Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2012, **10**, 5466

www.rsc.org/obc

PAPER

Chromobacterium violaceum ω-transaminase variant Trp60Cys shows increased specificity for (*S*)-1-phenylethylamine and 4′-substituted acetophenones, and follows Swain–Lupton parameterisation†

Karim Engelmark Cassimjee,[a] Maria Svedendahl Humble,‡[a] Henrik Land,[a] Vahak Abedi[b] and Per Berglund*[a]

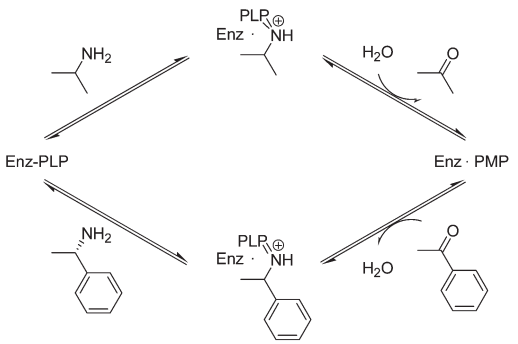
Received 11th February 2012, Accepted 31st May 2012
DOI: 10.1039/c2ob25893e

For biocatalytic production of pharmaceutically important chiral amines the ω-transaminase enzymes have proven useful. Engineering of these enzymes has to some extent been accomplished by rational design, but mostly by directed evolution. By use of a homology model a key point mutation in *Chromobacterium violaceum* ω-transaminase was found upon comparison with engineered variants from homologous enzymes. The variant Trp60Cys gave increased specificity for (*S*)-1-phenylethylamine (29-fold) and 4′-substituted acetophenones (~5-fold). To further study the effect of the mutation the reaction rates were Swain–Lupton parameterised. On comparison with the wild type, reactions of the variant showed increased resonance dependence; this observation together with changed pH optimum and cofactor dependence suggests an altered reaction mechanism.

Introduction

For the synthesis of chiral amines, transaminase enzymes are an attractive option. These enzymes interconvert amino and keto groups, generally with high enantioselectivity, assisted by their coenzyme pyridoxal-5′-phosphate (PLP).[1] The transaminase subgroup denoted ω-transaminases (ωTAs) can convert aliphatic ketones and amines without a carboxylate group in the α-position. The reaction mechanism, assumed to follow that of aspartate transaminase,[1,2] is composed of two half reactions. An amine reacts with the holoenzyme to produce the corresponding ketone and pyridoxamine-5′-phosphate (PMP), another ketone can then react to form its corresponding amine and the holoenzyme is reformed. A simplified scheme is shown in Scheme 1; see ESI Fig. S4† for a detailed mechanism. Generally, carboxylate-containing substrates, such as keto acids or amino acids, are faster reacting. This also applies for the enzyme used in this work, *Chromobacterium violaceum* ωTA (*Cv*-ωTA).[3] There are several examples of successful employment of ωTAs for synthesis of chiral amines of high enantiomeric excess,[4–15] for use in *e.g.* pharmaceutical preparations.[16] Successful reaction engineering approaches have dealt with the commonly unfavourable reaction equilibria.[17–19]

In general, the employment of an ωTA in a specific synthetic reaction requires enzyme engineering.[20] (Semi)rational design,[16,21–23] but mostly directed evolution[24–27] has been the method used for improvement of ωTA stability, activity and enantioselectivity for a desired reaction. Advances in computational methods for finding suitable ωTAs have also been made.[28] Previously, we designed ωTA variants of both *Cv*-ωTA[29] and of *Arthrobacter citreus* ωTA[22] that showed improved enantiospecificity or reversed enantiomeric preference. These point mutations were guided by using homology models.



Scheme 1 The ω-transaminase enzyme (Enz) catalysed reaction as half transamination reactions, of isopropylamine/acetone (top) and acetophenone/(*S*)-1-phenylethylamine (bottom).

[a]*KTH Royal Institute of Technology, Division of Biochemistry, School of Biotechnology, AlbaNova University Centre, SE-106 91 Stockholm, Sweden. E-mail: per.berglund@biotech.kth.se; Fax: +46 8 5537 8468; Tel: +46 8 5537 8366*
[b]*AstraZeneca R&D, Pharmaceutical Development, SE-151 85 Södertälje, Sweden*

†Electronic supplementary information (ESI) available: pH profiles, active site quantification, rate equations and examples of plots, Swain–Lupton parameterisation, reaction mechanism, materials and methods. See DOI: 10.1039/c2ob25893e

‡Present address: Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden.

Another example is the tailoring of the ωTA from *Vibrio fluvialis* (*Vf*-ωTA) by rational design to increase the activity for aliphatic amines.[21] In that work, a tryptophan residue in the active site was replaced by a glycine (*Vf*-ωTA Trp57Gly) to create a larger substrate binding pocket with space for larger hydrophobic groups on substrates in the (*S*)-configuration. In another work, a thermostable ωTA variant from *Arthrobacter citreus* denoted CNB05-01, where a cysteine replaced the corresponding tyrosine in the active site, was found by directed evolution.[26]

To measure and compare turnover numbers between different enzyme variants or enzymes from different species, an active site quantification method is required. Recently, we published such a method for *Cv*-ωTA.[30] This enzyme was now subject to protein engineering, and the active site quantification method made kinetic comparisons possible. Interestingly, the enzyme shows a time dependent activation in solution,[30] possibly due to structural rearranging upon cofactor binding.[31] For this reason, the enzymes used in this paper were after IMAC-purification incubated with an excess of cofactor, desalted, and then the amount of active enzyme was quantified.

In transaminase reactions with substituted acetophenones we observed significant rate differences depending on the substituent, hypothetically due to electrochemical effects. In chemical reactions with benzoic acid and analogues thereof, the Hammett equation may be used to predict *meta* and *para* substituent effects on reaction rate and reaction equilibrium.[32] The coefficients for various substituents are derived by studying the pK_a of substituted benzoic acids. Swain and Lupton extended the work of Hammett by deriving two coefficients for each substituent; by assuming that field and resonance are independent of each other these effects can be linearly combined with corresponding coefficients.[33–35]

We studied transaminase reactions of two common amino donors, and of chosen substituted acetophenones by their dependence on field and resonance effects by employing the Swain–Lupton equation, in the wild type and a variant derived from comparisons with engineered homologous enzymes. To determine specificity constants (k_{cat}/K_M) we chose substrate concentrations near the value of K_M (within the linear range), or derived relative specificity constants by allowing several substrates to compete for the active site. Then, we treated the specificity constants as first-order rate constants.

Results

In this work, homology models of *Vf*-ωTA and CNB05-01 were structurally aligned to the homologous *Cv*-ωTA to localize the corresponding amino acid residue position from previous engineering work by Cho *et al.*[21] and by Cambrex North Brunswick Inc.[26] In *Cv*-ωTA, Trp60 was thereby identified and targeted for site-directed mutagenesis. Previously, we showed that *Cv*-ωTA Trp60Cys has increased enantiospecificity for 1-phenylethylamine, 1-aminotetraline and 2-aminotetraline compared to the wild-type enzyme.[29] Additionally, *Cv*-ωTA Trp60Ala shows reduced activity and enantiospecificity and gives poor cultivation yields compared to the wild-type enzyme and was therefore not further explored.[29]

The effect of the mutation Trp60Cys (a cysteine residue at the corresponding position was found in CNB05-01) compared to the wild type was explored by measuring the absorbance spectra of the holoenzymes, the pH optima, the effect of excess PLP and the specificity constants for two amine substrates and several selected 4′-substituted acetophenones. The values obtained for the selected substituted acetophenones were used for Swain–Lupton parameterisation to investigate the presence of putative mechanistic differences in the variant compared to the wild type enzyme.

The wild type holoenzyme has an absorbance maximum at 395 nm.[30] Instead, *Cv*-ωTA Trp60Cys showed an absorbance maximum at 407 nm, after incubation with PLP and desalting. This is similar to free PLP and/or the hemimercaptal form of PLP.[36] This change of absorbance for the variant dictated the modification and reestablishment of the active site quantification method, since the method relies on the virtually irreversible reaction of the wild type holoenzyme with an absorbance maximum at 395 nm. The wild type was measured at 395 nm, as previously described.[30] The correlation between the Trp60Cys putative holoenzyme amount of active sites and the absorbance at 407 nm was measured, analogous to the said method, by measuring the amount of consumed (*S*)-1-phenylethylamine ((*S*)-1-PEA) by HPLC, in a half transamination reaction, see ESI† for details.

The pH rate dependence of the *Cv*-ωTA Trp60Cys variant was measured in synthesis mode, *i.e.* for the amination of acetophenone by isopropylamine (IPA). *Cv*-ωTA wild-type has optimum pH at 8.3,[30,37] while the Trp60Cys variant showed an optimum at pH 7.0, see ESI, Fig. S1.†

Cv-ωTA wild-type is inhibited by an excess of PLP,[30] but the Trp60Cys variant did not show this behaviour, see Fig. 1. Instead, *Cv*-ωTA Trp60Cys required an excess of PLP to achieve maximum rate; additionally, continuous measurements showed that reactions without excess PLP stopped after ~10 min.

The specificity constants for (*S*)-1-PEA and IPA were measured for a transamination reaction with pyruvate catalysed by *Cv*-ωTA wild type or variant Trp60Cys. The reaction of (*S*)-1-PEA was followed spectrophotometrically at 245 nm.[38] Initial rates for concentrations of (*S*)-1-PEA for transamination

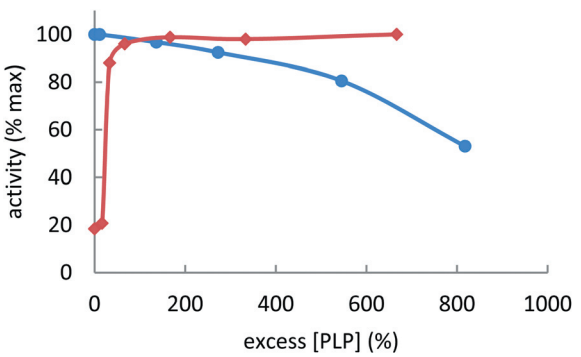
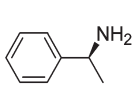


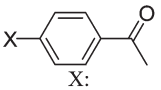
Fig. 1 The effect of excess PLP on the initial rate of *Cv*-ωTA wild type (blue dots) and variant Trp60Cys (red diamonds), obtained by following the consumption of acetophenone at 285 nm, in HEPES buffer (50 mM, pH 8.2 for the wild type and pH 7.0 for the variant) in a system with IPA (50 mM for the wild type and 200 mM for the variant) as the amino donor. As previously shown[30] the wild type enzyme is inhibited. The variant did not reach maximum rate below a two-fold excess of PLP.

Table 1 Kinetic constants for (*S*)-1-PEA and IPA for *Cv*-ωTA wild type and variant Trp60Cys[a]

				
	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	k_{cat} (s^{-1})
Cv-ωTA wild type	0.68	0.20±0.004	73	15±0.3
Cv-ωTA Trp60Cys	20	0.024±0.002	190	4.6±0.4

[a] Reactions performed in 50 mM HEPES buffer at pH 8.3 (wild type) and pH 7.0 (Trp60Cys). Excess PLP (200 μM) was added to the reactions with the variant, as dictated by its PLP-dependence curve (Fig. 1).

Table 2 Specificity constants for chosen 4′-substituted acetophenones for *Cv*-ωTA wild type and variant Trp60Cys[a]

 X:	k_{cat}/K_M (s^{-1} mM^{-1})	
	Cv-ωTA wild type	*Cv*-ωTA Trp60Cys
NO_2–	0.030	0.16
Cl–	0.029	n.d.[d]
Br–	0.028	0.039
CN–	0.026	0.10
F–	0.020	n.d.[c]
H–	0.0082[b]	0.034[b]
Me–	0.0050	0.024
OH–	0.0024	0.013
MeO–	n.d.[c]	0.011

[a] Reactions performed in 50 mM HEPES buffer pH 8.3 with 50 mM IPA (wild type) or pH 7.0 with 200 mM IPA (Trp60Cys). Excess PLP (200 μM) was added to the reactions with the variant, as dictated by its PLP-dependence curve (Fig. 1). [b] Experimentally determined values from which the remaining values were calculated from their relative values. [c] Not determined, no observed reaction. [d] Not determined, significant background reaction.

of pyruvate were collected. Rates which were linearly dependent on the concentration were used to calculate the specificity constant (k_{cat}/K_M). For an ordered ping-pong bi–bi reaction the true specificity constants can be deduced in this manner, even though pseudo-one-substrate kinetics are applied; see ESI† for the rate equations and examples of the rate plots.

The transamination reaction of IPA and pyruvate was measured by, first, treating IPA as a competing substrate in the above mentioned spectrophotometrically measurable reaction, by measuring rates with and without IPA. From this the K_I for IPA was obtained. Then, since in this case $K_I = K_M$, an appropriate concentration of IPA could be chosen for analysis of formed alanine with ninhydrin, IPA does not give a colour change upon incubation with ninhydrin.[39] As shown in Table 1, variant Trp60Cys showed a significantly increased specificity constant for (*S*)-1-PEA; for IPA the specificity was reduced.

Further, the relative specificity constants of a variety of 4′-substituted acetophenones were investigated; 4′-fluoro-, chloro-, bromo-, nitro-, cyano-, methyl-, hydroxyl-, methoxy-, and unsubstituted acetophenone were used. By employing several ketones simultaneously their relative reaction rates could be obtained. Ketone consumption was followed by HPLC with IPA as the amino donor. All ketones could not be used at once due to peak overlap in the HPLC-measurement. Instead, four separate reactions were prepared. Intercomparison was achieved by adding common ketones in the reactions. Ketone concentration was kept low (0.2 mM for each ketone, 0.6 mM total) to avoid inhibition.

The division of two or more rate equations for a competing substrate case, where the enzyme and substrate concentrations are equal, will simplify to the division of the specificity constants (k_{cat}/K_M), as described in ESI.† This applies irrespective of the amount of competing substrates. Our experiment with substituted acetophenones can therefore be regarded as a comparison of relative specificity constants. By experimentally determining the specificity constant for acetophenone, by the method described by Schätzle *et al.*,[38] the remaining specificity constants could be calculated from the relative values. Table 2 shows the specificity constants for the chosen ketones in the wild type and the mutant.

The values in Table 2 were used to construct Swain–Lupton plots,[33–35] shown in Fig. 2. The Swain–Lupton equation has fixed values for field and resonance for each substituent. The equation can be written as

$$\log\frac{k}{k_0} = fF + rR \qquad (1)$$

where k is the first order rate constant for a given substituted species, k_0 is the rate constant for the unsubstituted species, F and R are the predetermined values for field and resonance for a given substituent, and f and r are their coefficients which were subject to least squares fitting to the given set of rate constants.

We treated the specificity constant (k_{cat}/K_M) as a first order rate constant for this purpose. For the wild type enzyme, least squares fitting revealed two points that were outliers. These points were thereafter omitted from the fitting (but still shown in the figure) to achieve a more accurate calculation of the coefficients for the remaining points which were in accordance with the Swain–Lupton equation. Visually the plot appeared the same with or without the inclusion of these points for the least squares fitting. In the variant, in contrast, all points were included in the least squares fitting and provided a good linear regression.

The redox state of the introduced cysteine residue was not determined. Quantification of free cysteines, or disulfide bonds, with Ellman's reagent[40,41] was attempted but the absorbance of the holoenzyme together with the possibility of dissociation of PLP with concomitant oxidation, yielding the carboxylic acid, prevented accurate measurements; the absorbances of the said species are in the same range as for the Ellman's test.

Discussion

Cv-ωTA Trp60Cys shows an increased specificity constant for (*S*)-1-PEA, which is consistent with the previously observed increased enantiospecificity, compared to wild-type.[29] The effect is consistent with the hypothesis that a larger hydrophobic substrate binding pocket favours the substrate phenyl group in the (*S*)-configuration. However, this hypothesis cannot account for

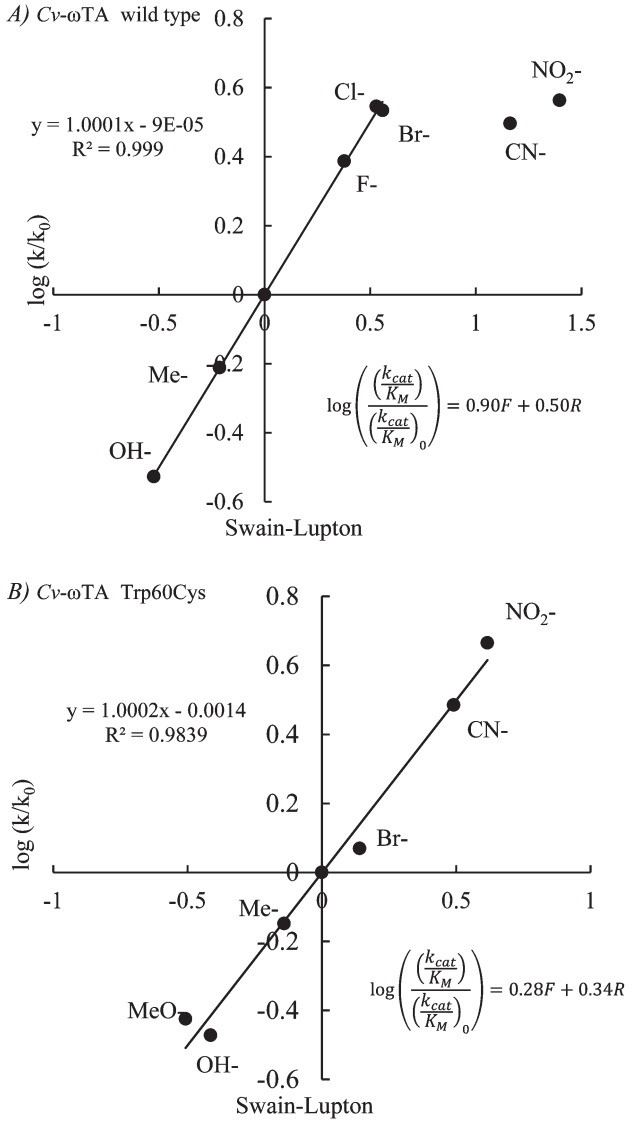


Fig. 2 Swain–Lupton plots for chosen 4′-substituted acetophenones from the specificity constants listed in Table 2, for (A) *Cv*-ωTA wild type and (B) variant Trp60Cys. The 4′-substitution of the acetophenones is marked by the corresponding points on the graph. The values of the coefficients *f* and *r* were calculated as 0.90 and 0.50 for the wild type, and 0.28 and 0.34 for Trp60Cys. The percent resonance is 36% in the wild type and 55% in the variant.

the successful mutation to a cysteine residue in contrast to an alanine residue.

In a transamination reaction catalyzed by *Cv*-ωTA wild type using IPA as the amino donor with any of the substituted acetophenones, the second half reaction (the amination of the ketone) can be regarded as rate determining. This is visible on comparison of the specificity constants; the values are the same for a system with IPA as the amino donor for any of the acetophenones. For the Cys variant, in contrast, the half reaction with IPA is only rate determining for the two slowest reacting ketones among the ones tested. One may also conclude that high yield synthesis of (*S*)-1-PEA from acetophenone with IPA as the amino donor is problematic since the enzymes favour the product amine from IPA; as the reaction proceeds the formed (*S*)-1-PEA will act as a competing substrate and virtually halt the reaction. Continuous removal of the product and/or a large excess of IPA are plausible solutions, if IPA remains the amino donor of choice. Applying the appropriate amino acceptor–donor pair has been shown to increase the yield.[20]

We observed a lower specificity for IPA in the variant compared to the mutant. In the work by Cho *et al.* with *Vf*-ωTA[21] the corresponding tryptophan was mutated to a glycine which yielded an enzyme with a two-fold increase of specific activity for the resolution of 1-PEA, in agreement with our results, and a 19-fold increase for IPA, differing from our observations. Even though we used a different enzyme similar results would be expected. It is noteworthy that in the work by Cho *et al.* the same pH was employed for both wild type and mutant. It is possible that the apparent increase of activity for IPA is mainly due to the conditions being more suitable for the mutant, and that *Vf*-ωTA Trp57Gly has similar properties to *Cv*-ωTA Trp60Cys in terms of pH optimum and PLP-dependence.

In our experiments with competing ketones the concentration of IPA was chosen to be high enough for the ketone reaction to be rate determining, but low enough for significant substrate inhibition to be absent, if present. Complete two-substrate kinetics were not performed, but pseudo-one-substrate reactions with acetophenone (1.0 mM) as the amino acceptor and varied concentrations of IPA as the amino donor were performed where no substrate inhibition by IPA was detected within the chosen range (5–100 mM for wild type and 5–250 mM for Trp60Cys). This verifies that the 4′-substituted acetophenones are competing for the enzyme : pyridoxamine-5′-phosphate-complex (E : PMP) in our experiments and that the specificity constants in Table 2 are valid (see ESI† for the reaction mechanism).

The Swain–Lupton parameterisation shows *Cv*-ωTA Trp60Cys to be a more general catalyst for these substituted acetophenones than the wild type since the agreement with the Swain–Lupton equation was better over the substrate range. It can be assumed that the enzyme contribution is approximately equal for all these substrates in the Trp60Cys variant. The rate differences can be attributed to the activation/deactivation by the substrate alone. Whereas, in the wild type, the specificities for the 4′-nitro and 4′-cyano substituents were lower than the Swain–Lupton parameterisation would predict. The relative value of the Swain–Lupton factors for the field and resonance effect is different for the variant, compared to the wild type. The resonance effect contribution has changed from 36% to 55%, in terms of the ratio of the Swain–Lupton factors. This, together with the change of pH optimum, indicates that the mechanism and/or the rate determining step in the amination reaction of the ketone have changed upon mutation Trp60Cys. The increase of the resonance contribution suggests that the increased space in the active site is not the sole cause for the increased specificity. The putative role of the introduced cysteine residue as a Lewis acid, or other electrochemical effects, can be regarded as a subject for further investigations, based on these findings. The change of pH optimum to 7.0 in the variant may suggest that the introduced cysteine residue actively partakes in the reaction. Possibly the cysteine residue provides a field effect which lessens the importance of field effects by the 4′-substituents; this is consistent with the observed increased importance of resonance effects from the substituents. The lack of inhibition of PLP in Trp60Cys, together

with the change in absorbance maximum, affords a hypothesis of a difference of binding of the cofactor. Hypothetically, PLP does not bind as a Schiff-base to the catalytic lysine, but is bound in the PLP-binding pocket and may form a hemimercaptal with the introduced cysteine residue. The impact of this cannot be explained without a deeper investigation of the reaction mechanism and putative changes thereof and/or a crystal structure.

Conclusions

We found a key point mutation in *Cv*-ωTA which increases the specificity constant for the aliphatic amine (*S*)-1-PEA and a range of 4′-substituted acetophenones. Plausibly, a more effective enzyme for larger aliphatic substrates has been created. The variant, Trp60Cys, has a more general nature than the wild type for 4′-substituted acetophenones since their specificity constants can be plotted in good agreement with the Swain–Lupton equation, in contrast to the wild type enzyme. The point mutation was found by structural alignments of homology models of other engineered ωTAs and is therefore of interest for understanding the mechanism and the development of rational design for this enzyme group. The straightforward variant Trp60Ala was a poor catalyst, the role of the cysteine residue in this position is not trivial and a suitable subject for further investigation.

Acknowledgements

The authors would like to acknowledge Prof. Wolfgang Kroutil (Graz University) for supplying the gene for Cv-ωTA, Dr Linda Fransson (KTH) for fruitful discussions and VINNOVA (The Swedish Governmental Agency for Innovation Systems) for financial support.

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