ACS Catalysis

RESEARCH ARTICLE

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Active Site Quantification of an ω-Transaminase by Performing a Half Transamination Reaction

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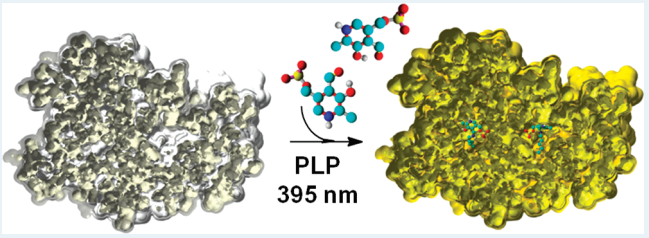
ABSTRACT: Measurement of the active enzyme fraction in a given enzyme preparation is a requirement for accurate kinetic measurements and activity comparisons of, for example, engineered mutants. ω-Transaminases, enzymes capable of interconverting ketones and amines by use of pyridoxal-5′-phosphate (PLP), can be used for the production of pharmaceutically important chiral amines but are subject to engineering to meet the practical requirements in synthesis reactions. Therefore, an active site quantification method is needed. Such a method was developed by quantifying the amount of consumed substrate in a virtually irreversible half transamination reaction. (*S*)-1-phenylethylamine was converted to acetophenone, while the holo enzyme (E-PLP) was converted to apo enzyme with bound pyridoxamine-5′-phosphate (E:PMP). Further, the mass of active enzyme was correlated to the absorbance of the holo enzyme to achieve a direct measurement method. The active *Chromobacterium violaceum* ω-transaminase with bound PLP can be quantified at 395 nm with an apparent extinction coefficient of 8.1 mM^{-1} cm^{-1}.



KEYWORDS: aminotransferase, chiral amines, pyridoxal-5′-phosphate, PLP, biocatalysis, enzyme kinetics

INTRODUCTION

Chiral amines are common motifs in pharmaceuticals and fine chemicals.[1,2] Compounds containing amines, such as amino acids, are produced in vivo by enantioselective enzyme-catalyzed transamination reactions.[3] Transaminases catalyze the reversible transfer of an amino group from an amino donor to an amino acceptor, a carbonyl compound, to yield an amine or an amino acid. Known transaminases require the coenzyme pyridoxal 5′-phosphate (PLP). When substrate amines or amino acids with more than one methylene between the carboxyl- and amino groups are accepted, the enzyme can be classified as an ω-transaminase (ω-TA). Generally, these enzymes display high enantioselectivity and are useful for asymmetric synthesis of chiral amines of high enantiomeric excess.[4−7] This attractive biocatalyst, nonetheless, requires engineering to function as an industrially feasible option. Obstacles, such as the commonly unfavorable thermodynamic equilibrium, substrate and product inhibition, poor thermostability, low solvent tolerability, insufficient enantioselectivity, or inadequate specific activity for desired substrates, need to be surmounted. For this purpose, engineered reactions and more efficient mutants have successfully been constructed,[4,7−12] as well as discovery of naturally occurring transaminase enzymes with desired properties.[13]

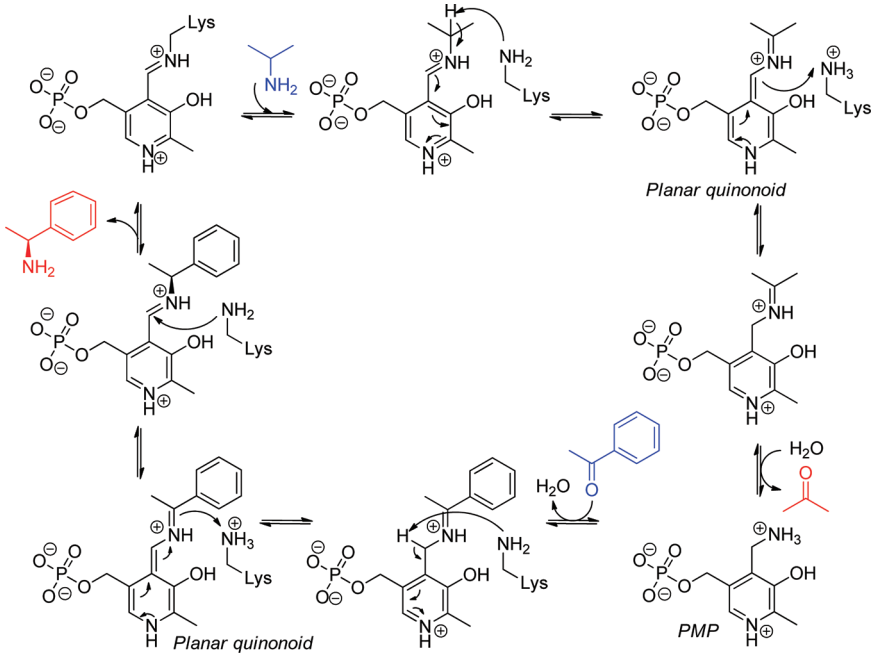
To correctly assess the activity a measurement of the amount of active enzyme is necessary. A comparison between different enzyme batches or mutants by mass alone will be misleading. The inactive fraction may differ in proportion or stability and activity may be misinterpreted. Hence, active site quantification is of paramount importance and it is a requirement for proper kinetic measurements. To our knowledge, a specific active site titration or quantification method for ω-transaminases has not been published to date. Cycloserine and gabaculine have been shown to be active site titrants (suicide inhibitors) for PLP-dependent amino acid transaminases.[14] In this paper the aim was to determine the amount of active enzyme through a half-transamination reaction per se, with substrate release.

The catalytically active enzyme coordinates PLP in the active site by hydrogen bonding on the pyridinium nitrogen atom to an acidic amino acid residue and by various hydrogen bond coordinations to the phosphate group. In the enzyme resting state, a covalent bond between a catalytic lysine and the PLP carbonyl carbon forms an internal aldimine. The apo enzyme is catalytically inactive,[3,15] the activity is proportional to the amount of PLP-enzyme complex, the holo enzyme. In addition, the employment of an excess of the cofactor (PLP) has been shown to be detrimental to the observed initial rate of an ω-transaminase variant of *Arthrobacter citreus*.[12] The ω-transaminases are assumed to follow a two step reaction mechanism revealed for α-transaminases.[15] According to this reaction mechanism (Scheme 1), an amino group is transferred from an amino donor to PLP yielding pyridoxamine-5′-phosphate (PMP), while the amino donor is released as the corresponding ketone. Then, an amino acceptor keto compound reacts with PMP to form the

Special Issue: Biocatalysis and Biomimetic Catalysis for Sustainability

Received: June 13, 2011
Revised: July 26, 2011
Published: July 29, 2011

Scheme 1. Transaminases Follow a Two-Step Reaction Mechanism Consisting of Two Half-Transamination Reactions[a]



[a] In resting state, a catalytic lysine coordinates to PLP to form an internal aldimine. In the first half-transamination reaction, an amino donor reacts with the internal aldimine to form pyridoxamine-5′-phosphate (PMP) and a keto by-product. In the second half-transamination reaction, an amino acceptor (a carbonyl compound) reacts with PMP to yield a chiral amine.[15]

corresponding chiral amine and thereby regenerates the PLP.[15] If no amino acceptor is present, only the first half-transamination reaction can occur. Accordingly, in a virtually irreversible reaction the holo enzyme will not be regenerated.

EXPERIMENTAL SECTION

Cloning, Protein Expression, and Purification. The gene for *Chromobacterium violaceum* ω-transaminase was a kind gift from Professor Wolfgang Kroutil, previously described by Koszelewski et al.[5] PCR amplification was performed with the following primers:

Forward primer with *Nhe*I restriction site, 5′-CATATGGC-TAGCCAGAAACAGCG-3′

Reverse primer with *Hind*III restriction site, 5′-GCAGAA-GCTTTTAGGCCAGACC-3′

After digestion (*Nhe*I, *Hind*III), the product was inserted in the plasmid pET28a(+) with an N-terminal His_6-tag and sequence verified (Eurofins MWG Operon, Ebersberg, Germany). The construct was transformed to *Escherichia coli* BL21(DE3) by electroporation. Expression was done by mixing a 20 mL overnight culture with 180 mL of Luria−Bertoni (LB) medium with 50 mg/L kanamycin and 0.4 mM IPTG and incubating for 24 h at 25 °C (150 rpm, baffled flask). The cells were thereafter separated from the medium by centrifugation, resuspended in IMAC binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4), and disrupted by addition of BugBuster 10× (Merck); cell debris was removed by centrifugation and filtration. The obtained solution was applied to a column with Chelating Sepharose FastFlow resin (GE Healthcare) pretreated with a saturated water solution of Cobalt(II) chloride. After it was washed with the binding buffer, the His_6-tagged enzyme was eluted with IMAC elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4). An excess of cofactor (PLP) was added and the solution was incubated at room temperature for 20 min, before desalting on a PD10 column (GE Healthcare). The holo enzyme, dissolved in 50 mM Na_2HPO_4, pH 7.0, was lyophilized.

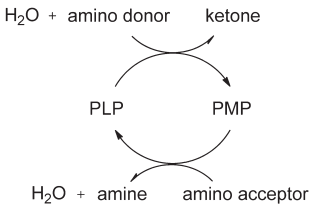
Active Site Quantification. The lyophilized enzyme was quantified by weight, with correction for the buffer content by lyophilization of an equal volume of pure buffer and subtracting its mass from that of the enzyme lyophilized in the same buffer. Milli-Q-H_2O was added and the lyophilized enzyme was redissolved, the absorbance at 280 and 395 nm was recorded. Racemic 1-phenylethylamine was added to 200 μM to solutions of different enzyme concentrations (6.57, 13.1, 19.7, 26.3, and 32.9 μM). The resulting mix could be spectrophotometrically studied at 245 nm to follow the formation of acetophenone. After completion of the reaction, when the absorbance no longer increased significantly, the solutions were acidified by addition of $HClO_4$ and filtered through 0.45 μm syringe filters. HPLC analysis was performed with UV detection at 254 nm (Waters 616 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. Retention times for the enantiomers of 1-phenylethylamine were 36 min (S) and 47 min (R).

Kinetic Experiments. Reaction rates for the amination of acetophenone (1.2 mM or otherwise specified) with isopropylamine (200 mM or otherwise specified) as donor were measured by following the consumption of acetophenone at 285 nm[12,16] on a dual-beam spectrophotometer (Cary300, Varian Inc.). Isopropylamine solutions were prepared by mixing Milli-Q-H_2O, the desired buffer salts, isopropylamine (1.0 M), and HCl (1.0 M). After heating to the desired temperature the pH was set. A pH profile was made by mixing different buffer salts (HEPES, TRIS, borate, and CHES, 50 mM) to afford a buffer mix suitable over a large range, thereafter the reaction rate was measured at pH 6.0,

dx.doi.org/10.1021/cs200315h |ACS Catal. 2011, 1, 1051–1055

Scheme 2. (A) Complete Two-Step Transamination Reaction and (B) a Half Transamination Reaction with 1-Phenylethylamine as the Amino Donor Forming Acetophenone and apo Enzyme[a]

A)



B)

[a] This equilibrium reaction is in aqueous media strongly shifted towards products because of the consumption of a water molecule, and also because of stabilizing electron resonance effects in the formed acetophenone.

6.5, 7.0, 7.5, 7.75, 8.0, 8.25, 8.5, 9.0 9.5 and 10.0. The individual effect of different buffer salts (HEPES, TRIS, diethanolamine or potassium borate, 50 mM) was performed at pH 8.3. The reaction rate and time dependent activity loss at different temperatures (25, 30, 35, 37, 40, and 45 °C) was evaluated at pH 8.3 in HEPES buffer (50 mM). Michaelis−Menten kinetics were performed using HEPES buffer (50 mM) at pH 8.3, the pseudo-one substrate kinetics were performed by keeping the isopropylamine concentration constant and varying the acetophenone concentration (0.3, 0.6, 0.9, 1.05, 1.2, 1.35, 1.5, 1.8, 2.1, 2.4, 2.7, 3.0, 3.3, and 3.6 mM), or keeping the acetophenone concentration constant and varying the isopropylamine concentration (2.0, 4.0, 8.0, 10, 20, 40, 50, 80, 100, and 200 mM).

RESULTS

Active Site Quantification for ω-Transaminase. A method for quantifying the amount of active sites of an ω-transaminase, based on a half transamination reaction per se, was constructed and verified with *Chromobacterium violaceum* ω-transaminase. A complete enzymatic transamination reaction occurs as an ordered ping-pong reaction.[15] After amination of the cofactor (PLP) the enzyme is in apo form (with bound PMP). Thereafter an amino acceptor, a ketone, can be aminated by PMP, and the holo enzyme is regenerated. Without an amino acceptor present the holo enzyme can only be regenerated by reformation of the amino donor. The quantification of the active sites can therefore be done by employing only an amino donor, if it results in a virtually irreversible reaction, by quantifying the remaining unreacted amino donor or formed ketone. The half transamination reaction is in water by itself strongly shifted toward the products since a water molecule is consumed. Scheme 2A shows a complete transamination reaction. The chosen half transamination reaction of 1-phenylethylamine to form acetophenone is depicted in Scheme 2B. In this case the formed ketone, acetophenone, is more stable than the corresponding amine due to electron resonance effects.

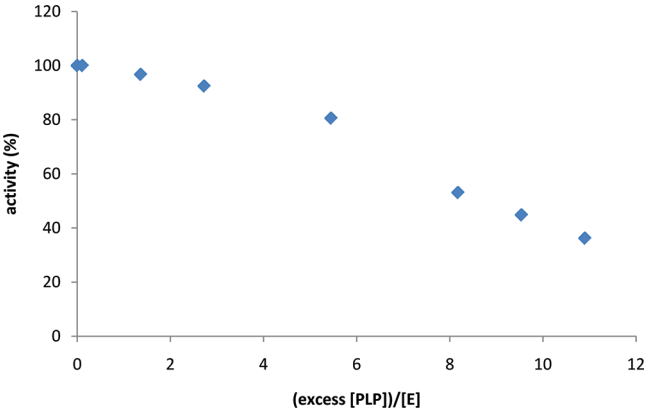


Figure 1. Activity (initial reaction rate as percent of maximum) of *C. violaceum* ω-transaminase versus the excess of the cofactor (PLP). The studied reaction, which was followed spectrophotometrically at 285 nm, was the amination of acetophenone by isopropylamine to produce (*S*)-1-phenylethylamine and acetone.

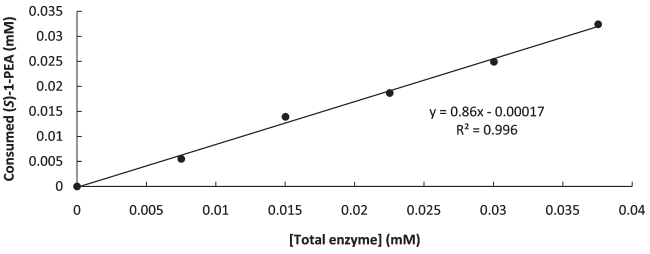


Figure 2. Consumed (*S*)-1-phenylethylamine ((*S*)-1-PEA) versus enzyme concentration. The fraction of active enzyme was in this case calculated to 86%.

Chromobacterium violaceum ω-transaminase was expressed in *E. coli* BL21(DE3) using the vector pET28a(+), with an N-terminal His_6-tag. The cultivation was done by adding a 20 mL overnight culture to 200 mL of LB-medium and directly inducing with IPTG. The average yield was 135 mg/L after 20 h of expression. The enzyme was, after IMAC purification, subjected to an excess of PLP and thereafter desalted. The procedure of desalting after adding excess cofactor ensures a balanced amount, no excess PLP remaining. This was shown to yield the most active enzyme in terms of initial rate measurements, well in accordance with previous results with an optimized variant of *Arthrobacter citreus* ω-transaminase in a similar expression system.[12] Figure 1 shows the initial rate as a function of the excess of cofactor.

Different amounts (<40 μM) of the *C. violaceum* enzyme were mixed with an excess of racemic 1-phenylethylamine (200 μM) at pH 7.0. The formation of acetophenone could be continuously followed spectrophotometrically at 245 nm.[16] The absorbance increased with decreasing rate over a period of approximately 3 h after which it reached a constant level. This was interpreted as the production of acetophenone and apo enzyme. Prolonged measurement (up to 24 h) resulted in a decrease, presumingly because of evaporation of acetophenone. Initially the absorbance change was measured and tested as a method for quantifying the active sites. However, it was found that the background absorbance from other compounds in the mix was high. This, combined with the volatile nature of acetophenone, gave inaccurate measurements. Instead the

dx.doi.org/10.1021/cs200315h |*ACS Catal.* 2011, 1, 1051–1055

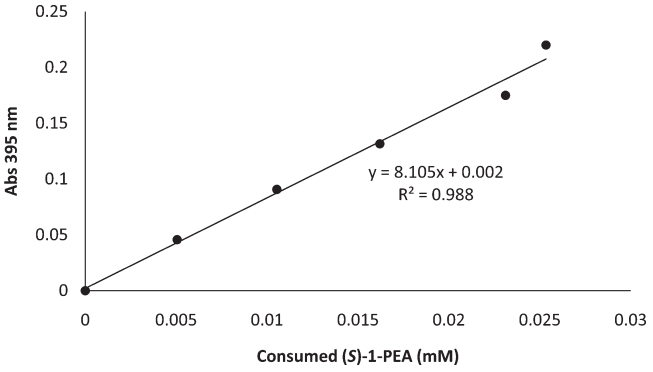


Figure 3. Absorbance at 395 nm versus consumed (*S*)-1-phenylethylamine. By assuming that the PLP−enzyme complex is proportional to active enzyme, the active site concentration can be measured by absorbance with an extinction coefficient of 8.1 mM^{-1} cm^{-1}. The absorbance measurement was performed prior to the proceeding of the reaction.

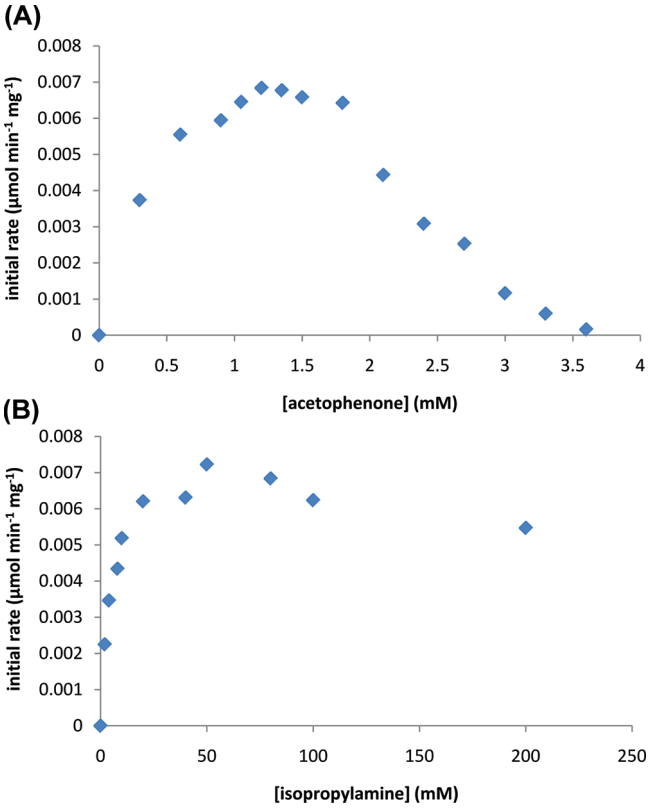


Figure 4. Pseudo-one substrate Michaelis−Menten kinetics showing substrate inhibition. Initial rates were measured spectrophotometrically as the consumption of acetophenone, with isopropylamine as amino donor. In A, the isopropylamine concentration was 200 mM; in B, the acetophenone concentration was 1.2 mM.

amount of consumed (*S*)-1-phenylethylamine was quantified by HPLC. In this case the *E*-value of the enzyme was high ($E = 154$, S-selective), so the peak corresponding to the R-enantiomer was used as internal standard. This procedure also corrected for the amount of imine formed by reaction of the acetophenone with the 1-phenylethylamine, which would occur in equal proportion with both enantiomers. Figure 2 shows the amount of consumed substrate versus the amount of enzyme. There is a clear linear relationship with a slope of 0.86, that is, an active enzyme fraction of 86%.

Figure 3 shows the absorbance at 395 nm (corresponding to where a peak was found in the absorbance spectrum of a solution of PLP-enzyme complex) plotted against the amount of consumed (*S*)-1-phenylethylamine. The absorbance was measured prior to the start of the reaction to ensure the presence of PLP-enzyme complex. Upon addition of 1-phenylethylamine to the enzyme solution the yellow color gradually cleared.

When the enzyme was left in phosphate buffer at room temperature aggregation was observed; after filtration, treatment with PLP and desalting the absorbance difference at 395 nm was proportional to the decrease in measured activity. When an excess of enzyme was added no (*S*)-1-phenylethylamine could be detected after completion of the reaction; this verifies that the equilibrium is shifted toward the products.

Kinetics. Previously, phosphate buffer has been shown to have a detrimental effect on enzyme activity.[17] Also borate lowered the observed initial rate drastically in our experiments. In HEPES buffer the observed specific activity increased significantly (129%) after incubation in room temperature overnight or 4 h in 37 °C, after the IMAC purification. This effect was observed without supplementary addition of PLP.

Pseudo-one substrate Michaelis−Menten kinetics for the amination of acetophenone by isopropylamine, yielding (*S*)-1-phenylethylamine and acetone, was measured. The curves (Figure 4) both show the presence of substrate inhibition. At the found optimum conditions, i.e. 50 mM HEPES pH 8.3, 37 °C, also found by Ward et al.,[17,18] at the found optimal substrate concentrations (1.2 mM acetophenone, 50 mM isopropylamine) the specific activity was 0.0072 μmol/min/mg.

DISCUSSION

The active site quantification method here described provides a means for measuring the active enzyme fraction based on a *half* transamination reaction. This includes the conversion of PLP to PMP and (*S*)-1-phenylethylamine to acetophenone. The equilibrium reaction on which the quantification is based is virtually completely shifted toward products. This allows accurate comparison of cultivation batches and mutants, and may be feasible for any ω-transaminase which is capable of performing the chosen reaction. If one assumes that only a negligible amount of inactive enzyme binds the cofactor, i.e. the amount of bound PLP is proportional to the amount of active enzyme after the desalting step described above; the active enzyme can be quantified by directly measuring the amount of PLP-enzyme complex. This is a fair assumption since unexpected binding modes of PLP, for example, to lysine residues, are likely to be disfavored or of constant amount. In other words, stabilized binding of PLP is assumed to be present in active enzyme and to negligible extent in inactive enzyme. This can be said to be consistent with the linear regression in the graph in Figure 3, which closely passes through origo. According to this graph and the given assumptions, the active enzyme can be measured as the absorbance at 395 nm, after desalting an enzyme solution with excess PLP, with an apparent extinction coefficient of 8.1 mM^{-1} cm^{-1}. This is only valid for freshly desalted enzyme, before a significant amount has been degraded. If this measurement would be performed with a mutant the stability of the PLP-enzyme complex would have to be assured, since poor cofactor retention is likely to lead to a measurement error regarding the active enzyme fraction; if PLP is released and

degraded in solution[19] before the desalting step the apo enzyme will not be included in the measurement. Still, any following rate measurements will yield the specific activity provided that no extra PLP is added. Correlation of the active enzyme amount to the absorbance reading is required. If the PLP-enzyme complex is stable during and before the desalting additional PLP may be added after the absorbance based recording of holo enzyme, should it be necessary.

When freshly purified enzyme was kept in HEPES buffer at room temperature overnight or at 37 °C for four hours the activity increased by 129%. This may be a result of complete dimerization to form active enzyme. The active site is formed by both subunits of the homodimer. Since no PLP had to be added to observe the activity increase, the monomer alone has to be able to bind PLP; the enzyme was submitted to an excess of PLP before being desalted, before the time-dependent activation was observed.

Possibly, the presence of phosphate or borate can disturb the binding of PLP, which contains a phosphate group, in the active site. The low activity in phosphate or borate buffer may be interpreted as a competitive inhibitor effect.

It is possible that apo enzyme with bound PMP is formed after expression of the enzyme in *E. coli* which, unless purged, will cause an error in the active site quantification. Intact E:PMP will not contribute to the consumption of the (*S*)-1-phenylethylamine, still, it should be considered as active enzyme. Seo et al. have recently shown that the E:PMP can in fact be used for amination of the keto substrate.[20] In our case significant abundance of PMP is improbable since the enzyme was effectively rinsed when bound to the IMAC column and then subjected to an excess of PLP, which binds more effectively than PMP. When necessary a suitable amino acceptor, for example, pyruvate, may be added together with the excess of PLP after the purification to ensure the complete formation of E-PLP even though E:PMP is present.

CONCLUSION

An active site quantification method for ω-transaminases was devised, based on the simple procedure of adding an amino donor, in this case 1-phenylethylamine, but no amino acceptor, to enzyme with a balanced amount of the cofactor (PLP). This results in the conversion of PLP to PMP, and production of the corresponding ketone, acetophenone in this case. This equilibrium reaction greatly favors the products. Hence, the amount of active sites can be measured by quantifying the substrate. The obtained data was compared to the absorbance reading at 395 nm, a quantification of the amount of PLP-enzyme complex. The absorbance at 395 nm can be used for quickly measuring the amount of active enzyme, once the relation between the absorption and the active site concentration has been determined, assuming that all PLP-enzyme complexes are active or inactive to a negligible amount.

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ACKNOWLEDGMENT

The authors express their gratitude towards VINNOVA (The Swedish Governmental Agency for Innovation Systems) for financial support and Prof. Wolfgang Kroutil, Graz University, Austria, for supplying the gene for *C. violaceum* ω-transaminase.

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