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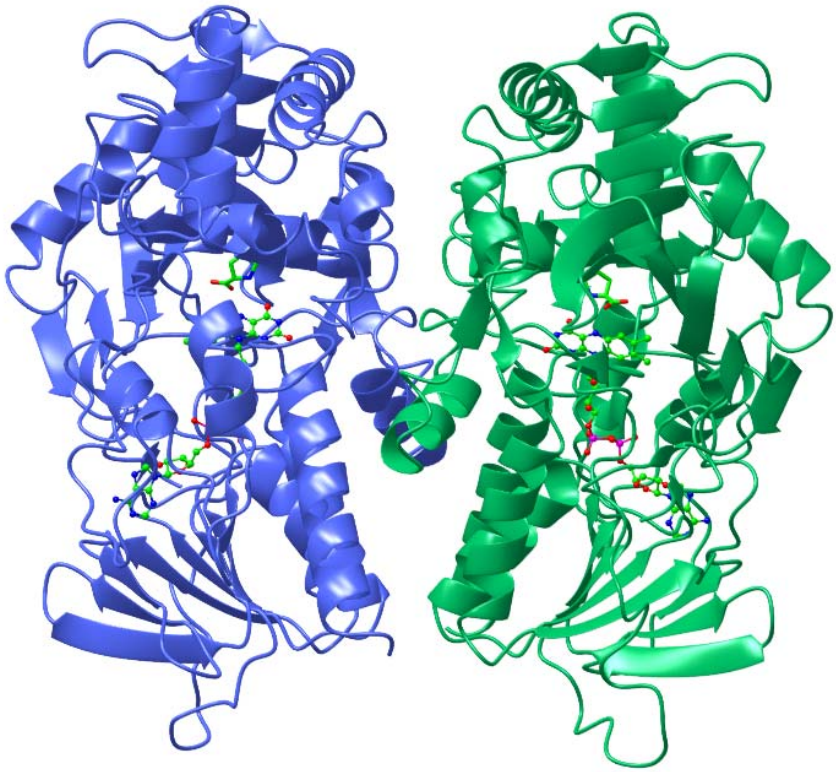

Transaminations with isopropyl amine: equilibrium displacement with yeast alcohol dehydrogenase coupled to *in situ* cofactor regeneration†‡

Karim Engelmark Cassimjee,[a] Cecilia Branneby,[b] Vahak Abedi,[c] Andrew Wells[d] and Per Berglund*[a]

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Enantiopure chiral amines synthesis using ω-transaminases is hindered by an unfavourable equilibrium, but when using isopropylamine as the amine donor the equilibrium can be completely displaced by using a specific dehydrogenase *in situ* for removal of formed acetone.

The production of chiral amines of high enantiomeric excess is of great interest since such motifs are present in a plethora of pharmaceuticals and other fine chemicals. Use of ω-transaminases (ω-TA) for this purpose has been shown to be feasible and effective.[1–5] All known transaminases use pyridoxal-5-phosphate (PLP) as a cofactor, providing a route for conversion of an amine to the corresponding ketone, yielding pyridoxamine-5-phosphate (PMP). The PMP thereafter functions as an amine donor by converting a ketone of choice to the corresponding chiral amine.[6] A thermodynamic analysis of such a system shows that the equilibrium is most often shifted towards the substrates since the ketone of choice is usually much more stable than the corresponding amine, due to electron resonance effects.[7] Therefore, different methods for shifting the equilibrium have been devised by coupling the reaction with secondary irreversible reactions. Then the yield can theoretically reach 100%. This has been shown to be effective with an amino acid, *e.g.* alanine, as the amine donor as the produced ketoacid can be further reacted with effective enzymes such as pyruvate decarboxylase.[7–12]

The use of isopropyl amine as the amine donor is, from an industrialist's point of view, more desirable since this chemical is easily obtainable and cost efficient. The formed acetone in such a case can be removed by distillation, thus shifting the equilibrium and good yields can be obtained.[9,13,14] However, in large scale equipment it is not always feasible to effectively remove highly soluble volatile substances like acetone from aqueous mixtures, also raising the temperature to assist this process requires a more heat stable enzyme. In this work, an alternative method for removal of formed acetone with a commonly available commercial dehydrogenase was used. The substrate ketone was not affected by the dehydrogenase due to its narrow substrate specificity, a good example of effective use of enzyme selectivity in a cascade reaction.

Yeast alcohol dehydrogenase (YADH; *Saccharomyces cerevisiae*) is a well described enzyme with a narrow substrate range. The active site of this enzyme is unable to accommodate larger groups, methanol/methanal and ethanol/ethanal are effectively converted, as well as isopropyl alcohol/acetone, although at lower rate. For substrates with four carbons the reaction is extremely slow, and for bigger substrates no reaction has been observed. YADH should therefore be effective for removal of the acetone in a transamination reaction where isopropyl amine is the donor (Fig. 1). The amine acceptor (acetophenone in Fig. 1) will not be a substrate for YADH as long as it is not small, which is rarely the case.[15]

Conversion of acetone to isopropyl alcohol by YADH requires NADH, which must be regenerated for a cost efficient synthesis. In this work we used formate dehydrogenase (FDH). Other reports include glucose dehydrogenase for the same purpose.[16]

The synthesis with the designed equilibrium displacement system was tested with the ω-transaminase from *Arthrobacter citreus* variant denoted S9 (Cambrex Karlskoga AB) in purified form after cloning and overexpression, and with the enzyme ATA-113 (Codexis). The gene for *A. citreus* variant S9 was cloned into the vector pET28a(+) with an N-terminal His_6-tag and effectively expressed in *E. coli* BL21(DE) at

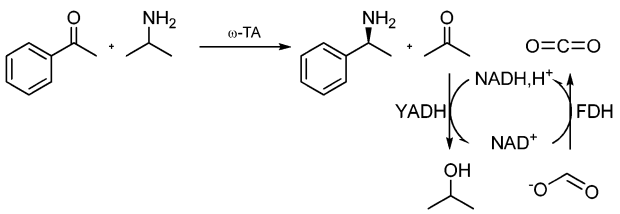

Fig. 1 Effective synthesis of (*S*)-1-phenylethylamine through the transamination of acetophenone with isopropylamine as donor, by using an *S*-selective ω-transaminase variant from *A. citreus*, denoted ω-TA S9 (Cambrex Karlskoga AB). The employment of an equilibrium displacement system consisting of yeast alcohol dehydrogenase (YADH, *S. cerevisiae*) and formate dehydrogenase (FDH, *Candida boidinii*) drives the reaction to completion.

[a] *Division of Biochemistry, School of Biotechnology, Royal Institute of Technology (KTH), 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] *Cambrex Karlskoga AB, SE-691 85 Karlskoga, Sweden. E-mail: cecilia.branneby@cambrex.com; Fax: +46 586 783129; Tel: +46 586 783000*
[c] *AstraZeneca R&D, SE-151 85 Södertälje, Sweden. E-mail: vahak.abedi@astrazeneca.com; Fax: +46 8 5532 4276; Tel: +46 8 5532 1766*
[d] *AstraZeneca Global Process R&D, Bakewell Road, Loughborough, Leics, UK LE11 5RH. E-mail: andrew.wells@astrazeneca.com; Fax: +44 (0)1509 645588; Tel: +44 (0)1509 644439*
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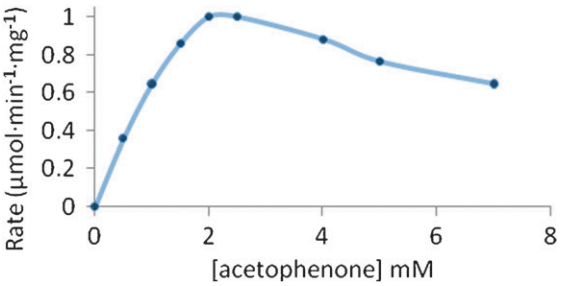


Fig. 2 Initial rate of the ω-transaminase at different concentrations of the amine acceptor acetophenone. The concentration of isopropylamine was kept at 750 mM.

19 mg l^{-1}, and purified by IMAC. Use of pure transaminase yields definite information regarding the enzyme kinetic function, as opposed to a whole cell system approach that is commonly used for this enzyme. This transaminase was extremely enantiospecific, no *R*-enantiomer could be detected.

The production of enantiopure (*S*)-1-phenylethylamine is seemingly straight forward when using an ω-transaminase and acetophenone as the amine acceptor. However, acetophenone has been found to be a less than effective substrate for many transaminases due to substrate inhibition.[17] The purified enzyme S9 was found to be virtually inactive at high acetophenone concentrations, with the highest reaction rate at approximately 2.5 mM (0.97 μmol min^{-1}·mg^{-1} in 50 mM sodium phosphate, pH 7.0, 37 °C). Fig. 2 shows the initial reaction rate *versus* concentration of acetophenone. The reaction was easily followed spectrophotometrically by tracking the consumption of acetophenone at 285 nm, in close accordance with Schätzle *et al.*[18] At 245 nm the molar extinction coefficient of acetophenone is 12 mM^{-1} cm^{-1} which is at a local maximum. Here 285 nm was used where the molar extinction is 1.0 mM^{-1} cm^{-1}. With the same assay the K_M for isopropyl amine was calculated to 260 mM (50 mM sodium phosphate, pH 7.0, 37 °C).

The concentration of the cofactor (PLP) was found to be crucial for the function of the enzyme. High concentrations led to inactivation. By adding an excess of PLP and then desalting the enzyme solution through a PD10 column (GE Healthcare), a balanced amount of PLP to enzyme was obtained. This was found to be the most efficient enzyme preparation, adding more PLP lowered the activity. Fig. 3 shows the effect of adding additional PLP, *i.e.* above the concentration of

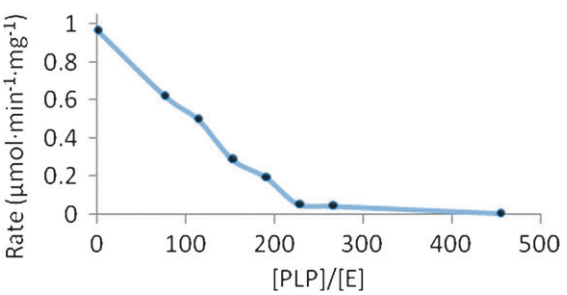


Fig. 3 The effect of excess [PLP], *i.e.* adding cofactor that exceeds the enzyme concentration. Enzyme concentration was kept constant at 0.16 g l^{-1}. With no excess PLP ([PLP]/[E] = 1) the maximum rate of 0.97 U mg^{-1} was obtained, where 1 U is defined as the formation of 1 μmol of (*S*)-1-phenylethylamine per minute.

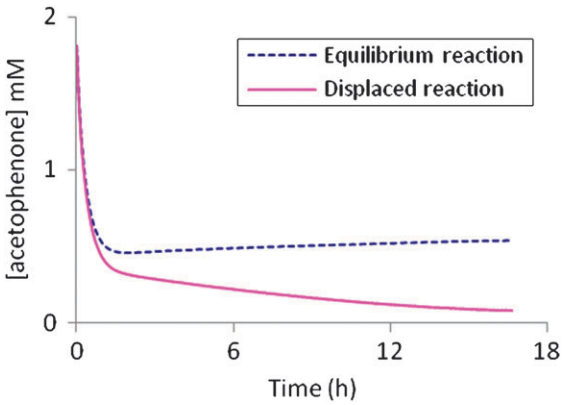
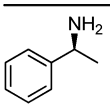
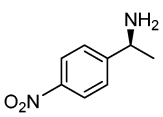
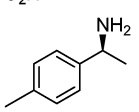
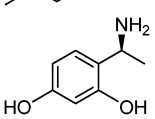


Fig. 4 Comparison between the equilibrium displaced reaction and a system without YADH/FDH, continuously followed by the consumption of acetophenone at 285 nm.

enzyme, on the activity. It is clear that an excess of cofactor is detrimental to the reaction rate.

Even at a large excess of amine donor the reaction failed to reach completion due to the unfavourable equilibrium. The viability of the equilibrium displacement system was tested by comparison with a reaction without YADH/FDH. The reactions were continuously followed on a spectrophotometer by tracking the consumption of acetophenone (285 nm). Fig. 4 shows the continuation of the displaced reaction, while the non-displaced one stops at equilibrium conversion. Table 1 shows the conversion and enantiomeric excess of the products from a range of acetophenone amine acceptors as determined by HPLC. In the equilibrium displaced system the conversion virtually reaches completion while the enantiomeric excess is unchanged.

Table 1 Comparison of the displaced and non-displaced reactions at 24 h with 560 mM isopropyl amine and ω-transaminase S9. The data were obtained by HPLC and the product structures verified by GC-MS

		Conversion (%)	
Product amine	ee_p (%)	No displacement	With displacement
	> 99.9	82[a]	> 99[a]
	> 99.9	63[b]	> 99[b]
	> 99.9	89[c]	> 99[c]
	> 99.9	68[c]	> 99[c]

[a] 1.8 mM acetophenone. Conversions were calculated based on formed product. [b] 20 mM 4-nitroacetophenone (two phase system as saturation is reached at ~2 mM). Conversions were calculated based on formed product. [c] 20 mM ketone substrate. Conversions were calculated based on consumed substrate.

To further explore the versatility of the system the transaminase ATA-113 from Codexis was also tested. Together with YADH and the cofactor regeneration the reaction went to a conversion of >99% with (*S*)-1-phenylethylamine of >99.9% ee, as previously shown.[9] Similar to the data in Table 1, the non-displaced reaction showed 82% conversion.

In conclusion, the formed acetone was effectively removed by employment of YADH, *S. cerevisiae*, when using isopropyl amine as amine donor in a transamination reaction. Complete conversion was achieved by regenerating the cofactor (NADH) with formate dehydrogenase *in situ*. Furthermore, the concentration of the cofactor PLP was found to be of great importance since a higher than equimolar amount resulted in markedly decreased enzyme activity.

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