-KG should be converted to 2.5 mM in 2.5 min by 1U α-TA (assuming an equilibrium of 50%). Two 1 ml samples containing 21 U of L-Asp:α-KG TA, 5 mM α-KG/5 mM L-Asp or 10 mM α-KG/10 mM L-Asp, 100 mM HEPES pH 7.5, 0.1 mM PLP were incubated for 10 min at 37 °C and aliquots analysed in 2.5 min steps by HPLC. Under these conditions the equilibrium was expected to settle within less than 1 min and it had indeed been established after the first 2.5 min.

All data were obtained from measurements of two independent samples which differed by less than 5% from each other.

*2.2.3. Bioconversions*

For studies on PLP turnover to PMP (pyridoxal turnover to pyridoxamine), 1 ml samples contained 5 or 10 mM of each PLP (or pyridoxal) and amine donor (MBA, L-Ala or L-Asp), up to 12% (v/v) enzyme and – if not stated otherwise – 100 mM HEPES pH 7.5. Bioconversions were performed at 37 °C for up to 24 h. Aliquots were quenched with 2% (v/v) formic acid and diluted 1:1 with solvent tetra-*n*-butyl ammonium hydrogen sulfate (TBAS). The final pH was ca. 2.5. All data were obtained from measurements of two independent samples which differed by less than 5% from each other.

*2.2.4. HPLC analysis*

The depletion of α-KG by α-TA was analysed with an Aminex HPX-87H Ion exclusion column (300 mm × 7.8 mm; Bio-Rad, Hemel Hempstead, UK). 0.1% (v/v) TFA was used as mobile phase for isocratic elution with an oven temperature of 65 °C and a flow rate of 0.8 ml/min. α-KG was detected by UV at 210 nm and had a retention time of 6.2 min.

Acetophenone and MBA were analysed using an ACE 5 C18 reverse phase column (150 mm × 4.6 mm, 5 μm particle size; Advance Chromatography Technologies, Aberdeen, UK). A gradient was run from 15% acetonitrile/85% 0.1% (v/v) trifluoroacetic acid (TFA) to 72% acetonitrile/28% TFA over 8 min, followed by a re-equilibration step for 2 min (oven temperature 30 °C, flow rate 1 ml/min). UV detection was carried out at 210 and 250 nm. The retention times (in min) under these conditions were: MBA 3.6 and acetophenone 7.9.

The same column was used for detecting PLP, PMP, pyridoxal and pyridoxamine. As existing HPLC methods for the determination of PLP and PMP were based on reversed-phase chromatography at strongly acidic pH and postcolumn derivatization [31], we have developed a new rapid and straightforward direct HPLC assay for these two vitamin B6 vitamers under physiological conditions. A gradient was run from 100% TBAS pH 7 at $t = 2$ min to 27.5% acetonitrile/72.5% TBAS pH 7 at $t = 13$ min, followed by a requilibration step for 1 min (oven temperature 35 °C, flow rate 1 ml/min). UV detection was performed at 254 nm. Typical retention times: Pyridoxamine 2.1 min, PMP 2.6 min, pyridoxal 5.6 min, PLP 13.6 min. Glass vials were used for all analytical measurements.

## 3. Results and discussion

### 3.1. Activity of purified His-tagged CV2025

The purification and concentration of His-tagged CV2025 yielded a highly active enzyme preparation of 80 mg/ml and a volumetric activity of 575 U/ml. The obtained enzyme was stable for at least two months at −20 °C without detectable loss of activity. The purchased *V. fluvialis* preparation was not a pure enzyme, but the highly expressed ω-TA represented about 50% of the soluble protein [26]. Its protein concentration was determined to be 51 mg/ml (actual TA concentration: 25 mg/ml) and its volumetric activity was 171 U/ml. Under the conditions specified above, both ω-TAs had a similar specific activity of 7.2 (for CV2025 TA) and 6.7 U/mg (for *V. fluvialis* TA).

### 3.2. Optimal pH and temperature

His-tagged CV2025 TA was characterized with respect to its pH and temperature optimum for the MBA/pyruvate substrate pair (Fig. 3A and B). The enzyme had a pH optimum of pH 8.5–9.0 (similar to the *V. fluvialis* enzyme: 9.2) and a temperature optimum of 55–60 °C (which is much higher than the optimum of the *V. fluvialis* TA: 37 °C).

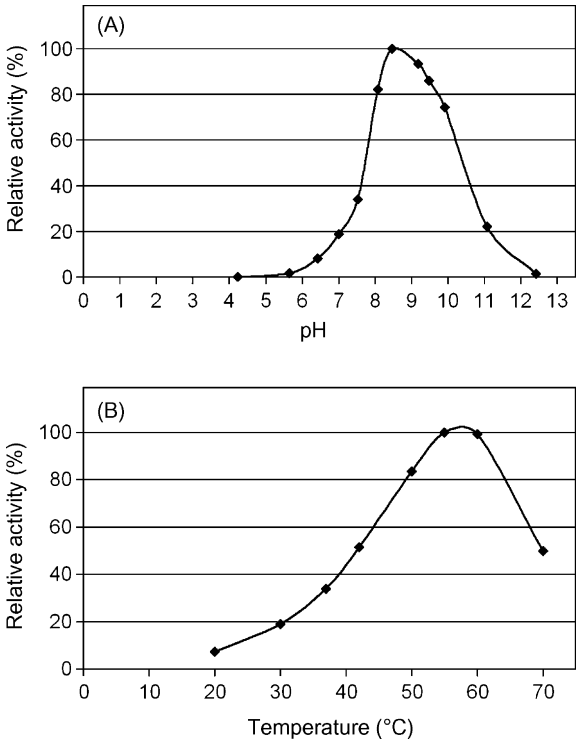


**Fig. 3.** Effect of pH (A) and temperature (B) on the activity of purified His-tagged CV2025 TA. Conditions were: 10 mM MBA, 10 mM pyruvate, 0.41 U/ml enzyme. (A) The following buffers (100 mM) were used at 37 °C: pH 5–6 citrate, pH 7 potassium phosphate, pH 8–10 borate, pH 11–12 potassium phosphate. (B) Reactions were performed in 100 mM potassium phosphate buffer pH 7 at 20 to 70 °C. Activities were determined by measuring AP formation after 3 min reaction time and are shown as relative values.

### 3.3. Detection of pyridoxamine:pyruvate and PLP:pyruvate conversion by ω-TAs

The investigation of the conversion of PLP by the two (*S*)-specific MBA:pyruvate converting ω-TAs from *C. violaceum* (CV2025) and *V. fluvialis* resulted in the interesting observation that this class of enzymes is capable of PLP amination (Fig. 4). In an initial experiment with 5 mM MBA and 5 mM PLP as the substrates and 2 U/ml enzyme, we detected within 1 h the formation of 0.26 mM PMP by CV2025 TA and of 0.12 mM PMP by *V. fluvialis* TA (Fig. 4). A parallel experiment with 5 mM MBA and 5 mM pyridoxal resulted in the

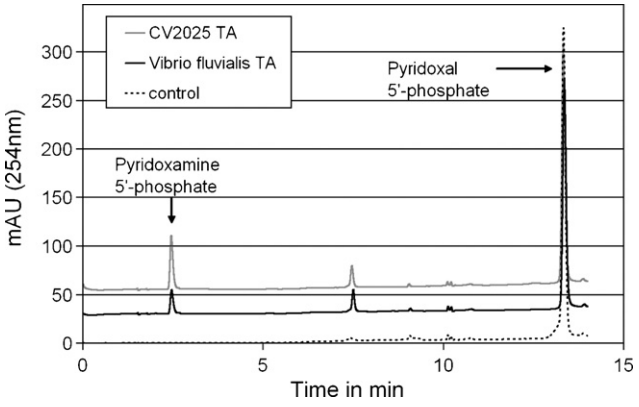


**Fig. 4.** HPLC traces showing the production of PMP from PLP by two ω-TAs. Reaction conditions: 5 mM PLP and 5 mM MBA as the amine donor, 100 mM phosphate buffer pH 7, 2 U/ml of either His-tagged purified CV2025 TA or purchased *V. fluvialis* TA (U determined at pH 7), 37 °C, 1 h reaction time. The control reaction contained TA but no amine donor. HPLC conditions as described under Section 2.2.4.

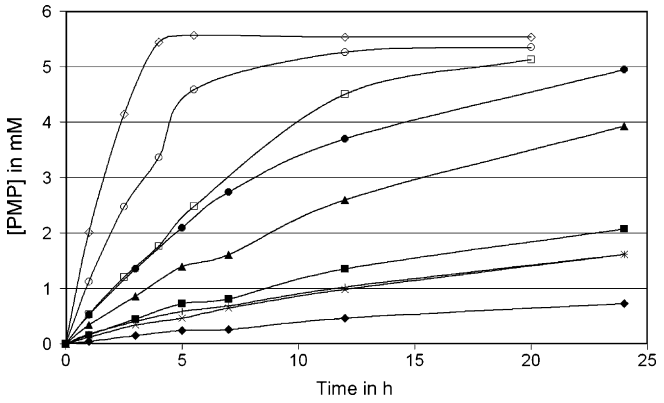


**Fig. 5.** Time course of 5 mM PLP/5 mM MBA conversion with increasing amount of CV2025 enzyme. Samples of 1 ml contained 100 mM HEPES pH 7.5 and 5 mM MBA/PLP. The following enzyme concentrations were tested (quantities in mg/ml): 0.08 (♦), 0.24 (■), 0.48 (▲), 0.80 (●), 1 (□), 2 (○), 3 (◊). These concentrations correspond to 0.57, 1.73, 3.45, 5.75, 7.19, 14.4, 21.6 U/ml, respectively. Volumes of added enzyme were below 4% (v/v). Sample (+) contains 5 mM L-Ala, 5 mM PLP, 0.48 mg/ml enzyme. Sample (x) contains 0.5 mM gabaculine; MBA was added after preequilibration of enzyme (0.48 mg/ml) with gabaculine and PLP for 1 min. Controls were set up without MBA and did not contain any detectable amounts of PMP at the end of the experiment.

formation of 0.14 mM pyridoxamine by CV2025 TA, and of 1.37 mM by *V. fluvialis* TA indicating that the latter prefers pyridoxal over PLP as substrate. Since pyridoxamine:pyruvate TAs are known among the ω-TAs but none of these would convert PLP significantly we focused specifically on the capability of PLP amination.

### 3.4. Time courses of PLP:pyruvate conversion with CV2025 TA

The initial observation of PMP formation was followed up further by performing time courses over 20–24 h using different amounts of CV2025 enzyme and using 5 mM of both PLP and MBA (Fig. 5). We kept the pH at 7.5 and the temperature at 37 °C during all our investigations. Substrate concentrations were kept low since substrate and product inhibition are known for *V. fluvialis* like ω-TAs [29,30,32] and since the effect of high PLP concentration on enzyme activity was not clear. Enzyme concentrations were increased up to 3 mg/ml or 21.6 U/ml. All conversions with enzyme concentrations of 0.8 mg/ml (5.75 U/ml) and above were complete within 24 h. The fastest completion was achieved with 3 mg/ml enzyme within 4 h.

### 3.5. Inhibition by gabaculine

An MBA/pyruvate reaction resulting in the formation of 1.61 mM acetophenone from 10 mM substrates within 3 min in the presence of 0.54 U/ml CV2025 TA was shown to be completely inhibited in the presence of 0.5 mM gabaculine. To assess whether PMP formation is affected by this inhibitor, a sample containing 0.48 mg/ml (3.45 U/ml) CV2025 TA was set up (Fig. 5). The reaction was started by the addition of 5 mM MBA after 10 min preincubation with 0.5 mM gabaculine and 5 mM PLP. After 24 h, 1.61 mM PMP were detected instead of 3.93 mM (as measured without gabaculine). There might be several explanations for the incomplete inhibition of the PLP amination by CV2025 in the presence of gabaculine. Firstly, the gabaculine concentration might not be high enough. However, the applied molarity of gabaculine exceeds the enzyme molarity by factor 50. Secondly, in the conditions above gabaculine degradation could occur over 24 h. But then PMP formation would not start right away as observed here. The most evident explanation would be the fact that PLP amination is affected by gabaculine binding but not completely inhibited. Further mechanistic and structural studies are necessary to shed some light on

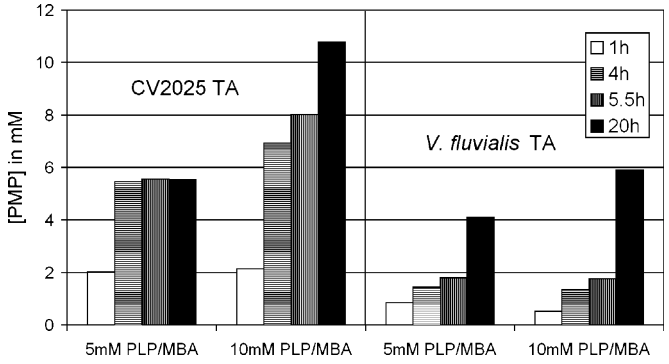


**Fig. 6.** PMP formation within 20 h by two ω-TAs, CV2025 TA and *V. fluvialis* TA. The 1 ml samples contained 100 mM HEPES pH 7.5, 5 or 10 mM of each PLP and MBA and 3 mg/ml TA (21–22 U/ml), and were incubated at 37 °C. Volumes of added enzyme were 4% (v/v) for CV2025 TA and 12% (v/v) for *V. fluvialis* TA. 100 μl aliquots were quenched at 1 h, 4 h, 5.5 h, 20 h. Controls without MBA showed no PMP formation.

this observation. The recently described pyridoxamine:pyruvate TA from *Mesorhizobium loti* [20] is not inhibited by the typical inhibitors of PLP-dependent enzymes, whereas the pyridoxamine phosphate TA of *E. coli* was shown to be inhibited by hydroxylamine [25].

### 3.6. Alternative amine donors

L-Ala was tested as an alternative amine donor. L-Ala proved to be an excellent donor, when CV2025 assays were performed with glyoxylate as the amine acceptor [26]. A reaction was set up containing 0.48 mg/ml CV2025 TA (3.45 U/ml), 5 mM PLP and 5 mM L-Ala instead of MBA. 1.61 mM PMP were formed after 24 h (Fig. 5) which is about 40% of the amount of PMP formed with MBA in the same time period. Since the CV2025 enzyme showed also specificity towards other donors like isopropylamine [26], choosing a different donor is a further degree of freedom for reaction optimization.

### 3.7. PLP amination by *V. fluvialis* ω-TA and porcine L-Asp:α-KG TA

It was of interest to us to know whether the ability to catalyze PLP amination was unique for CV2025 TA, or whether it was also found for other ω-TAs and whether a typical α-TA would be capable to aminate its cofactor. For this purpose we compared PMP formation over time by CV2025 TA, *V. fluvialis* TA and porcine L-Asp:α-KG TA (Figs. 6 and 7). MBA was used as the donor for the ω-TAs and L-Asp for α-TA. For the ω-TAs, an enzyme concentration corresponding to 3 mg/ml or, due to the similar specific activities, 21–22 U/ml, was chosen. In the case of the α-TA we decided to apply the same amount of Units. Starting with a substrate concentration of 5 mM, 4.10 mM PMP was obtained within 21 h with *V. fluvialis* TA, 5.53 mM with CV2025 TA. With 10 mM of substrates 5.91 mM PMP was produced by *V. fluvialis* TA and 10.8 mM by CV2025 TA (Fig. 6). The reaction catalysed by CV2025 was significantly faster. The *V. fluvialis* preparation used at that time had been stored for ca. 6 months at −20 °C before use. Final PMP concentrations obtained with CV2025 enzyme appeared reproducibly slightly higher than expected from the start concentrations of PLP. One explanation would be evaporation over the period of the experiment. In case of the L-Asp:α-KG TA with PLP and L-Asp as the substrates, we saw some PMP in detectable amounts, i.e. 0.30 mM with 5 mM substrate concentration and 0.99 mM with 10 mM substrate concentration – however, after 21 h only. Similarly, low but detectable pyridoxamine:α-keto acid transamination by porcine L-Asp:α-KG TA had been reported before [33,34] and it was speculated that this might reflect its evolutionary descendance from a PLP-independent prototype [20].

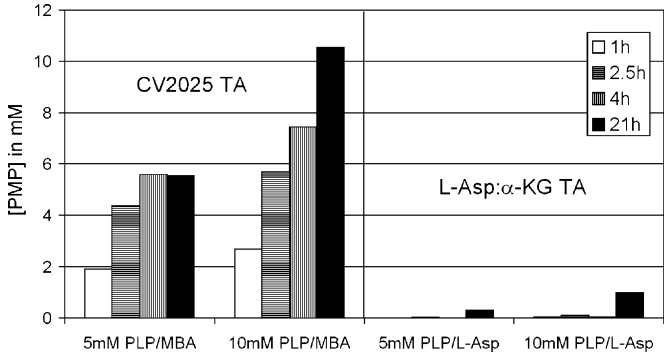


**Fig. 7.** Comparison of PMP formation by ω-TA CV2025 and porcine L-Asp:α-KG TA. The 1 ml samples contained 100 mM HEPES pH 7.5, 5 or 10 mM of each PLP and donor (MBA or L-Asp, respectively) and 21 U of enzyme and were incubated at 37 °C. Volumes of added enzyme were below 4% (v/v). 100 μl aliquots were quenched at 1, 2.5, 4, and 21 h. Controls without MBA showed no PMP formation.

Under the above conditions PMP biosynthesis appears to be more productive with ω-TA as catalyst than with α-TA. The complete conversion of PLP and MBA to PMP and acetophenone suggests a high equilibrium constant which may be due not only to a thermodynamically favoured forward reaction but also to severe inhibition of the reverse reaction, as observed before for the resolution of α-methylbenzylamine by *B. thuringiensis* ω-TA [30]. Detailed kinetic parameters including inhibition constants as well as thermodynamic characterizations need to be obtained in the future to establish which of both enzymes converts PLP more efficiently. However, for the purpose of quantitative biocatalytic PMP formation, CV2025 ω-TA is the more useful enzyme and this bioprocess might be further intensified by substrate feed and product removal (SFPR).

## 4. Conclusions

The study shows that among the TAs, the *V. fluvialis* like ω-TAs seem to have a potential as biocatalysts for PLP amination. The biocatalytic synthesis of PMP via ω-TA CV2025 offers an attractive route to PMP without extensive downstream processing because of the complete and selective conversion. For large scale production the biocatalytic route needs to be further optimized. Higher yields could be achieved by product removal, the use of alternative amine donors or optimized temperature and pH conditions. To our knowledge this is the first example of a PLP converting transaminase with an attributed gene and the first demonstration of biocatalytic pyridoxamine 5′-phosphate synthesis to completion.

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