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Enzymatic Network for Production of Ether Amines From Alcohols

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ABSTRACT: We constructed an enzymatic network composed of three different enzymes for the synthesis of valuable ether amines. The enzymatic reactions are interconnected to catalyze the oxidation and subsequent transamination of the substrate and to provide cofactor recycling. This allows production of the desired ether amines from the corresponding ether alcohols with inorganic ammonium as the only additional substrate. To examine conversion, individual and overall reaction equilibria were established. Using these data, it was found that the experimentally observed conversions of up to 60% observed for reactions containing 10 mM alcohol and up to 280 mM ammonia corresponded well to predicted conversions. The results indicate that efficient amination can be driven by high concentrations of ammonia and may require improving enzyme robustness for scale-up.

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KEYWORDS: biocatalysis; alcohol dehydrogenase; aminotransferase; amination; ether amines

Introduction

Ether and polyether amines are valuable synthetic compounds with applications in diverse areas such as surface chemistry, colloid chemistry, and bioconjugate chemistry (Bayat and Akarsu, 2002;

Hedberg, 1970; Mongondry et al., 2003). Polyether amines may be used as curing agents, silica deposition agents, in paintings, or as intermediates in the synthesis of polyamides or polyureas (Brandl et al., 2010; Harris et al., 1984; Lin et al., 2010). Ether amines can be produced chemically by a reaction of haloalkyl ethers with ammonia or diamines at high pH and high temperature (Buckmann et al., 1981), or by the cyanoethylation of ether alcohols with acrylonitrile followed by hydrogenation of the corresponding cyanoethers (Froimowicz et al., 2005). A more cost-effective process would be the direct conversion of an ether alcohol, using ammonia as the amine donor. Chemical manufacturing processes have been developed for such conversions employing metal catalysts (Lafrance et al., 2012) or by making use of other well-known chemical reactions (Swamy et al., 2009). However, the lack of enantio- and regioselective control often hampers these processes by formation of undesired side-products. Furthermore, the catalysts are toxic and the reactions typically require harsh conditions involving high energy use and environmental costs (Imm et al., 2011). Hence, a biocatalytic process would be an attractive alternative if it affords high efficiency and selectivity with a low energy consumption and little use of raw materials.

As there is no enzyme known to catalyze the conversion of an alcohol to the corresponding amine in a single step, multi-enzyme routes have been considered to access amines (Kohls et al., 2014; Kroutil et al., 2013; O'Reilly and Turner, 2015; Simon et al., 2014; Villegas-Torres et al., 2015). One attractive solution emulates a short metabolic pathway via an enzymatic network process that oxidizes an alcohol to an aldehyde or ketone catalyzed by an alcohol dehydrogenase (ADH), and subsequent transamination of the carbonyl compound to an amine by an aminotransferase (AT). If L-alanine is used as amino donor and NAD as electron-accepting cofactor for the dehydrogenase, their recycling is required for an efficient process. This can be done with an alanine dehydrogenase (AlaDH), which oxidizes the reduced cofactor and synthesizes alanine from pyruvate (Sattler et al., 2012). Theoretically, the recycling would allow the use of only low concentrations of NAD and alanine. In practice, a large excess of alanine has been used

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(Sattler et al., 2012), which makes it possible to overcome equilibrium or product inhibition (Shin and Kim, 2002) issues that might prevent efficient cycling in the network.

Variations of this network have been described, including, the use of other enzymes for cofactor recycling (Klatte and Wendisch, 2014, 2015; Klatte et al., 2014; Kroutil et al., 2013). For example, Tauber et al. (2013) examined regeneration of the reduced nicotinamide cofactor formed during alcohol oxidation by an NADP-dependent alcohol dehydrogenase and an NADPH oxidase, while the NADH required by alanine dehydrogenase was recycled by a formate dehydrogenase. The same group investigated the use of a lactate dehydrogenase to reduce pyruvate to lactate in order to shift the conversion of the aminotransferase step to completion. Such modifications can increase yields, but conversion becomes dependent on auxiliary reactions, that is, formate oxidation and pyruvate reduction. The two-step transformation of an alcohol to the corresponding amine has also been applied for the conversion of caprolactone to 6-aminohexanoic acid (Sattler et al., 2014), in which case a methyl ester was transiently generated to prevent formation of 6-hydroxyhexanoic acid. Recently, a variation of such a network was described in which an engineered amine dehydrogenase rather than an aminotransferase was used for direct incorporation of ammonia into ketones (Mutti et al., 2015). These studies indicate that various measures can be undertaken to improve conversion in an amination network, and raise the questions which factors restrict conversion by a self-sufficient network when no auxiliary reactions are included for recycling of intermediates.

To examine the suitability of a self-sufficient network for ether amine formation and study the factors that determine conversion, we investigated a biocatalytic process that uses ether alcohols and ammonia as the substrates to produce the ether amines. For this purpose, a redox-neutral enzymatic network of three enzymes was used (Sattler et al., 2012). Different alcohol dehydrogenases, aminotransferases, and alanine dehydrogenases were tested, and the characteristics of the system were studied by measurement of reaction equilibria, which are important parameters for the design and optimization of such biocatalytic networks (Tufvesson et al., 2012). Only few equilibrium constants for amination and alcohol oxidation reactions have been reported in the literature (Goldberg and Tewari, 1994; Goldberg et al., 2004, 2007; Tufvesson et al., 2011). We selected a primary ether alcohol (butyldiglycol, **1a**) and a secondary ether alcohol (1-butoxy-2-propanol, **2a**) as model substrates, as both the alcohols and amines are miscible with water and ether amines derived from similar compounds are used in industrial products. From experimentally determined conversion curves and reaction equilibria, it appeared that very high concentrations of ammonia can drive conversion toward effective synthesis.

Materials and Methods

Substrates and Chemicals

Butyldiglycol (**1a**), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide (NADH), L-alanine, ammonium carbamate, and sodium carbonate were purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands). Compounds **1b,c** and **2a–c** were prepared by BASF (Ludwigshafen, Germany;

see Supporting Information). Substrate **2a** was the (S)-enantiomer, in agreement with the enantioselectivity of the ADH that was selected. Pyridoxal phosphate (PLP) was purchased from Fisher Scientific (Landsmeer, the Netherlands) and pyruvic acid was acquired from Acros Organics (Geel, Belgium).

Enzymes

Enzymes tested as catalysts for the enzymatic network were alcohol dehydrogenases from *Geobacillus stearothermophilus* 1 (GsADH1, accession number BAA14411), *G. stearothermophilus* 2 (GsADH2, CAA80989), *Pseudomonas putida* (PpADH, ADR59556), or *Rhodococcus jostii* RHA1 (RjADH, ABG94302.1). Aminotransferases were from *Chromobacterium violaceum* (CvAT, NP_901695), *Vibrio fluvialis* (VfAT, AEA39183), and *Escherichia coli* putrescine aminotransferase (EpTA, NP_417544). The two alanine dehydrogenases examined were from *Archeoglobus fulgidus* (AfAlaDH, AIG98665.1) and *Vibrio proteolyticus* (VpAlaDH, AF070716). The coding DNA segments were obtained by gene synthesis or PCR and cloned into the expression vector pET28b or pBAD to add a hexahistidine tag (Table I). The constructs were confirmed by DNA sequencing.

The enzymes were produced in *E. coli* C41(DE3) or *E. coli* TOP10 (Table I). For this, cells were grown at 37°C in TB medium with 50 µg/mL kanamycin. Expression was induced by addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG; pET system) or 0.02% arabinose (pBAD system) when the optical density (OD₆₀₀) reached ca. 0.6. Cultivation was continued for 16 h at 28°C and 135 rpm. Cells were obtained by centrifugation and disrupted by sonication at 4°C, followed by centrifugation for 45 min at 15,000 rpm to obtain the cell-free extract. The enzymes were purified in a two-step procedure using metal affinity chromatography (HisTrap HP column; 5 mL; GE Healthcare, Eindhoven, the Netherlands) and a desalting step (HiPrep 26/10 column, 53 mL, GE Healthcare). The enzymes were stored frozen at –20°C in 50 mM potassium phosphate, pH 8.0.

Alcohol Dehydrogenase Reactions

Activities of different alcohol dehydrogenases were measured by following NAD reduction at 340 nm ($\epsilon_{\text{NADH}} = 6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). The reaction mixtures of 1 mL contained ammonium carbamate (40 mM, pH 9), alcohol dehydrogenase (1 mg/mL), NAD⁺ (1 mM) and alcohol **1a** or **2a** (10 mM). For estimating reaction equilibria, the assay system consisted of the same buffer, a suitable amount of GsADH (ca. 0.1 mg/mL), NAD⁺ (0.4 mM), and alcohols **1a** (5 mM) or **2a** (0.4 mM) in a final volume of 1 mL. For the reverse reaction, the same enzyme concentration and buffer system were used with NADH (0.4 mM) and aldehyde **1b** (5 mM) or ketone **2b** (0.4 mM). The formation or depletion of NADH was followed at 340 nm at 30°C. Equilibria were calculated from Equation (2), assuming stoichiometric relationships between the change in NADH and alcohol or aldehyde.

Aminotransferase Reactions

Activities of different aminotransferases were determined by following acetophenone formation at 245 nm ($\epsilon = 12 \text{ mM}^{-1} \text{ cm}^{-1}$)

Table I. Expression and catalytic activities of enzymes for use in amination network.

Reaction	Enzymes	Gene ^a	Expression system	Expression (mg/L)	Activity ^c (mU/mg)	
					1a/1b/L-Ala	2a/2b/L-Ala
ADH	<i>Rhodococcus jostii</i> RHA1	PCR	pET/C41(DE3)	6	<1	<1
	<i>Geobacillus stearothermophilus</i> 1	PCR	pET/C41(DE3)	100	74	834
	<i>Geobacillus stearothermophilus</i> 2	PCR	pET/C41(DE3)	200	55	483
	<i>Pseudomonas putida</i>	Syn ^b	pET/C41(DE3)	10	12	46
AT	<i>Vibrio fluvialis</i>	Syn ^b	pET/C41(DE3)	160	3	62
	<i>Chromobacterium violaceum</i>	PCR	pET/C41(DE3)	200	5	78
	<i>E. coli</i> putrescine AT	Syn ^b	pET/C41(DE3)	80	<1	<1
AlaDH	<i>Archeoglobus fulgidus</i>	PCR	pBAD/TOP10	20		2 × 10 ⁴
	<i>Vibrio proteolyticus</i>	PCR	pBAD/TOP10	300		1 × 10 ⁴

^aGene obtained by PCR amplification of genomic DNA or as synthetic gene.

^bSynthetic gene.

^cOne unit of enzyme activity corresponds to a conversion of 1 μmole/min under the assay conditions used. Data represent averages of measurements done in duplicate or triplicate. Average standard deviations estimated from multiple duplicate measurements were less than 10% of the reported values.

as described by Schätzle et al. (2010). The reaction mixtures contained 1-phenylethylamine (2 mM), **1b** or **2b** (10 mM), sodium phosphate (50 mM, pH 7), and aminotransferases (0.2 mg/mL).

Equilibria were estimated by incubating purified enzyme in ammonium (sodium) carbamate buffer (40 mM, pH 9) with CvAT (0.35 mg/mL), L-Ala (10 mM), ketone **2b** (1 mM), and PLP (0.35 mM). Samples (1350 μL) were taken and quenched by adding NaOH (150 μL, 10 N) and ethyl acetate (500 μL). The mixture was stirred with a Vortex mixer for 30 s, sonicated, centrifuged for 1 min at 13,200 rpm and analyzed. The concentrations of the ketone **2b** and amine **2c** were determined by GC analysis, whereas the concentrations of pyruvate and L-Ala were calculated from the stoichiometry. Equilibria were calculated with Equation (3).

Alanine Dehydrogenase Reactions

Reaction mixtures contained enzyme (0.002 mg/mL), ammonium carbamate (40 mM, pH 9), NADH (0.2 mM), pyruvate (10 mM), and ammonium carbonate (125 mM). Activities were calculated from the rates of pyruvate-dependent NADH oxidation, measured at 340 nm.

The assay system for establishing the equilibrium of the alanine recycling reaction consisted of sodium carbonate (40 mM, pH 9), NAD (0.4 mM), VpAlaDH (0.004 mg/ml), and L-alanine (2.5 mM) in a final volume of 1 mL. The conversion was followed by monitoring the formation of NADH at 340 nm, while the concentrations of the other components were calculated from the reaction stoichiometry.

Network Reaction Conditions

Three-enzyme conversions were routinely performed in reaction mixtures (15 mL) containing ammonium carbamate buffer (40 mM, pH 9), alcohol **1a** or **2a** (10 mM, final concentration), NAD⁺ (1 mM), L-Ala (0.5 mM), PLP (0.35 mM), GsADH (0.1 mg/mL), VpAlaDH (0.004 mg/mL for **1a** and 0.04 mg/mL for **1b**) and CvAT (0.064 mg/mL). The mixtures were incubated in 50 mL glass bottles at 30°C, and samples were taken at different times for analysis by gas chromatography (GC). Apparent equilibrium constants (K') were

calculated with Equation (1). For these calculations and in Tables and Figures, we used nominal concentrations of ammonia representing the sum of protonated and unprotonated species and assuming complete dissociation of carbamate to 2 equivalents of ammonia (Wang et al., 1972).

Optimization was studied by individually varying concentrations of ammonium (80 mM, 200 mM, 400 mM, 1 M, or 2 M), alcohol **1a** (5, 10, 20, 50, and 100 mM), L-Ala (0.5, 1, 2.5, and 5 mM), and NAD⁺ (0.125, 0.25, 0.5, and 1 mM). PLP was kept at 0.35 mM and enzyme concentrations were GsADH at 0.1 mg/mL, VpAlaDH at 0.004 mg/mL, and CvAT at 0.064 mg/mL.

Results and Discussion

Enzyme Production and Selection

To establish a three-enzyme network for alcohol to amine conversion, we first examined several enzymes for each reaction step. We cloned and expressed four alcohol dehydrogenases for the oxidation of **1a** and **1b**, three transaminases for the transamination of the corresponding aldehyde (**1b**) and ketone (**2b**), and two alanine dehydrogenases for the recycling of the cofactors and amino donors (Table I). All enzymes were expressed in soluble form and were purified by His-tag affinity chromatography.

Activity assays with the purified enzymes were performed as described under Materials and Methods to select suitable enzymes for the designed network. The results in Table I show that the thermostable ADH from *G. stearothermophilus* 1 (GsADH1) and the aminotransferase from *C. violaceum* (CvAT) had the highest activity toward **1a** and **2a**, and **1b** and **2b**, respectively. These were, therefore, selected for further use. The thermostable and highly active AlaDH from *V. proteolyticus* (VpAlaDH) was used for cofactor recycling because of its high activity and high overexpression in *E. coli*.

Network Reactions

Having selected suitable enzymes, we tested the possibility for a network reaction with **1a** and **2a** as substrates (10 mM) in the presence of all three enzymes (Fig. 1). In the initial tests, eight

Figure 1. Enzymatic network with **1a** and **2a** as substrates. The proposed enzymatic cycle encompasses three enzymes: an alcohol dehydrogenase (*GsADH1*) that converts the alcohol to a ketone or an aldehyde; a transaminase (*CvAT*) that converts the ketone or the aldehyde to the amine with L-Ala as the amino donor; and an alanine dehydrogenase (*VpAlaDH*) that converts pyruvate to L-Ala with simultaneous recycling of NADH to NAD⁺.

equivalents of ammonia and one equivalent of L-Ala were added (final concentrations 80 and 10 mM, respectively). For both substrates, conversion of ether alcohol to ether amine reached a promising 40%, using the network reaction conditions described in Materials and Methods.

We next examined the effect of varying concentration of substrates on the conversion, aiming at a higher yield of the ether amines. For this, we performed time course experiments in which supposedly important conditions were varied, especially the concentrations of alcohol substrate, cofactor (NAD⁺), L-Ala, and ammonium. First, different concentrations of alcohol **1a** were tested in the range from 5 to 100 mM. The highest yields and conversions rates were observed with the lower concentrations of **1a** (Fig. 2A). The highest accumulation of product **1b** was obtained with 50 mM alcohol, but the conversion of substrate to amine decreased at the highest concentration of **1a** almost to zero. Further reactions were carried out with alcohol concentrations in the range of 5 to 10 mM because these gave the best conversion (Fig. 2A).

Next, the effect of increasing ammonium concentrations was measured as this might shift the equilibrium of the reaction. However, increasing the concentration by adding different level of ammonium (80 mM, 200 mM, and 1M) did not improve the percentage of conversion of **1a–c**, and conversion was significantly reduced above 200 mM ammonia while precipitation of enzymes was observed. The best conversion was observed at 80 mM of ammonia (Fig. 2B).

We also examined the effect of varying L-Ala concentrations. The conversion to amine **1c** was only slightly affected by the level of L-Ala added (Fig. 2C). Importantly, at a low concentration of L-Ala (0.5 mM) conversion to amine already reached 30%, and conversion was not better at higher levels. Furthermore, L-Ala was indeed recycled, as the level of amine produced (3 mM) was sixfold higher than the level of L-Ala added (0.5 mM). Subsequent

experiments on the amination of **1a** and **2a** were carried out with 0.5 mM L-Ala.

Finally, the level of NAD was varied. Good conversion was obtained with 1 mM NAD added (Fig. 2D), whereas conversion was reduced when the cofactor concentration was lower. As the final concentration of product **1c** (4 mM) was higher than that of cofactor (1 mM), also AlaDH-mediated cofactor recycling was working properly. Further experiments were carried out with 1 mM NAD⁺.

A series of optimization measures were tested, in which substrate, amino donor, ammonium, and cofactor concentrations were varied in different combinations. These attempts did not lead to identification of reaction conditions that improved conversion beyond 40% for **1a–c** and conversion also did not exceed 40% for **2a–c**. This suggests that equilibrium factors, in combination with effects of ammonia on enzyme activity, were limiting the degree of conversion.

Reaction Equilibria

In agreement with observations on other reactant systems (Höhne et al., 2008), the thermodynamic equilibrium was postulated to limit conversion in the three-enzyme amination network. To examine this possibility, we measured the apparent equilibrium constants of the three-enzyme network and of separate reactions. Following the notation of Goldberg (2014), the equilibrium for the overall conversion of alcohol to amine (K'_N) is given by Equation (1), while Equation (2) gives the apparent equilibrium constant for alcohol oxidation (K'_o), Equation (3) for the aminotransferase reaction, and K'_r in Equation (4) stands for the recycling step or alanine dehydrogenase reaction. The numbers are pH-dependent. All measurements were done at pH = 9.0.

$$K'_N = \frac{[\text{Ami}]}{[\text{Alc}][\text{NH}_4^+]} \quad (1)$$

$$K'_o = \frac{[\text{NADH}][\text{Ald}]}{[\text{NAD}^+][\text{Alc}]} \quad (2)$$

$$K'_t = \frac{[\text{Pyr}][\text{Ami}]}{[\text{Ala}][\text{Ald}]} \quad (3)$$

$$K'_r = \frac{[\text{NAD}^+][\text{Ala}]}{[\text{Pyr}][\text{NH}_4^+][\text{NADH}]} \quad (4)$$

In these equations, [Ami] stands for the equilibrium concentrations of amines **1c** or **2c**, [Alc] stands for the alcohols **1a** or **2a**, [Ald] stands for aldehyde **1b** or ketone **2b**, [Pyr] for pyruvate, and [NH₄⁺] is the total nominal concentration of ammonia. The other abbreviations have their usual meaning. By convention, water is fixed at 1 and does not appear in the equilibrium constants (Goldberg, 2014). All concentrations are lumped for different protonation states, which are obviously pH dependent.

Figure 2. Time course of ether amine formation under different reaction conditions. Panels **A**, varying substrate alcohol **1a**; **B**, varying ammonium; **C**, varying amino donor (L-alanine); **D**, varying cofactor (NAD) concentration.

The apparent equilibrium constant of the whole network should match the equilibrium constants of the separate reactions (Equation (5)).

$$K'_N = K'_o \cdot K'_t \cdot K'_r \quad (5)$$

Although prior studies have shown that the equilibrium of transamination reactions with a ketone or aldehyde as substrate and L-Ala as amino donor is in the direction of the amino acid (Koszelewski et al., 2010), little is known about the equilibrium of the oxidation reaction of ether alcohols to ether ketones or ether aldehydes. Some equilibrium data of alcohol/ketone redox reactions have been reported in the literature (Goldberg et al., 1993, 2007), but the equilibrium constants were calculated at different temperatures, buffer conditions, and with different compounds. In order to establish the position of the various equilibria, reactions with the three enzymes were performed separately as described in Materials and Methods.

All the equilibrium constants (K'_N , K'_o , K'_r and K'_t) shown in the Table II were calculated from experimental measurements, except for the value of K'_t for the reaction of **1b**–**c**. Due to the reactivity of **1b** with product **1c** under the conditions of sample preparation, the

analysis of **1c** was not reliable. A value for K'_t was estimated from the other experimentally measured constants.

The apparent equilibrium constants K'_o calculated with Equation (2) from conversion data (Fig. 3A) showed that alcohol oxidation is unfavorable for substrates **1a** and **2a** (Table II). Similar equilibrium constants were reported for 1-butanol ($1.8 \cdot 10^{-3}$) and for 1-octanol oxidation ($1.1 \cdot 10^{-3}$) confirming that NAD-coupled alcohol oxidation is thermodynamically rather unfavorable (Goldberg et al., 1993). Especially for ether alcohol **1a**, the equilibrium is strongly in the direction of the alcohol.

The aminotransferase equilibrium (Equation (3)) of **2b** and **2c** was estimated by GC analysis (Fig. 3B). The equilibrium constants appeared to be highly unfavorable both for ether aldehyde and ether ketone amination (Table II). Especially the K'_t value for conversion of ether ketone **2b** to amine **2c** illustrates poor acceptance of an amino group. The observed unfavorable equilibrium of the aminotransferase reaction is in agreement with the equilibrium constant reported for the transamination of 2-oxoglutarate and L-Ala ($2.9 \cdot 10^{-3}$; Goldberg and Tewari, 1994). Additionally, the equilibrium constant of the transamination of acetophenone with alanine to produce 1-phenylethylamine is reported to be $8.81 \cdot 10^{-4}$ (Koszelewski et al., 2010), which is also strongly favorable toward the

Table II. Experimental equilibrium constants at 30°C and pH 9.0. Mean values are reported $\pm$ the standard error of the mean.

Substrate	K'_o (ADH)	K'_t (AT)	K'_r (M^{-1}) AlaDH	K'_N (M^{-1})	
				Experimental	Calculated
1a, 1b	$2.6 \times 10^{-4} \pm 2.9 \times 10^{-6}$	6.1×10^{-2a}	$8.23 \times 10^{+5} \pm 4.23 \times 10^{+4}$	13.1 ± 0.45	–
2a, 2b	0.14 ± 0.004	$4.0 \times 10^{-4} \pm 4.7 \times 10^{-6}$		12.1 ± 1.28	46^b

^aThis value was calculated from the experimental values of K'_o , K'_r , and K'_N , from Equation (5).

^bThis value was calculated with Equation (5).

substrates. Obviously, transfer of the charged (at neutral pH) amino group from the zwitterionic L-Ala to an apolar acceptor is not favored.

The alanine dehydrogenase equilibrium was measured by following NADH formation (Fig. 3C). The equilibrium constant appeared highly favorable for recycling of alanine as amine donor and NAD^+ as electron acceptor for alcohol oxidation, and predicts an L-alanine/pyruvate ratio of 82,000 per M of ammonia present at equal concentrations of NAD^+ and NADH. The equilibrium constants obtained here are in the same range as those reported for similar substrates by Goldberg et al. (1993, 2007).

From these individual equilibria, it appears that conversion of **1a–c** and of **2a–c** are both thermodynamically feasible (Table II),

mainly due to the alanine recycling reaction, which strongly favors alanine and NAD^+ formation thereby driving the alcohol oxidation and aminotransferase reactions.

Implications for Network Reactions

The relationship between the overall reaction equilibrium K'_N and the theoretical maximal conversion in the three-enzyme network was examined. As a very low concentration of NAD^+ was used, the accumulation of aldehyde or ketone is negligible in Equation (1), and ammonia-dependent conversion of alcohol to amine is given by

Figure 3. Estimation of reaction equilibria. **Panel A:** Formation of NADH by GsADH acting on the oxidation of alcohol **2a**. **Panel B:** Production of ketone **2b** catalyzed by CvAT. **Panel C:** NADH formation in the conversion of L-Ala to pyruvate, catalyzed by VpAlaDH. **Panel D:** Progress curves for alcohol **2a** consumption and amine **2c** production in the complete enzymatic network. Reaction conditions are described in Materials and Methods. Curves are representative examples of experiments that were performed in duplicate.

Figure 4. Maximal conversion in amination networks based on equilibria. **Panel A:** Predicted maximal conversion of 10 mM ether alcohol to the corresponding ether amine with different concentrations of ammonia. The solid line represents $K'_N = 13.1$ for reaction **1a–c**. The dashed line represents $K'_N = 12.1$ for reaction **2a–c**. **Panel B:** Feasibility of operation window of the amination network as a function of K'_N and ammonia concentration. Lines: dotted line, $[\text{alcohol}]_0 = 0.1$ M; short dashed line, $[\text{alcohol}]_0 = 0.5$ M; long dash, $[\text{alcohol}]_0 = 1$ M. **Panel C:** Surface representing the theoretical maximal conversion (%) dependent on the ammonia concentration and the K'_N of the network.

$$K'_N = \frac{[\text{Ami}]}{([\text{Alc}]_0 - [\text{Ami}])([\text{NH}_4^+]_0 - [\text{Ami}])} \quad (6)$$

where $[\text{Alc}]_0$ is the initial concentration of the ether alcohol and $[\text{NH}_4^+]_0$ is the initial concentration of ammonia (sum of ionization states). Conversion in the whole network can be calculated from

Equation (7) describes the theoretical maximal conversion as a function of the overall reaction equilibrium K'_N and the initial substrate concentrations, and was used to calculate the maximum conversion at different concentrations of ammonia (Fig. 4A). To visualize the conditions at which amination becomes feasible, we also plotted the $>90\%$ conversion window as a function of K'_N and the initial ammonia loading (Fig. 4B). The plot shows that K'_N should be at least 10–100 to obtain a product yield exceeding 90%.

$$c = \frac{[\text{Ami}]}{[\text{Alc}]_0} = \frac{\left([\text{Alc}]_0 + [\text{NH}_4^+]_0 + \frac{1}{K'_N}\right) - \sqrt{\left([\text{Alc}]_0 + [\text{NH}_4^+]_0 + \frac{1}{K'_N}\right)^2 - 4[\text{Alc}]_0[\text{NH}_4^+]_0}}{2[\text{Alc}]_0} \quad (7)$$

Figure 5. Time course of alcohol to amine conversion by a three-enzyme network. **Panel A:** Observed conversion of alcohol **1a** (squares) to amine **1c** (closed circles) as compared to predicted maximum conversion (inverted triangles, calculated using Equation (7)). The concentration of aldehyde **1b** remained below the detection limit (<0.03 mM). **Panel B:** observed conversion of alcohol **2a** (squares) to ketone **2b** (triangles) and amine **2c** (closed circles) and theoretical maximum conversion (inverted triangles). The reactions were carried out at 30°C in ammonium carbamate buffer (40 mM, pH 9), substrate alcohol (**1a**) or (**2a**) (10 mM), NAD^+ (1 mM), PLP (0.35 mM), L-Ala (0.5 mM), *GsADH* (0.1 mg/mL), *VpAlaDH* (0.004 mg/mL), and *C ω AT* (0.064 mg/mL). Pulses of ammonium and re-addition of enzyme took place after sampling at 4 days (40 mM ammonium), 6 days (80 mM), 7 days (40 mM), and 8 days (80 mM) for **1a** and **2a**. Samples were analyzed in duplicate. Data show a representative example of an experiment carried out in duplicate.

The dependence of the yield on K'_N and ammonia concentration is further plotted in Figure 4C, again illustrating the requirement of a high K'_N (>10) and high ammonium concentrations (>0.5 M) for effective amination.

These results show that the conversion of the three-enzyme amination networks can be limited by an unfavorable equilibria of the final amination steps yielding amines **1c** and **2c** and of the formation of the intermediates aldehyde **1b** and ketone **2b**. The use of a high ammonium concentration can drive the equilibrium to product formation.

Reaction Optimization

Using Equation (7) as a guide, we attempted to further improve conversion of alcohol to amine. For reactions **1a–c** and **2a–c**, at least 0.7 M of ammonia is predicted to be required for a good conversion ($>95\%$ yield, Fig. 4A). Therefore, conversion was tested with a range of ammonia concentrations varying from 80 mM to 1 M. However, addition of ammonia to concentrations exceeding 200 mM caused precipitation of the enzymes, as indicated by visual turbidity and SDS–PAGE analysis of the precipitate. The latter indicated that all three enzymes formed precipitating aggregates (data not shown). Therefore, reactions were run with repeated addition of smaller amounts of ammonia (concentration 80 mM) and of enzyme (*GsADH* [0.1 mg/mL], *VpAlaDH* [0.004 mg/mL] and *CvAT* [0.064 mg/mL]), and the production of ether amine was followed over time. This strategy resulted in improved yields. The highest conversion was found with stepwise addition of a total 280 mM ammonium, which afforded a conversion of 60% for **1a–c** and for **2a–c** (Fig. 5). To the best of our knowledge, these are the highest conversions reached in such a three-enzyme network for secondary amine production using purified enzymes as catalysts (Tauber et al., 2013).

The time course experiment with stepwise addition of ammonium and enzyme showed that the accumulation of amine correlated reasonably well with predictions for maximum conversion based on reaction equilibria (Fig. 5), suggesting that conversions indeed became equilibrium-limited. The final product concentrations of amines **1c** and **2c** were 6 mM, exceeding the level of added nicotinamide cofactor (1 mM NAD^+) and L-Ala (0.5 mM for **1a**), again indicating effective recycling in the alanine dehydrogenase reaction. This is in agreement with the equilibrium calculations, which showed that cofactor recycling is an essential component of the network since it drives the overall conversion toward amine synthesis.

Although cofactor recycling networks were reported earlier (Findrik and Vasic-Racki, 2009; Kragl et al., 1996; Yun et al., 2003), amination networks are usually operated in the presence of an excess of L-Ala, which may be added up to a 25-fold excess (Klatte and Wendisch, 2014; Sattler et al., 2012). Alternatively, amination is driven by an additional redox reactions, such as the conversion of NADH to NAD^+ by an NADH oxidase (Findrik et al., 2008), or the conversion of pyruvate to lactate (Koszelewski et al., 2010). For example, Klatte and Wendisch (2014) used whole cells expressing the three enzymes of the network with addition of cofactors, amino donor, ammonia source, and substrates. They described a conversion of 100% with 1,10-diaminodecane (10 mM) with addition of 250 mM (25 equivalents) of L-alanine. Equimolar concentrations of alanine and alcohol substrate were effective when

transamination was stimulated by oxidase-mediated conversion of the produced pyruvate (Klatte and Wendisch, 2015).

In conclusion, the biocatalytic conversion of ether alcohols to the corresponding amines with ammonia as the sole additional substrate would be an attractive process. In the current study, we describe an amination cycle using an alcohol dehydrogenase, an aminotransferase, and an alanine dehydrogenase that recycles both the nicotinamide cofactor (NAD^+) for the alcohol dehydrogenase and the amine donor alanine (L-Ala) for the aminotransferase. Equilibrium measurements showed that conversion of alcohol to aldehyde or ketone is disfavored, as is the amination of aldehyde and ketone. The overall conversion of alcohol to amine is based on the alanine dehydrogenase-mediated formation of L-Ala and NAD^+ . Using measured and computed equilibrium constants, theoretical maximum conversions and feasible operation windows were calculated. High ammonia concentrations can drive the overall reaction, giving conversions of up to 60%, in agreement with equilibria. The results further suggest that enzyme robustness, especially resistance and maintenance of activity at high concentrations of ammonia and high pH, is a key factor when pursuing practical application of the suggested amination network.

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