Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Supplementary Information

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

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Properties for *Chromobacterium violaceum* ω-transaminase (*Cv*-ωTA) wild type and variant Trp60Cys were measured; the pH rate dependence, concentration effect of the cofactor pyridoxal-5'-phosphate (PLP), and specificity constants (k_{cat}/K_M) for isopropyl amine (IPA), (*S*)-1-phenylethylamine ((*S*)-1-PEA) and a variety of 4'-substituted acetophenones.

More detailed results regarding the pH optima, the active site quantification of the variant Trp60Cys, rate equations with examples of plots, and Swain-Lupton parameterisation for the reactions with the 4'-substituted acetophenones are found below. The interested reader may find this information valuable for understanding and discussion; we also included a putative reaction mechanism for clarity. Materials and methods are found at the end of this document.

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pH profile

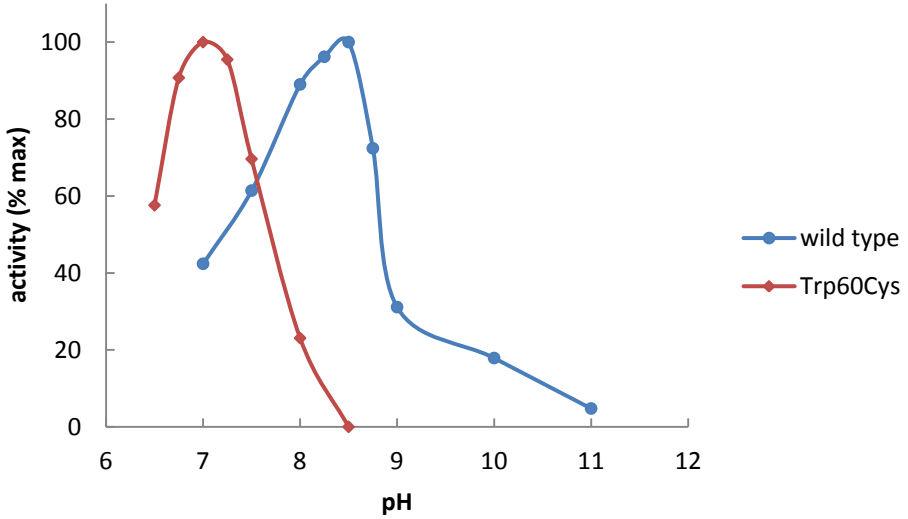

Fig. S1 The pH rate dependence of *Cv*-ωTA wild type and variant Trp60Cys, obtained by following the consumption of acetophenone at 285 nm, in a system with IPA(50 mM for the wild type and 200 mM for Trp60Cys) as amino donor. The optimum was measured to 8.3 for the wild type and 7.0 for Trp60Cys.

Active site quantification of *Cv*-ωTA Trp60Cys

As previously described,[1] the active sites can be quantified by performing a half transamination reaction with (*S*)-1-PEA, without an amino acceptor. Racemic 1-phenylethylamine was used, the consumed (*S*)-1-PEA was quantified by HPLC where the peak corresponding to the *R*-enantiomer was used as internal standard (the E-value was known to be high[2]). After incubating *Cv*-ωTA Trp60Cys with an excess of PLP for 4 h in 30 °C it was desalted and the absorbance spectrum was collected. A maximum at 407 nm was found. The absorbance at 407 nm was thereafter correlated with the amount of consumed (*S*)-1-PEA, shown in Fig. S2. According to these results the amount of active sites can, for this variant, be quantified by absorbance at 407 nm with an apparent extinction coefficient of 4.7 $mM^{-1}cm^{-1}$.

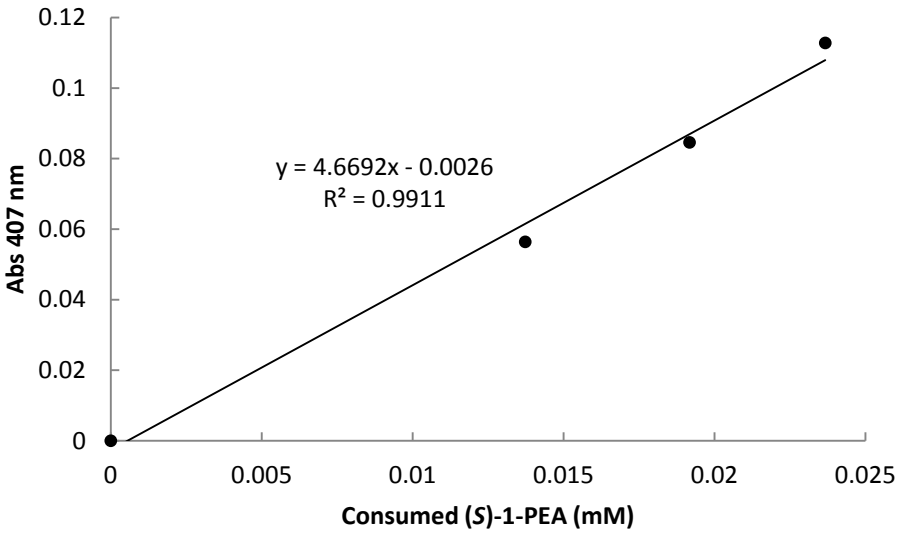

Fig. S2 The absorbance at 407 nm versus the amount of consumed (*S*)-1-PEA, in a half transamination reaction with *Cv*-ωTA Trp60Cys.

Rate equations and measurement of specificity constants

The ω-transaminase catalyses a two-substrate reaction with an ordered ping-pong bi-bi mechanism, presumed to be analogous to that of aspartate transaminase,[3] where the rate equation for substrates A and B at a given enzyme (Enz) concentration is written as:

$$v = \frac{k_{cat}[\mathrm{Enz}][\mathrm{A}][\mathrm{B}]}{K_M^A[\mathrm{B}]+K_M^B[\mathrm{A}]+[\mathrm{A}][\mathrm{B}]} \qquad \text{(eq. S1)}$$

When employing a pseudo one-substrate model with substrate B at a constant concentration eq. S1 can for simplification be rewritten as follows:

$$v = \frac{k_{cat}[\mathrm{Enz}][\mathrm{A}]}{K_M^A+[\mathrm{A}]\left(1+\frac{K_M^B}{[\mathrm{B}]}\right)} \qquad \text{(eq. S2)}$$

The bracketed expression in the denominator of eq. S2 will be treated as a constant. When comparing enzymes or enzyme variants pseudo one-substrate kinetics may be applied but the results cannot directly be regarded as general since the affinity of the constant substrate may vary and give arbitrary results only valid at the given reaction conditions. However, eq. S2 can be further simplified by ignoring [A] in the denominator, which is valid in the linear range in the beginning of the curve (low [A]). Analogous to the Michaelis-Menten equation for a single substrate reaction at substrate concentrations below or close to K_M, the rate is given by:

$$v = \frac{k_{cat}}{K_M^A}[\mathrm{Enz}][\mathrm{A}] \qquad \text{(eq. S3)}$$

Thereby the true specificity constant of one substrate of an ordered ping-pong bi-bi reaction can be deduced by studying the linear concentration range of the rate dependence with the other substrate at any fixed concentration.

In a situation where two substrates, C and D, are allowed to compete for an enzyme, in a one-substrate reaction, the rate equation for the individual substrate C is given by:

$$v_C = \frac{k_{cat}^C[\mathrm{Enz}]K_M^D[\mathrm{C}]}{K_M^D[\mathrm{C}]+K_M^C[\mathrm{D}]+K_M^CK_M^D} \qquad \text{(eq. S4)}$$

The ratio of the reaction rates for substrates C and D, given by the division of eq. S4, will simplify to the division of the specificity constants if the substrate concentrations are equal:

$$\frac{v_C}{v_D} = \frac{\left(k_{cat}/K_M\right)^C}{\left(k_{cat}/K_M\right)^D} \qquad \text{(eq. S5)}$$

This is also valid in the pseudo one-substrate case in an ordered ping-pong bi-bi reaction with a second common substrate at a fixed concentration. When the substrates in eq. S5 are enantiomers the equation is commonly denoted as the E-value.[4, 5] The same rational can be applied for studying relative rates of competing substrates, where the relative rates will correspond to the relative specificity constants.

The specificity constants for (*S*)-1-PEA of *Cv*-*ω*TA wild type and variant Trp60Cys was calculated from spectrophotometric measurements of the initial rates of transamination reactions with pyruvate (1 mM) by the production of acetophenone,[6] rates within the linear range were used where eq. S3 applies. Fig S3 shows rate-concentration plots for the wild type and the variant, analogous to Michaelis-Menten plots.

The relative specificity constants for chosen 4'-substituted acetophenones were calculated by allowing the substrates to compete, by application of eq. S5. The time dependent consumption of the 4'-substituted acetophenones was followed by HPLC. Upon division of the rate equations the time, the enzyme concentration and the substrate concentrations cancelled since they were equal; rate plots were therefore not necessary for the calculation of the constants. However, rate plots were made to ensure that the reactions had not approached equilibrium concentrations for the samples chosen for calculation. Blank reactions without enzyme were made to study the background reactions which were significant for the more reactive ketones, particularly for 4'-chloro-acetophenone in the blank reaction for Trp60Cys with 200 mM of IPA. Fig. S4 shows an example of a plot with data for three of the ketones from two different reaction mixtures after multiplication with a correlation factor deduced by employing a common ketone (4'-methyl-acetophenone) in both reactions. The slopes of the graphs thereby represent the relative specificity constants. The specificity constant for acetophenone was calculated by

spectrophotometrical measurement of initial rates by the consumption of acetophenone,[6] in a reaction with IPA as previously published.[1] This value was thereafter used to correct the relative specificity constants from the competition reactions.

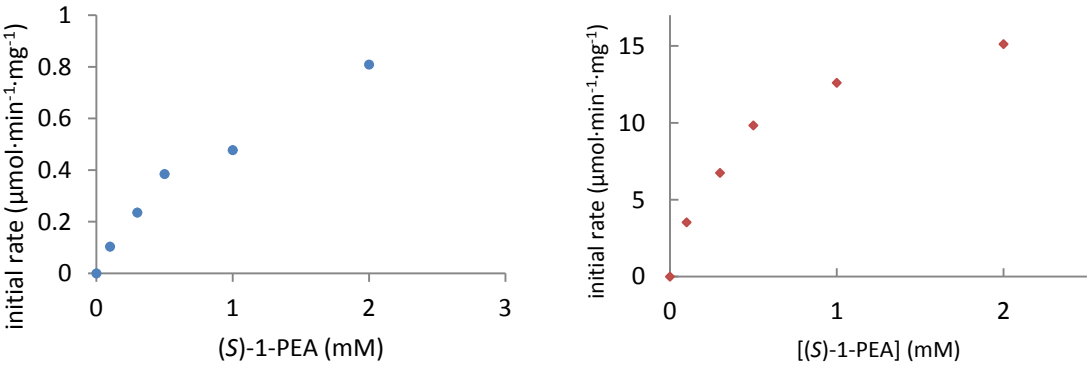

Fig. S3 Initial reaction rate for the amination of pyruvate (1.0 mM) with different concentrations of (*S*)-1-PEA with *Cv*-*ω*TA wild type (blue dots, on the left) and variant Trp60Cys (red diamonds, on the right).

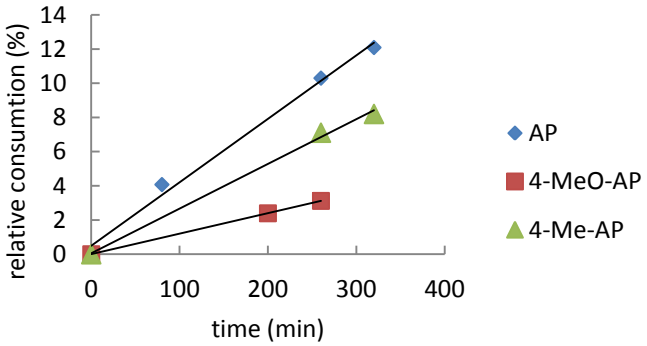

Fig. S4 Relative consumption of acetophenone (AP), 4'-methyl-acetophenone (4-Me-AP) and 4'-methoxy-acetophenone (4-MeO-AP) over time, with Cv-ωTA Trp60Cys.

Swain-Lupton parameterization

When several substrates are allowed to compete for the same enzyme species their relative rates can be directly correlated to their relative specificity constants. This is obvious when division of their individual rate equations are performed, similar to the reaction of two enantiomers. In our case the enzyme performs a ping-pong bi-bi mechanism. To obtain the relative specificity constants we allowed several ketones, 4'-substituted acetophenones, to compete for the same enzyme species, the E:PMP complex after amination by IPA. We chose concentrations of IPA to be high enough for the ketone reaction to be regarded as rate determining. The specificity constant for acetophenone was experimentally determined; from this value the remaining values could be calculated. By treating the specificity constants as first-order rate constants they could be used for Swain-Lupton parameterisation. This is valid since the concentrations of enzyme, pyruvate and IPA were equal for all ketones.

The values of *f* and *r* in the equation:

$$\log\left(\frac{\left(\frac{k_{cat}}{K_M}\right)}{\left(\frac{k_{cat}}{K_M}\right)_0}\right) = f * F + r * R \qquad \text{(eq. S6)}$$

where found by least squares fitting, the values for *F* and *R* are found in Table S1. The specificity constant for acetophenone was used as $(k_{cat}/K_M)_0$.

Table S1: Swain-Lupton parameters[7-9] for the chosen 4'-substituents of acetophenone.

4'-substituent	Field parameter (F)	Resonance parameter (R)
NO_2	1.0	1.0
CN	0.9	0.71
F	0.74	-0.6
Br	0.72	-0.18
Cl	0.72	-0.24
H	0	0
Me	-0.01	-0.41
OH	0.46	-1.89
MeO	0.54	-1.68

Putative reaction mechanism

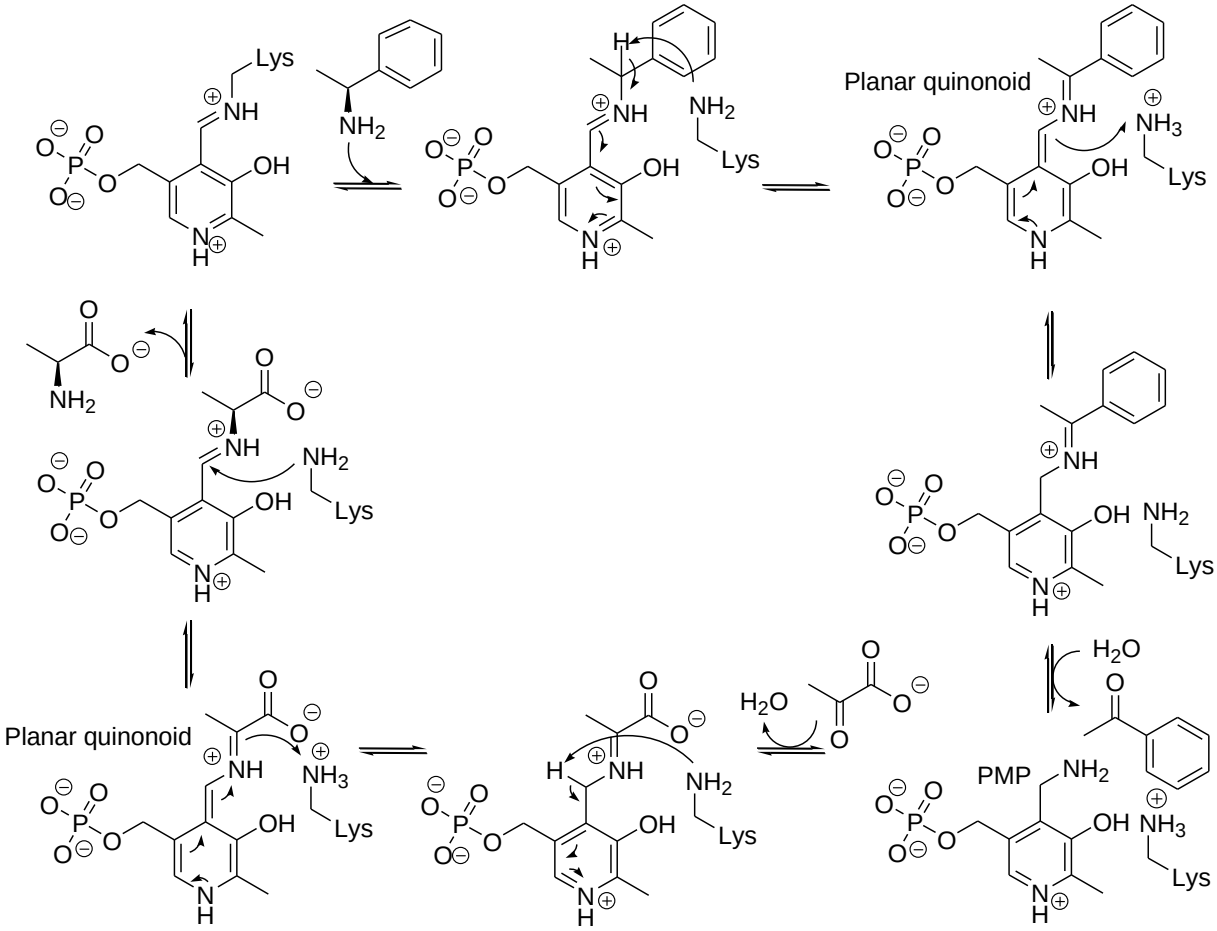

Fig. S5 The putative reaction mechanism of *Cv*-ωTA for the transamination of pyruvate by (*S*)-1-PEA, forming acetophenone and L-alanine, adapted from Silverman[10] from the reaction mechanism of aspartic transaminases.[3] The holoenzyme has PLP bound as a Schiff-base to Lys288, which is then aminated by (*S*)-1-PEA through a planar quinonoid intermediate. The reaction of a water molecule thereafter forms pyridoxamine-5'-phosphate (PMP) and acetophenone; upon reaction with pyruvate and the pass through another quinonoid intermediate L-alanine is formed with the reformation of a water molecule.

Materials and Methods

Chemicals were purchased from SigmaAldrich, Sweden. Enzyme cloning, mutagenesis and overexpression was performed as previously described.[1, 2]

Active site quantification of *Cv*-ωTA Trp60Cys was done by mixing racemic 1-phenylethylamine (200 µM) with different enzyme amounts after collecting the enzyme's absorbance at 407 nm, subsequent to identifying 407 nm as an absorbance maximum of the putative holoenzyme after incubation with an excess of PLP for 4 h in 30 °C, and desalting with a PD10 column (GE Healthcare). The samples were studied spectrophotometrically at 245 nm; when the absorbance seized to increase they were subject to HPLC analysis after acidification with $HClO_4$ and filtering through 0.45 µm syringe filters, with UV detection at 254 nm (Waters616 pump, Waters 600S Controller, Waters 486 tunable absorbance detector) with a Crownpak CR(+) column (Daicel), isocratically with $H_2O/HClO_4$ pH 1.6 as mobile phase at 0.6 mL/min. Retention times for the enantiomers of 1-phenylethylamine were 36 min (*S*) and 47 min (*R*). By using the (*R*)-enantiomer as internal standard the amount of consumed (*S*)-1-PEA was recorded and correlated to the absorbance at 407 nm.

The specificity constant for (*S*)-1-PEA was obtained by using the initial rate for three concentrations (0.1, 0.3 and 0.5 mM) within the linear range of the rate equation, with pyruvate (1 mM), and *Cv*-ωTA wild type (5.3µg, quantified at 395 nm) or Trp60Cys (0.39µg, quantified at 407 nm, with added excess PLP (200 µM)) in HEPES buffer (50 mM) pH 8.3 (wild type) or pH 7.0 (Trp60Cys). The amount of formed acetophenone was continuously measured at 245 nm in a dual beam spectrophotometer (Cary300, Varian Inc.) with an extinction coefficient of 12 $mM^{-1}cm^{-1}$.[6] Samples without enzyme were used as blank reactions in the continuous measurement. The specificity constant for IPA was obtained by first finding the inhibition constant, K_I, by adding it (50 mM) to the reactions with (*S*)-1-PEA and treating it as a competitive inhibitor to this ordered ping-pong bi-bi reaction. Since K_I equals the K_M in this case, a concentration of IPA within the linear range could be chosen. Reactions with IPA (50 mM), pyruvate (1 mM) and *Cv*-ωTA wild type (0.71 mg, quantified at 395 nm) or Trp60Cys (0.60 mg, quantified at 407 nm, with added PLP (200 µM)) was thereafter performed in HEPES buffer (50 mM) pH 8.3 (wild type) or pH 7.0 (Trp60Cys).The amount of formed alanine after 5 min was quantified after taking samples (400 µL) and stopping the reactions by addition of EtOH (400 µL) with ninhydrin (50 g/L), and Bis-Tris buffer (200 µL, 1.0 M, pH 6.2). The absorbance at 570 nm was measured after heating to 70 °C for 10 min and cooling to 21 °C. Blank reactions without enzyme and without either substrate were also performed. The amount of formed alanine was calculated by use of a standard curve.

The relative specificity constants for 4'-fluoro-, chloro-, bromo-, nitro, cyano-, methyl-, hydroxyl-, methoxy-, and unsubstituted acetophenone were calculated by performing four reactions for each enzyme, in HEPES buffer (50 mM) pH 8.2 (wild type) or pH 7.0 (Trp60Cys, with excess PLP (200 µM)), with IPA (50 mM for wild type or 200 mM for Trp60Cys) and three of the substituted acetophenones (0.2 mM each); 4'-cyano-, 4'-fluoro-, and 4'-bromo-acetophenone; 4'-cyano-, 4'-nitro-, and 4'-chloro-acetophenone; 4'-bromo-, 4'-methyl-, and unsubstituted acetophenone or 4'-hydroxy-, 4'-methoxy-, and 4'-methyl-acetophenone, and four identical reactions without enzyme to study the background reactions. Up to 12 samples (50 µL) from each reaction were taken at regular intervals during 6 h; the reactions were stopped by adding 450 µL acetonitrile/water 90/10 (v/v) with 0.03% v/v TFA with toluene as internal standard, which were after filtering (0.45 µm) subject to HPLC analysis with UV detection at 210, 225 and 254 nm (Dionex Ultimate 3000) with an XBridge C18, 2.5µ, 50x3.0 mm column, 40 °C, 0.85 mL/min acetonitrile/water 5/95 (v/v) + 0.03% v/v TFA with a 10.5 min gradient to reach acetonitrile/water 90/10 (v/v) + 0.03% v/v TFA. Retention times were (min): 4'-fluoro-acetophenone (3.9), 4'-chloro-acetophenone (4.6), 4'-bromo-acetophenone (4.8), 4'-nitro-acetophenone (3.8), 4'-cyano-acetophenone (3.3), 4'-methyl-acetophenone (4.3), 4'-hydroxyl-acetophenone (2.4), 4'-methoxy-acetophenone (3.7), acetophenone (3.5), and toluene (5.3). The reactions within 10% conversion were used for calculating the relative initial rates. From the relative values the true values could be calculated by comparison with the spectrophotometrically measured rates for acetophenone.[1]

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