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Abstract

The combination of enzymatic reactions in a simultaneous or sequential fashion by designing artificial synthetic cascades allows for the synthesis of complex compounds from simple precursors. Such multi-catalytic cascade reactions not only bear a great potential to minimize downstream processing steps but can also lead to a drastic reduction of the produced waste. With the growing toolbox of biocatalysts, alternative routes employing enzymatic transformations towards manifold and diverse target molecules become accessible. In vitro cascade reactions open up new possibilities for efficient regeneration of the required cofactors such as nicotinamide cofactors or nucleoside triphosphates. They are represented by a vast array of two-enzyme cascades that have been designed by coupling the activity of a cofactor regenerating enzyme to the product generating enzyme. However, the implementation of cascade reactions requires careful consideration, particularly with respect to whether the pathway is constructed concurrently or sequentially. In this regard, this chapter describes how biocatalytic cascades are classified, and how such cascade reactions can be employed in order to solve synthetic problems. Recent developments in the area of dynamic kinetic resolution or cofactor regeneration and showcases are presented. We also highlight the factors that influence the design and implementation of purely enzymatic cascades in one-pot or multi-step pathways in an industrial setting.

Keywords

Biocatalysis · Enzymatic cascades · In vitro biotransformations · Enzymes · Cofactor regeneration

3.1 Introduction

Reaction systems combining two or more chemical steps in one pot without isolation of reaction intermediates are commonly referred to as cascades [1]. In such systems, individual chemical steps can be enzyme catalyzed or involve chemical (metal or organo-) catalysts. Accordingly, multi-enzymatic cascades include several

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biocatalytic steps, which can be either performed simultaneously or sequentially. In the first case, also called concurrent cascade or tandem reaction, all enzymes and reagents are present from the beginning of the reaction, meaning that all reaction steps take place at the same time. In contrast, in a sequential multi-enzymatic cascade, certain enzymes and/or reagents are added at a later point in time after a certain sequence is completed. Hence, all reaction steps of a sequential cascade are still performed in one pot but must be separated in time. The latter might be necessary if, e.g., two or more enzymes of the same cascade require different reaction conditions, one enzyme is inhibited by a compound appearing prior or later in the sequence, or to prevent undesired side reactions if cross-reactivities of the involved enzymes occur. Moreover, in vitro and in vivo multi-enzymatic cascades are distinguished depending on the biocatalyst preparation (compare also Chap. 4). Whereas in in vivo cascades all enzymes of the cascade are included in whole living cells, in vitro cascades make use of isolated enzymes in purified form, as cell-free extracts, freeze-dried preparations, immobilized versions, etc. [2].

In addition, cascade reactions in general can exhibit different topologies (Fig. 3.1) [1, 3]. In a linear cascade (Fig. 3.1a), the product of one chemical step serves as substrate of the subsequent chemical step. This is probably the most straightforward type of cascade reaction as it avoids the isolation of (unstable) reaction intermediates with the final goal to increase the overall product yield while saving time and resources.

Additionally, a linear cascade can be used to shift an unfavorable reaction equilibrium of one step by combination with a subsequent irreversible reaction step that pulls the product out of the reaction. A recent example is the combination of the hydroxynitrile lyase from *Manihot esculenta* for the synthesis of optically pure (*S*)-4-methoxymandelonitrile with the *Candida antarctica* lipase A-catalyzed acylation of the formed α-cyanohydrin yielding a stable ester product (Scheme 3.1) [4]. This way, the equilibrium of the hydrocyanation reaction could be shifted towards product formation and isolation of the unstable cyanohydrin intermediate was avoided.

Next to linear cascades, also orthogonal (Fig. 3.1b), cyclic (Fig. 3.1c), and parallel, interconnected (Fig. 3.1d) cascades have been described. In an orthogonal enzyme cascade, the conversion of a substrate into the desired product is coupled with a second reaction to remove one or more by-products. An example is the combination of a transaminase with lactate dehydrogenase, where the by-product pyruvate (when using alanine as amine donor) of the transaminase-catalyzed reaction is further converted to lactic acid in order to shift the equilibrium of the transaminase reaction (Scheme 3.2) [5]. In a cyclic cascade, one enantiomer out of a racemic substrate mixture is converted to an intermediate product, which is then transformed back to the racemic starting material yielding the unreacted substrate enantiomer as final product. The same applies if the unreacted substrate enantiomer is racemized to yield enantiomerically pure product (dynamic kinetic resolution). Hence, cyclic cascades are commonly applied in deracemization processes, e.g. of amino acids, hydroxy acids, or amines [6, 7]. Finally, in a parallel, interconnected cascade, two separate biocatalytic reactions are connected by complementary cofactor requirements of the two enzymes. Therefore, parallel, interconnected cascades are commonly associated with cofactor recycling systems. While in one biocatalytic step a substrate is transformed into the desired product, a cheap co-substrate is converted to a co-product in the parallel enzyme-catalyzed step to recycle the required cofactor for the first enzyme.

As briefly mentioned, cascade reactions offer several advantages compared to conventional reaction schemes [1]. This includes the avoidance of operational work up steps, which saves time, resources, and reagents, can reduce waste formation and, at the same time, allows for higher final product yields. Additionally, different reaction steps can be smartly combined in a cascade to solve synthetic problems of a reaction sequence such as cofactor regeneration, shift of reaction

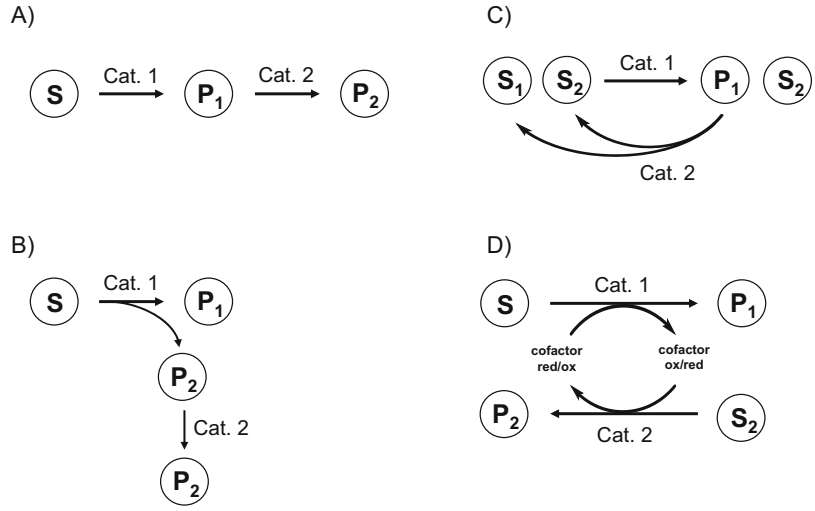
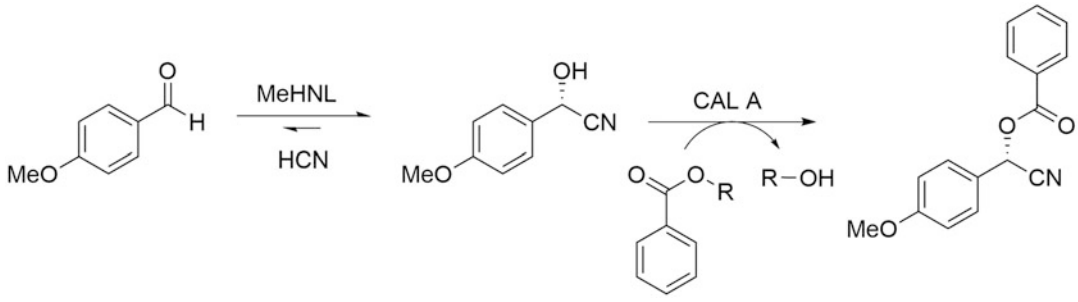


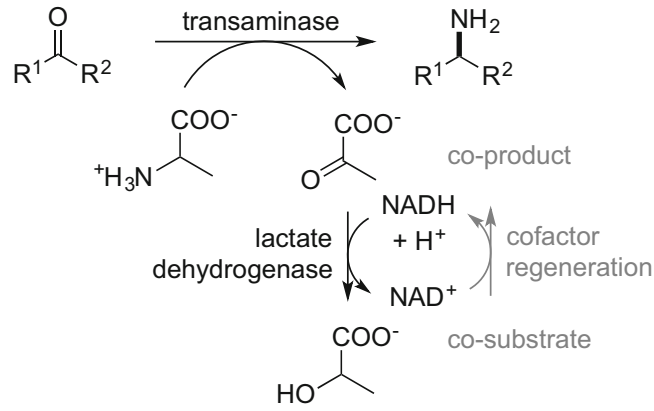
Fig. 3.1 Possible topologies of cascade reactions. (**a**) Linear. (**b**) Orthogonal. (**c**) Cyclic. (**d**) Parallel, interconnected. The scheme was adapted from [1]

equilibria, in situ generation of toxic or instable reagents, etc. On the other hand, the setup of efficient cascade reactions is usually a complex and challenging task [1]. Compatible reaction conditions have to be identified and, in case of a simultaneous cascade, the reaction rates of individual steps have to be balanced. Moreover, possible problems, such as the formation of undesired side products due to cross-reactivities of catalysts or the inhibition of an enzyme by a compound appearing earlier or later in the reaction sequence, can occur that have to be addressed. Multi-enzymatic cascades are usually easier to establish than chemoenzymatic or chemo-catalytic cascade reactions as enzymes commonly work in aqueous reaction media and often display similar temperature and pH requirements for optimal performance. Nevertheless, also several examples for the successful combination of enzymatic and chemical reaction steps in a cascade have been described in literature (compare also Chap. 5) [8].

In an impressive way, nature successfully evolved a multitude of highly complex concurrent cascade reactions. Living organisms built every minute thousands of highly complex molecules from simple precursors in an astonishing variety and efficiency. To achieve such high efficiencies, individual enzymatic transformations are arranged in cascading sequences (biosynthetic



Scheme 3.1 Combination of *Manihot esculenta* hydroxynitrile lyase (MeHNL) and *Candida antarctica* lipase A (CAL A) in a linear cascade to shift the reaction equilibrium of the first hydrocyanation reaction [4]



Scheme 3.2 Combination of transaminase and lactate dehydrogenase in an orthogonal cascade to shift the reaction equilibrium of the transaminase-catalyzed reaction

pathways) in living cells [9]. This strategy can be mimicked in vitro by the design of artificial metabolic pathways [10] through combination of multiple isolated enzymes in a homogeneous phase or by combination of two or more catalytic activities in a single protein, e.g., by fusion of genes encoding different enzymes or by crosslinking several enzymes [11–14]. Recently, a novel approach has been mentioned in literature named systems biocatalysis, which aims for the in vitro setup of synthetic metabolic cycles for the production of valuable compounds [15].

To illustrate the synthetic potential of multienzyme cascades, but also potential challenges in the development of cascade reactions, different cascade examples have been selected and are described in more detail in Sect. 3.2. Perhaps the most frequent motivation for currently used enzyme cascades is the concurrent regeneration of (redox-) cofactors or expensive reagents. A second application is the combination of isomerizing enzymes or catalysts with highly enantioselective enzymes for dynamic kinetic resolutions, which allow to overcome the yield limitation of 50% of kinetic resolutions. Cofactor regeneration and dynamic kinetic resolutions require concurrent cascades, which often makes it very challenging to provide optimal reaction conditions for both catalysts used. To overcome compatibility issues, compartmentalization is a possibility to enable different operating conditions for all reaction steps of a concurrent or step-wise cascade [8].

3.2 Cascades to Solve Synthetic Problems

3.2.1 Combination of Selective Enzymatic Steps with Isomerizing Reactions

Due to their availability and ease to use, hydrolases mainly constituted the first wave of biocatalysts in the first biocatalytic processes [16]. Kinetic resolution of racemic mixtures was often the preferred reaction form (Fig. 3.2) [17]. While this reaction is simple and robust, it suffers from an intrinsic limitation of 50% yield, which in turn requires the physical separation of substrate and product. The addition of a second catalyst, which racemizes the unreacted substrate, but not the product (Fig. 3.2) in a cyclic cascade allows for the complete conversion of starting material to the desired product enantiomer. The depletion of the faster-reacting substrate enantiomer leads to an increased conversion of the slower-reacting substrate enantiomer as its relative concentration increases, which reduces the optical purity of the product. To prevent this, the racemization should be one order of magnitude faster than the enantioselective reaction. This makes the racemization steps often the bottleneck of dynamic kinetic resolution reactions.

In 1997, Bäckvall et al. combined Ruthenium-catalyzed hydrogen transfer reactions for the racemization of aryl aliphatic secondary alcohols with their lipase-catalyzed kinetic resolution in a

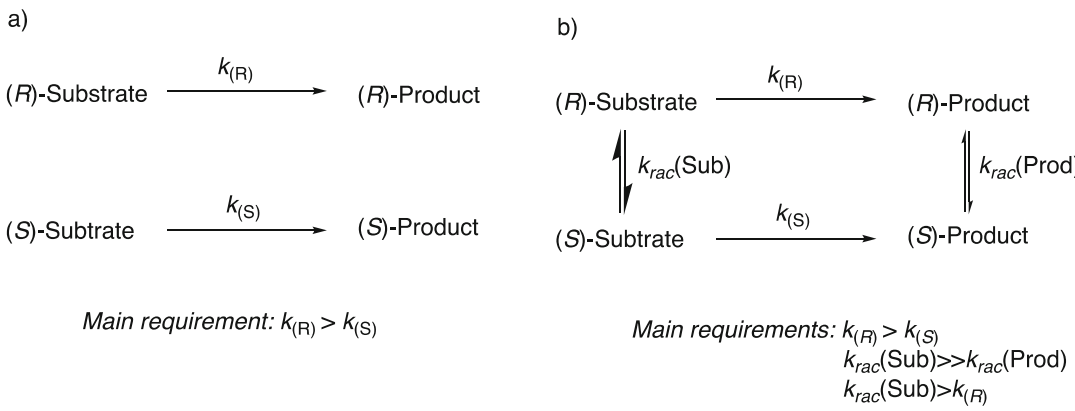
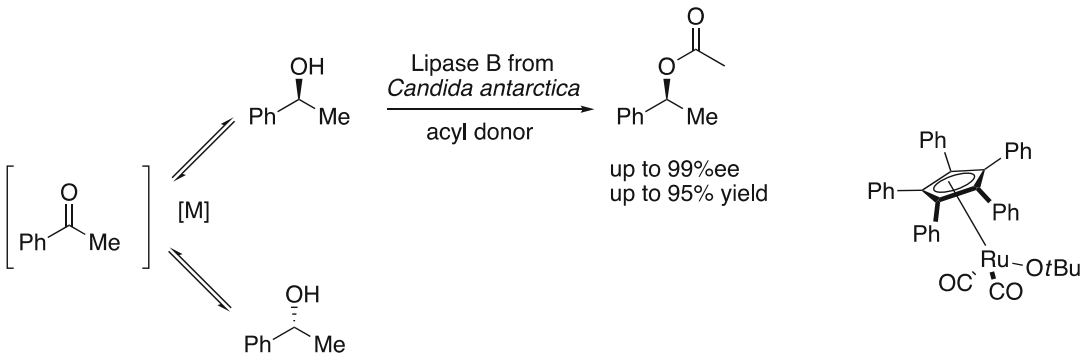


Fig. 3.2 Schematic representation of kinetic resolution (**a**) and dynamic kinetic resolution (**b**)

concurrent fashion to obtain the corresponding esters in high optical purity and yield (Scheme 3.3) [18]. The racemization of the secondary alcohol proceeds via intermediary oxidation to the corresponding ketone. Important parameters are the activity of the Ruthenium-catalyst and its stability. A high stability is desirable particularly against highly reactive acyl donors such as vinyl acetate that drive the transesterification towards ester formation [19]. By choosing the appropriate metal catalyst, excellent reaction rates, yields, and optical purities were achieved. The preference of most lipases for the formation of the (*R*)-enantiomers of secondary alcohols limited this approach to the formation of the respective (*R*)-esters. Instead, the use of proteases allowed to access the opposite (*S*)-enantiomers. The reaction concept was later extended to the transesterification of primary alcohols and aliphatic amines [20]. Lipases are frequently used for the kinetic resolution of esters either from chiral alcohols or chiral carboxylic acids. As shown, the racemization of secondary alcohols via redox reactions allowed the establishment of highly efficient dynamic kinetic resolution (DKR) reactions. The analogous DKR of the esters of α-chiral carboxylic acids, however, is much more challenging. Here, racemization of the stereocenter by a redox reaction is difficult to achieve. Racemization by acid-base catalysis is possible, but needs very special catalysts and conditions to avoid the base-catalyzed hydrolysis of the carboxylic ester. It is also not trivial to find conditions for an efficient base-catalyzed racemization that are compatible with the stability of the lipase [21]. This example underlines that concept for reaction sequences is often highly substrate-dependent. While the combination of metal-catalyzed racemization and lipases could be demonstrated with high yields and optical purities, the applicability to other enzyme classes beyond lipases and proteases is somewhat limited due to the requirement of the metal catalyst for organic solvents.

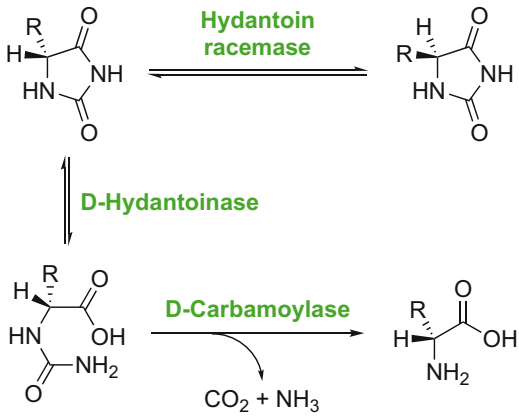
A prominent example for a fully enzymatic dynamic resolution is the so-called Hydantoinase process for the production of optically pure D- or L-amino acids, for which in vitro as well as in vivo approaches have been developed [22]. In general, D- or L-amino acids can be synthesized by chemical or enzymatic procedures. However, the chemical synthesis gives racemic mixtures of amino acids with low yield. The production of specific amino acids by fermentation of microorganisms is only useful for a few natural amino acids and depends on the microorganism. Thus, many chemical companies have embraced biocatalysis for manufacturing enantiomerically pure amino acids. This strategy involves aminoacylases, amidases, and hydantoinases. In 1980, the company Ajinomoto was first in purifying and immobilizing the enzyme that hydrolyzes hydantoins to optically pure amino acids. The mechanism was not understood at that time and they noted that more than one enzyme might be involved. Thereafter, several patent applications reported the production of



Scheme 3.3 Linear chemoenzymatic cascade for the dynamic kinetic resolution of secondary alcohols combining hydrolases with metal-catalyzed racemization

D- or L-*N*-carbamoyl-α-amino acids indicating that the reaction takes place in two steps [23–25]. In 1982, Olivieri et al. described in detail how D-amino acids are produced from the corresponding hydantoins catalyzed by three enzymes (Scheme 3.4) [26]. The enzymatic racemization of the inexpensive racemic hydantoin is crucial to achieve a complete conversion of the starting material to the product.

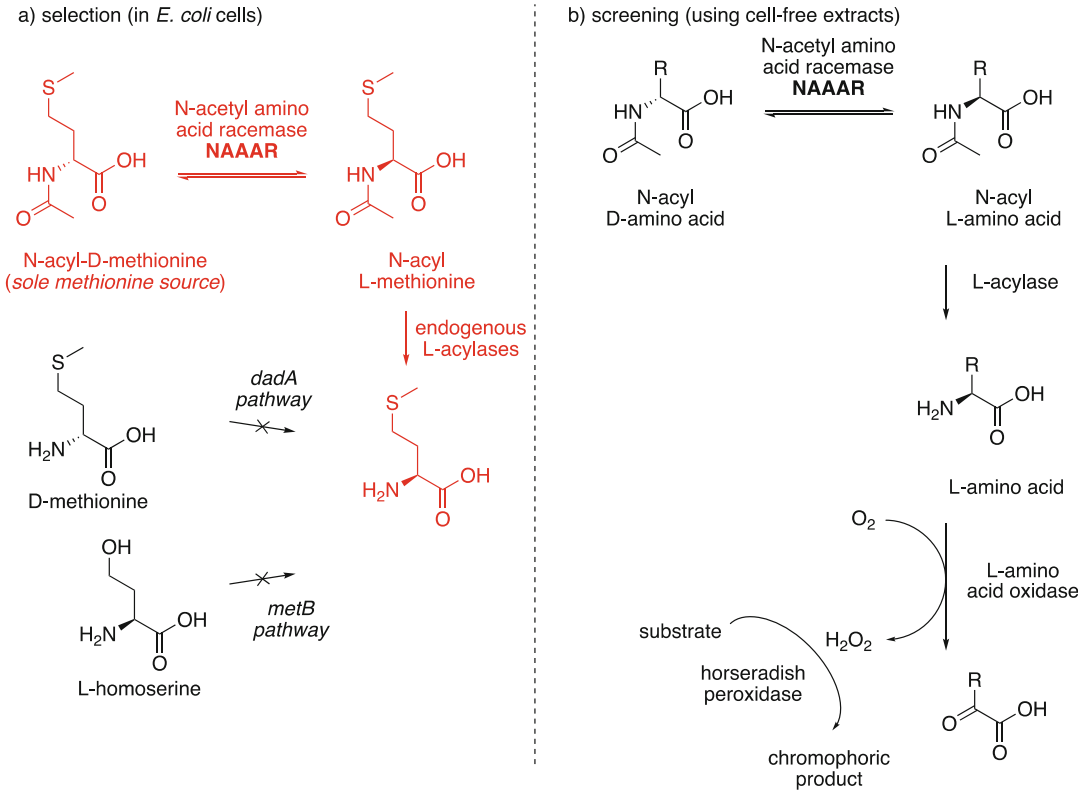
An alternative strategy for the synthesis of optically pure amino acids lies in the combination of N-acyl amino acid racemases (NAAAR) with stereoselective N-acetyl amino acid acylases (Scheme 3.5). Similar to the hydantoinase process, enzymes for the production of both enantiomers are available, which also allows the synthesis of the D-enantiomers. The bottleneck is often the low activity of the NAAAR. By performing a selection assay for racemizing activity, Campopiano et al. increased the specific activity of a microbial racemase [27, 28]. They used an *E. coli* strain with deleted ability to produce endogenous L-methionine. As the strain had several strictly L-selective acylases, feeding of N-acetyl-D-methionine allowed to couple racemizing activity to L-Met availability and hence growth. A variant with sixfold activity identified in the selection assay allowed the complete conversion of a 50 g/L solution of N-acetyl-DL-allylglycine into D-allylglycine within 18 h. For a more accurate characterization of the reaction in high-throughput format the same group also developed an in vitro assay that coupled formation of an L-amino acid to formation of hydrogen peroxide by use of an L-amino acid oxidase. The hydrogen peroxide could be easily detected by horseradish peroxide. This example underlines the importance of protein engineering to provide catalysts with optimal performance for their function in a cascade.



Scheme 3.4 Linear cascade for the production of optically pure amino acids by the hydrolysis of racemic D,L-5-mono substituted hydantoins. In the initial hydantoin molecule, R could be an aliphatic or aromatic residue, either substituted or unsubstituted. The scheme was adapted from Olivieri et al. [26]

3.2.2 Cascade Reactions for Cofactor Regeneration

Redox enzymes usually require expensive cofactors like NAD(P)H for the electron transfer



Scheme 3.5 Enzyme cascade reactions for selection (left) and screening (right) assays for amino acid racemases

from one molecule (reductant) to another molecule (oxidant). In organic synthesis, such biocatalysts can be employed either as isolated enzymes in combination with an appropriate cofactor recycling system or in whole cells, expressed in their original microorganism or recombinantly [29–32]. Generally, for such reactions various concepts have been developed that rely on electron supply via the metabolism of living heterotrophic cells. In synthetic applications, the nicotinamide cofactors are recycled by using energy-rich organic molecules as electron donors. In most cases, only a small fraction of the electrons provided by these sacrificial cosubstrates is utilized, resulting in a poor atom efficiency. Moreover, when glucose is supplied as sacrificial substrate for the recycling of NADPH, the often-used glucose dehydrogenase utilizes only a part of the electron pairs supplied by each glucose molecule. In contrast, many enzymatic in vitro cascade reactions have been developed in order to achieve an efficient regeneration of the required cosubstrates such as nicotinamide cofactors or nucleoside triphosphates. A vast array of two-enzyme cascades have been designed by coupling the activity of a cofactor regenerating enzyme to the product generating enzyme [1, 33]. By using this approach, the unfavorable reaction equilibrium is shifted by the second enzyme and thus drives the reaction to the desired product [34]. However, the necessity for sacrificial cosubstrates as well as an additional enzyme makes these cascade strategies less favorable. On the other hand, in vitro cascades can be designed in a way that they are redox neutral, i.e. the cofactor consumed in the first enzymatic step is regenerated by the second enzyme reaction. That offers the advantage that such redox-neutral cascades are simplified since no additional recycling system has to be supplemented.

One example for such redox-neutral cascade reaction has been recently reported by Turner and

coworkers [35]. In this example, an alcohol dehydrogenase (ADH) was employed to oxidize the alcohol starting material to the corresponding ketone. The cofactor NADH regenerated during the first step was used in the subsequent step, in which an amine dehydrogenase (AmDH) catalyzed a reductive amination of the intermediate ketone. Overall, the cascade is redox neutral and the hydrogen abstracted in the first step was reinstalled in the second in order to regenerate the NADH [36, 37]. However, it was necessary to run the cascade reaction in vitro with purified enzymes instead of cell-free extract since side reactions catalyzed by endogenous proteins in the crude cell preparation occurred. These side reactions sequestered and oxidized the NADH cofactor required for the second step and thus disrupted the hydrogen-borrowing nature of the cascade. Overall, moderate to excellent yields (30–91%) and high *ee* values (82 to >99%) could be achieved when running the reactions on preparative scale (100–126 mg of the ketone).

An example where the concept of a "mini" metabolic pathway was successfully applied is the synthesis of 6-aminohexanoic acid, which is the open-chain form of ε-caprolactam (the precursor for Nylon-6) reported by the Kroutil and coworkers [38]. In their work, a two-step system consisting of cascading enzyme sequences was build. In the first module an alcohol dehydrogenase (ADH) and a Baeyer–Villiger monooxygenase (BVMO) were combined to synthesize ε-caprolactone (ε-CL). This first reaction module can be considered as redox neutral, since the NADPH resulted from the ADH reaction was directly used by the BVMO. In the second module the produced ε-CL was further converted to 6-aminohexanoic acid via an in situ capping and uncapping step. The biocatalysts were either used as purified enzymes (in lyophilized form) or as freeze-dried cells containing overexpressed enzyme.

The same group reported a cascade for the amination of primary alcohols [39]. In this work, a thermostable ADH (ADH-hT) from *Bacillus stearothermophilus* was combined with an ω-transaminase (ω-TA) from *Chromobacterium violaceum* and an L-alanine dehydrogenase from *Bacillus subtilis* in order to recycle the amine donor in a strictly "non" buffered system. It could be shown that this cascade is redox neutral by applying a proper cofactor recycling system. Thus, the equilibrium of the cascade was shifted toward the product side by adding ammonia as cheap amine donor in excess. Under optimized conditions, they were able to fully convert 50 mM 1-hexanol to 1-hexylamine and 3-phenyl-1-propanol to 3-phenyl-1-propylamine. After further optimization of the reaction conditions, the authors were able to convert up to 50 mM octanediol or 1,10-decanediol to the corresponding diamines.

The direct oxidation of cycloalkanes to cycloalkanones employing a P450 monooxygenase and an ADH represents another successful enzyme cascade that is redox neutral or redox self-sufficient [40]. Key to success of this enzymatic cascade was the protein engineering of the P450 monooxygenase (BM3 from *Bacillus megaterium*). Two variants (19A12 and F87V) were identified as best candidates for cycloalkane hydroxylation. Although the initial proof-of-concept was successful, and the combination of the P450 variants with the ADH from *Lactobacillus kefir* resulted in the production of 6.3 mM of cyclooctanone, the low activity of the P450 monooxygenase turned out to be the bottleneck of this cascade reaction. Pennec et al. reported an extension of this cascade consisting of a cytochrome P450 monooxygenase for the initial oxyfunctionalization of a cycloalkane coupled with an alcohol dehydrogenase for ketone production and a Baeyer–Villiger monooxygenase for the subsequent conversion to the corresponding lactone [41]. By varying the cofactor dependence of the biocatalysts and the cofactor regeneration system, final product concentrations of around 3 g/L enantholactone from cycloheptane could be obtained within 12 h of reaction.

The combination of an ADH with a BVMO for the conversion of cyclohexanol to ε-CL has been strongly investigated since ε-CL is a valuable precursor for polymer synthesis [42–48]. The resulting biodegradable polymers, such as polycaprolactone, are of interest for applications

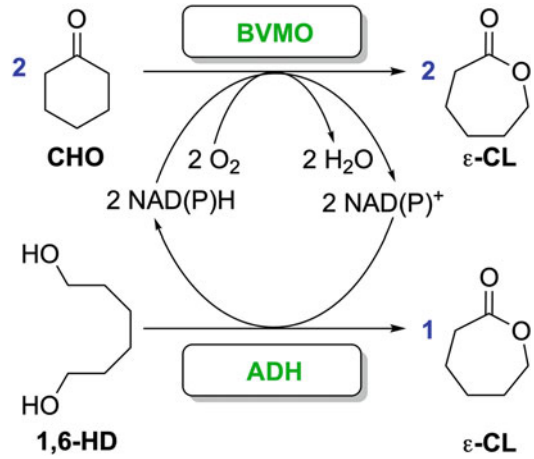
such as tissue engineering and drug delivery [49]. In all these examples, the ADH and the BVMO have been coupled in a redox-neutral fashion with respect to the nicotinamide cofactor. Advantageously, molecular oxygen is the only stoichiometric cosubstrate in the reaction. Two reports in 2013 showed the general feasibility of the cascade (94% conversion of 60 mM substrate in one case, 80% conversion of 10 mM in the other) [42, 43]. However, the cascade itself is limited since at high substrate concentrations, substrate as well as product inhibition has been observed. This limitation was addressed in a follow-up study by combining the ADH/BVMO reaction with lipase A from *Candida antarctica*, which converted the produced ε-CL in situ to oligomers of ε-CL and thereby removing it from the reaction mixture [45]. However, this cascade has been performed as hybrid in vivo/in vitro cascade.

Kara and coworkers developed a new class of redox-neutral reactions designated as convergent cascade involving a bi-substrate and a single product without intermediate formation was described [50]. This system involves a Baeyer–Villiger reaction catalyzed by a BVMO for the oxidation of cyclohexanone to ε-CL and an ADH for the simultaneous regeneration of NAD(P)H by oxidation of 1,6-hexanediol which serves as "double-smart cosubstrate" (Scheme 3.6).

In a follow-up study, the reaction parameters have been optimized by using design of experiments and an aqueous/organic biphasic reaction system [51]. These improvements led to an increase of the NADPH turnover number and the ADH by a factor of 50 and 10, respectively.

In order to gain as much redox equivalents as possible out of a single molecule for the regeneration of the cofactors, Kara et al. also reported a three-step oxidation of methanol to carbon dioxide (Scheme 3.7) [52].

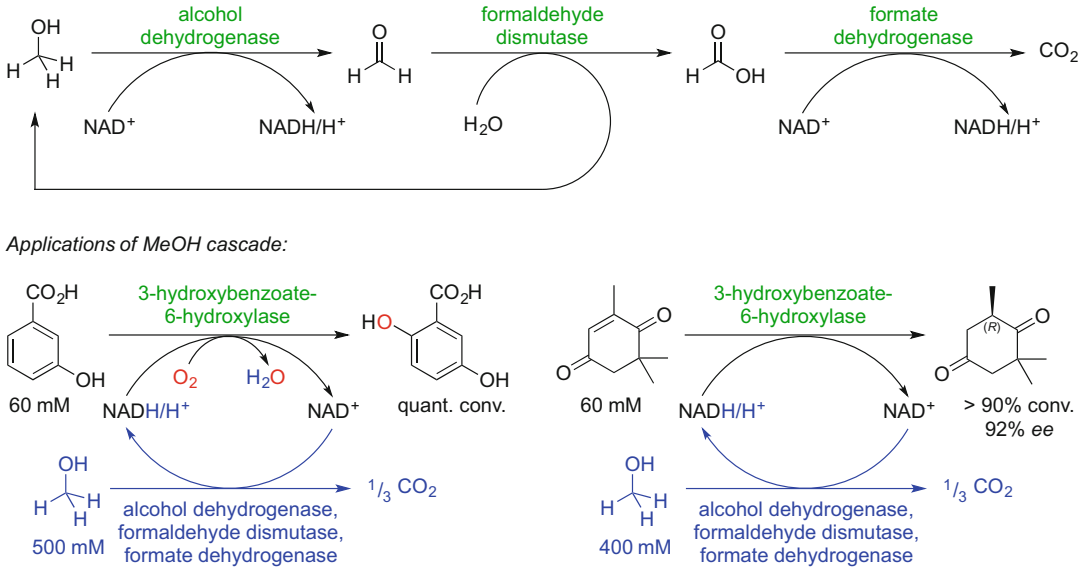
In this study, an ADH from yeast, a formate dehydrogenase (FDH) from *C. boidinii,* and a formaldehyde dismutase from *P. putida* were combined. The formaldehyde dismutase catalyzes the redox-neutral dismutation of formaldehyde into methanol and formic acid thereby providing a link between the ADH and FDH reactions. The



Scheme 3.6 "Double-smart cosubstrate" approach for the synthesis of ε-CL through a convergent cascade system. A BVMO-catalyzed oxidation of cyclohexanone (CHO) is coupled with an ADH-catalyzed oxidation of 1,6-hexanediol (1,6-HD) to ε-CL for the regeneration of two equivalents NAD(P)H. The scheme was adapted from Bornadel et al. [50]

overall cascade produces three molecules of NADH from NAD^+ for each molecule of methanol that is oxidized. This cofactor recycling system has been coupled on the one hand to an oxyfunctionalization reaction catalyzed by a monooxygenase and on the other hand to a C=C-bond reduction catalyzed by an ene-reductase. However, in both cases 500 mM MeOH must be added to convert up to 60 mM substrate. It has been assumed that this limitation can be attributed to the exceedingly high K_m value of the yeast ADH for methanol (>300 mM).

An intrinsic challenge for an efficient regeneration of the cofactors (recycling between 100 and 10^6 times) is given by the usually low long-term stability of the cofactors, even if a complete cascade is cofactor neutral and regenerates the cofactor during the course of its reaction [33, 53]. The stability of the cofactors can be influenced by the temperature, pH values, buffers, and salts of the reaction. Especially in terms of industrial applications, the pressure to perform enzymatic reactions at higher temperatures (50 °C and above) rises in the last years [54]. Possible solutions to solve this problem could be either the use of nicotinamide cofactor analogues

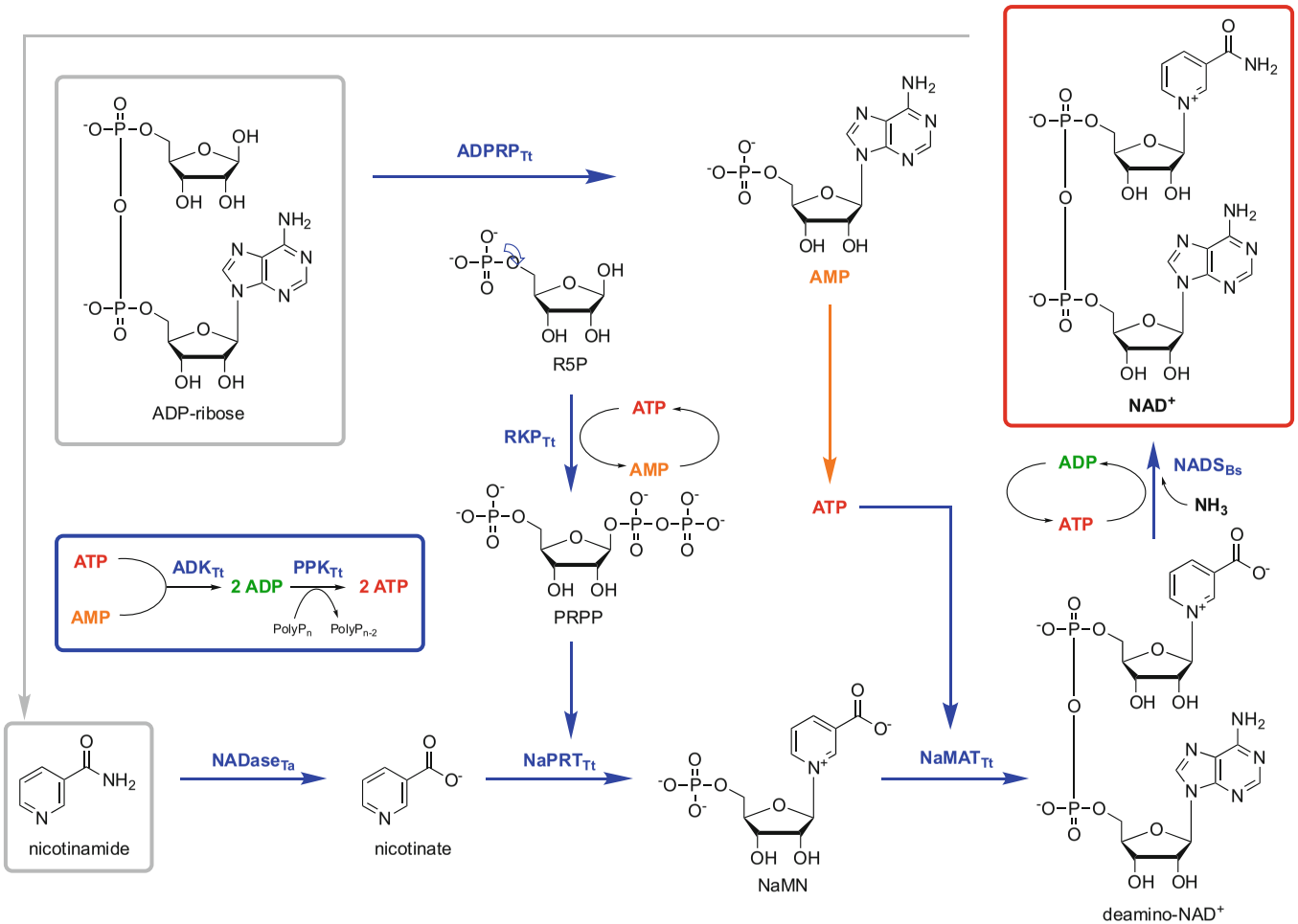


Scheme 3.7 Biocatalytic three-step cascade for the transformation of methanol to carbon dioxide for the regeneration of three equivalents NADH. The scheme was adapted from Kara et al. [52] and Schrittwieser et al. [1]

(so-called mimics), but their general applicability is limited since only a small fraction of enzymes is able to bind and convert them [55]. A promising cascade strategy to tackle this problem has been designed by Honda and coworkers, that combined eight different enzymes from thermophilic origins in order to construct an artificial metabolic pathway for the synthesis of NAD^+ from its degradation products [56]. With that approach, it was possible to keep the NAD^+ concentration constant for almost 15 h at a temperature of 60 °C.

Although this enzyme cascade replenishes the NAD^+ pool that is diminished by unwanted side reactions, there is still the problem that even perfectly balanced pathways loose reducing equivalents (e.g. NADH) over time by spontaneous oxidation [57]. Opgenorth et al. recently developed a molecular purge valve module as a strategy to overcome this problem (Scheme 3.8) [57]. This purge valve module was created in order to keep a balanced production and consumption of NADPH and NADH by applying two different pyruvate dehydrogenases that selectively accept either $NADP^+$ or NAD^+ in combination with an NADH oxidase that does not oxidize NADPH. With that system, high NADPH concentrations were maintained for reduction purposes while simultaneously allowing an independent carbon flux from pyruvate to polyhydroxybutyrate (PHB) or isoprene by purging of excess NADH.

Not only regeneration systems for nicotinamide cofactors have been studied intensively over the past couple of years, but also recycling approaches for adenosine-5′-triphosphate (ATP)-dependent reactions have gained increasing attention recently. ATP-dependent enzyme-catalyzed reactions can be widespread found in nature. Thus, ATP-dependent enzymes have an intrinsic potential for use in synthetic applications. Although regeneration systems for ATP starting from adenosine-5′-diphosphate (ADP) are in general available, certain limitations exist for in vitro applications [58]. Most available ATP regeneration systems start from ADP although methods for the regeneration of ATP from adenosine-5-′-monophosphate (AMP) have a high potential for cascade reactions. However, such systems are not well established yet. Those would enable ATP-dependent catalytic processes such as S-adenosylmethionine-dependent reactions or reactions where pyrophosphate (PP_i) is transferred to acceptor molecules. Most ATP



Scheme 3.8 In vitro enzyme cascade reaction for the salvage synthesis of NAD^+ from nicotinamide and ADP-ribose. Blue arrows: ATP regeneration reactions, which are shown in detail in the inner panel. *NaMATTt* Nicotinate mononucleotide adenylyltransferase, *NaPRTTt* nicotinate phosphoribosyltransferase, *ADPRPTt* ADP-ribose pyrophosphatase, *RPKTt* ribose-phosphate pyrophosphokinase, *ADKTt* adenylatkinase. The scheme was adapted from Honda et al. [56]

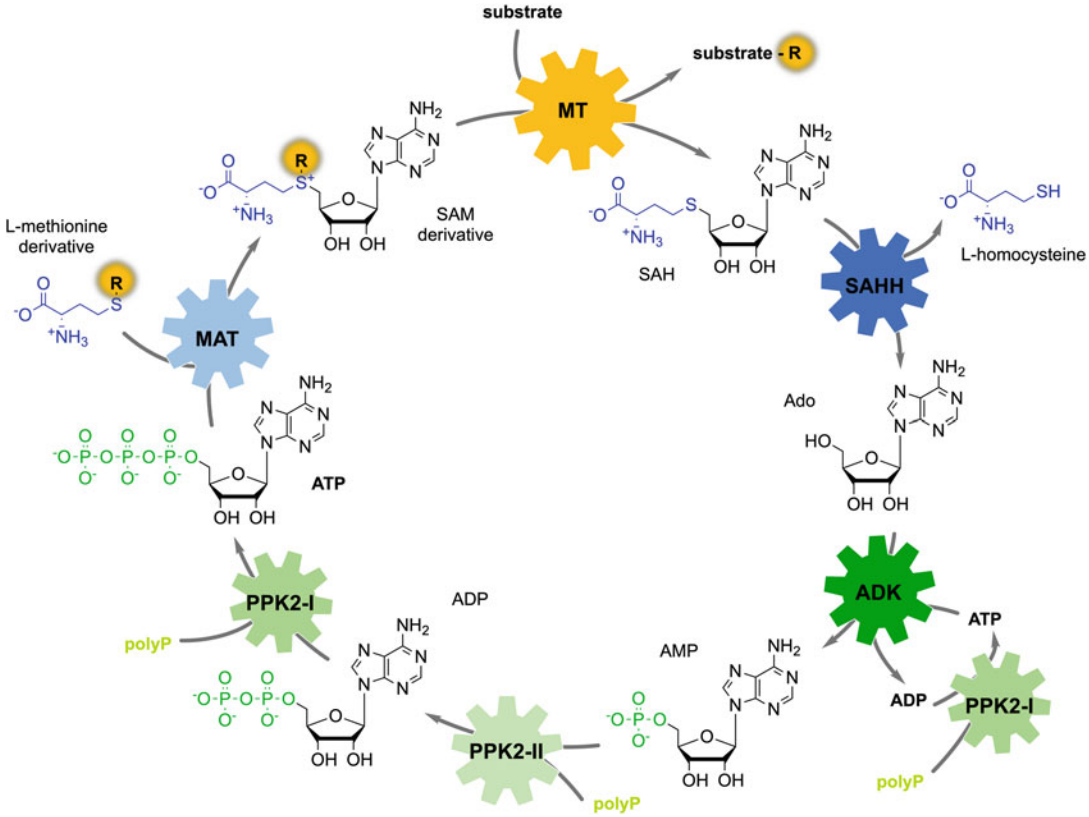
regeneration approaches rely on kinases starting from cheap raw materials. These enzymes usually transfer the γ-phosphate group of ATP or any other nucleotide to an acceptor molecule. The reverse reaction is commonly applied to phosphorylate ADP to yield ATP. Most important for an efficient regeneration approach based on these enzymes is the availability of a cheap and stable phosphate donor as well as the kinetic properties of the kinase. In order to create a recycling system starting from AMP, usually two or more enzymes are combined in a cascade. A very early example has been reported by Whitesides and coworkers that used a combination of adenylate kinase, adenosine kinase, and acetate kinase with acetyl phosphate as donor to achieve the triple phosphorylation of adenosine to ATP [59]. The system using an acetate kinase and a hexokinase or a glycerol kinase with acetyl phosphate as donor is one of the most frequently used examples for ATP regeneration in biocatalytic in vitro reactions [60–62]. Furthermore, pyruvate kinase and phosphoenolpyruvate (PEP) as phosphate donor can be employed for ATP regeneration [63, 64].

Andexer and coworkers reported an enzymatic reaction that combines the SAM-dependent methylation of several catechol derivatives with a coupled cyclic cascade that fuels the SAM-dependent O-methyltransferases (O-MTs) with the required cofactor ATP starting from the more stable SAM precursor adenosine (Scheme 3.9) [65]. The developed SAM regeneration cycle starts with the SAM-dependent alkylation of the substrate catalyzed by the O-MT resulting in the alkylated product as well as S-adenosylhomocysteine (SAH). The SAH is converted by a SAH hydroxylase, resulting in the formation of adenosine and the release of L-homocysteine. In subsequent steps, ATP was generated by a cascade reaction comprising adenosine kinase (ADK) from *Saccharomyces cerevisiae*, polyphosphate kinase (PPK2-II) from *Acinetobacter johnsonii* and polyphosphate kinase (PPK2-I) from *Sinorhizobium meliloti*. In order to drive the ATP formation and to shift the reaction equilibrium, an excess of polyphosphate and catalytic amounts of AMP (1:50 relative to the O-MT substrate) were added. Thus, to close the SAM regeneration cycle, a methionine adenosyltransferase (MAT) from *Escherichia coli* was used to catalyze the conversion of ATP to SAM using methionine as substrate. Overall, up to 25% conversion were achieved. This corresponds to a more than 10-fold regeneration of SAM and was observed for all tested methylation and ethylation reactions.

3.3 Examples of In Vitro Cascades from Industry

Biocatalysts are nowadays employed in a wide variety of industrial processes ranging from bulk chemical manufacture to fine chemical synthesis [66–71]. In particular, enzymes provide a powerful tool in order to produce enantiomerical pure compounds mainly through their high chemoselectivity, regioselectivity, and stereoselectivity [72]. The majority of these processes involve a single-step transformation catalyzed by one enzyme, followed by product isolation and purification. Industrial examples of de novo multi-step and multi-enzyme reactions being truly concurrent are still rare, but recent progress in cascade processes is paving the way for a greater industrial scope of the processes in the future [2]. However, a small number of cascade reactions have already been successfully implemented and reported in industrial laboratories [73]. One example involves a ketoreductase (KRED) for the synthesis of hydroxynitrile that is a key intermediate for atorvastatin using a multi-enzyme process.[69] This drug is a member of the statin family and lowers the cholesterol level by inhibiting the cholesterol synthesis in the liver. Atorvastatin is currently marketed by Pfizer under the trade name Lipitor® [69]. Codexis developed a two-step process consisting of three pre-evolved enzymatic steps, namely halohydrin dehalogenase (HHDH), glucose dehydrogenase (GDH), and KRED (Scheme 3.10).

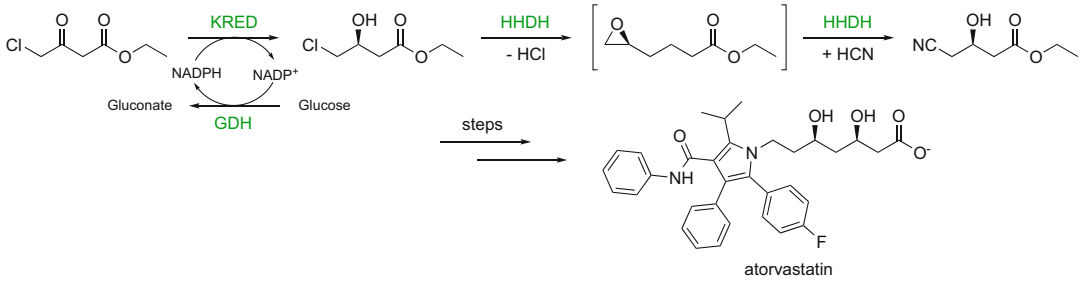
In the first step, the KRED catalyzes the reduction of ethyl-4-chloroacetoacetate that is coupled with the regeneration of the cofactor NADPH by the GDH. In a subsequent reaction catalyzed by



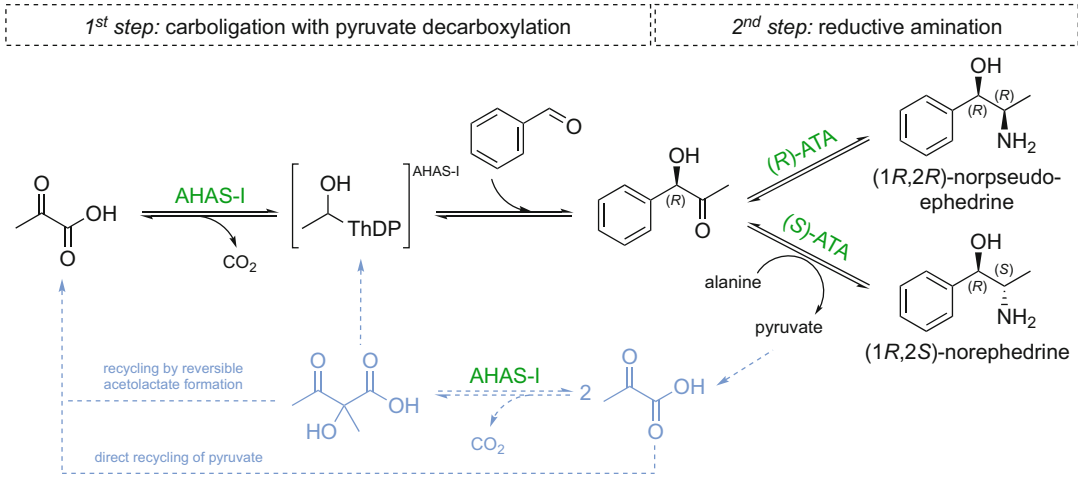
Scheme 3.9 The biocatalytic regeneration cycle of SAM. The enzyme-catalyzed alkylation combined with cofactor regeneration is powered by polyP and uses L-methionine (or a derivative) as alkyl donor. Methionine (or ethionine) and the corresponding substrate for the methyl transferase (MT) are added in stoichiometric amount, along with an excess of polyP and a catalytic amount of AMP as a precursor for the respective cofactor. The scheme was adapted from Mordhost et al. [65]

the HHDH, the product of the KRED is further converted to ethyl (*S*)-4-chloro-3-hydroxybutanoate that is used as a precursor for atorvastatin.

Due to the growing number of enzymes that can be utilized for versatile reactions to produce pharmaceuticals or fine chemicals, attempts at developing one-pot processes based on multi-enzyme reactions are increasing [1–3, 70, 74]. One-pot processes in general offer the advantage of high enantioselectivities while circumventing the need for multiple steps, thus being highly efficient. Especially amine transaminases (ATAs) have been used in a multitude of cascade reactions [75], and are mostly coupled to redox enzymes for cofactor regeneration. A recent example is an one-pot cascade comprising an ATA and a monoamine oxidase (MAO) for the synthesis of chiral 2,5-disubstituted pyrrolidines [76]. Another advantage of one-pot cascade reactions is represented by the possibility to start from simple, inexpensive, and achiral starting materials. The synthesis of nor-pseudoephedrine (NPE) and norephedrine (NE) has been recently demonstrated starting from simple materials such as benzaldehyde and pyruvate through a combination of an ATA and acetohydroxyacid synthase I (AHAS-I) (Scheme 3.11) [77]. By using an (*R*)- or (*S*)-ATA, the stereoisomers of NPE and NE were synthesized with high enantioselectivity (>99%). The by-product from



Scheme 3.10 Multi-enzyme in vitro cascade for the production of the hydroxynitrile intermediate for atorvastatin synthesis using engineered KRED, GDH, and HHDH. The scheme was adapted from Ma et al. [69]



Scheme 3.11 Synthesis of (1*R*,2*R*)-norpseudoephedrine and (1*R*,2*S*)-norephedrine, respectively, by an one-pot two-step cascade reaction combining acetohydroxyacid synthase I (AHAS-I) and a (*R*)-or (*S*)-selective amine transaminase (ATA) with internal pyruvate recycling. The scheme was adapted from Sehl et al. [77]

the ATA reaction (pyruvate) was recycled by the AHAS-I reaction. Moreover, a multi-enzyme network comprising an ADH, an ATA, and an alanine dehydrogenase (AlaDH) connected through redox-recycling has been reported [78].

Another unique example of the power of multi-enzymatic reactions is the commercial production of trehalose from starch. Trehalose, which is a disaccharide consisting of (α-1,1)-linked two glucose units with a relative sweetness of 45% compared to sucrose, while being more thermostable and having a wide pH-stability range compared to other saccharides. Moreover, trehalose does not undergo the Maillard reaction, which makes it an attractive material in the food industry [79]. In 1995, Murata and coworkers paved the way for the industrial production of trehalose by an enzymatic cascade reaction. The necessary enzymes, namely maltooligosyl trehalose synthase (MTSase) and maltooligosyl trehalose trehalohydrolase (MTHase) have been identified and isolated from the trehalose producing bacterium *Arthrobacter* sp. strain Q36. On the basis of this, the Hayashibara Company (Okayama, Japan) started the commercial production of trehalose from starch by using two additional enzymes (isoamylase and cyclodextrin glucanotransferase). The current production scale is assumed to be more than 30,000 tons/year [73].

Thanks to the power of synthetic biology, many patents have focused on optimizing this process by building recombinant plasmids for the co-expression of these three enzymes in one microbial host. Moreover, protein engineering enables further optimization of the biocatalysts by improving their activity, stability, stereoselectivity, and yield. Despite the highly motivating achievements that have been made for multi-enzymatic in vitro reactions, the industrial application of such systems has yet been limited to three biocatalysts in the cascading sequence. This can be mainly attributed to the limitations caused by the complexity of the process including the enzyme-purification procedures. However, the recent advances in genetic and enzyme engineering offer the potential to provide more convenient and less expensive approaches for the purification and the assembly of such multi-enzymatic artificial pathways in vitro [73].

3.4 Conclusion and Outlook

Enzyme cascade reactions have emerged as a widely used synthetic tool. Saving unit operations for the isolation and purification of intermediate products allows for tremendous savings in terms in cost, energy, and waste formation. As most enzymatic reactions require rather similar reaction conditions—they proceed in water and at moderate temperatures—the combination of enzymes in cascades is a generally applicable principle. Yet, the complexity of cascades as well as frequently encountered cross-reactivities and the still limited compatibility of the biocatalysts and their optimal reaction conditions have somewhat delayed the implementation of the concept. Cascade reactions have been routinely applied for redox cofactor regeneration and dynamic kinetic resolutions since decades. More recently, the application of cascades has been expanded to a wide range of different reaction types, and the number of industrial processes utilizing cascades is increasing. An intensive interdisciplinary collaboration between molecular biotechnology, biocatalysis, protein engineering, and process engineering facilitates overcoming the difficulties associated with cascades and is expected to pave the way towards a general application of the concept.

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