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Strategies for regeneration of nicotinamide coenzymes emphasizing self-sufficient closed-loop recycling systems

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Dedicated to Professor Karl-Erich Jäger on the occasion of his 60th birthday.

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ABSTRACT

Biocatalytic reduction reactions depending on nicotinamide coenzymes require an additional reaction to regenerate the consumed cofactor. For preparative application the preferred method is the simultaneous coupling of an *in situ* regeneration reaction. There are different strategically advantageous routes to achieve this goal. The standard method uses a second enzyme and a second co-substrate, for example formate and formate dehydrogenase or glucose and glucose dehydrogenase. Alternatively, a second substrate is employed which is converted by the same enzyme used for the primary reaction. For example, alcohol dehydrogenase catalyzed reactions are often coupled with excess 2-propanol which is oxidized to acetone during the regeneration of NAD(P)H. A third method utilizes a reaction-internal sequence by the direct coupling of an oxidizing and a reducing enzyme reaction. Neither an additional substrate nor a further regenerating enzyme are required for the recycling reaction. This kind of "closed-loop" or "self-sufficient" redox process for cofactor regeneration has been used rarely so far. Its most intriguing advantage is that even redox reactions with unstable precursors can be realized provided that this compound is produced *in situ* by an opposite redox reaction. This elegant method is applicable in special cases only but increasing numbers of examples have been published during the last years.

1. Introduction

During the last years, a large amount of enantio- and regioselective dehydrogenases have been developed covering a wide range of substrates (Gröger et al., 2012a,b; Matsuda et al., 2009). Such enzymes are widely distributed in nature, they have been found in many microorganisms, plant and animal tissues. Both the reduction of C=O-bonds by alcohol (Chen et al., 2012; Gröger et al., 2012a; Müller et al., 2005) or amino acid dehydrogenases (Hummel and Gröger, 2012) and of C=C-bonds by ene or enoate reductases (Winkler et al., 2012; Wohlgemuth, 2014) are of great interest for the production of various chiral compounds such as hydroxy acids, amino acids or alcohols from prochiral precursors. Such products have a high economic value and are applied in food and feed industry, or serve as building blocks in the synthesis of therapeutics, herbicides, insecticides and many more.

Most of these reaction pathways involve reductive steps converting a prochiral precursor compound into a chiral product. Dehydrogenase-catalyzed reactions are most advantageous as nearly all reactions are carried out with quite high enantioselectivity. However, in some cases oxidation reactions with dehydrogenases are suitable, too, for example the regioselective oxidation of one specific hydroxy group in the presence of further oxidizable groups. In all procedures using dehydrogenases it is unavoidable to consider an additional step required to regenerate the consumed coenzyme. This step is a crucial one as the use of such cofactors in stoichiometric amounts is far too expensive for any application. Depending on the kind of the involved coenzyme and the direction of the redox reaction in question, four different methods for the regeneration have to be considered, namely the regeneration of the reduced coenzyme NADH or NADPH, respectively or of the oxidized form NAD^+ or $NADP^+$, respectively.

In order to select an appropriate regeneration system, the following requirements should be noted: the reaction should be kinetically favored, neither the co-product nor the unreacted residual co-substrate of the regeneration reaction should interfere with the enzyme itself or with the subsequent isolation method for the desired product. The development of capable regenerating

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processes has been subject to intensive studies (Kara et al., 2014; Weckbecker et al., 2010). Generally, regeneration of nicotinamide cofactors can be accomplished through enzymatic, chemical, photochemical or electrochemical steps. An appropriate regeneration system should be practical and inexpensive. It must be stable over a long period of time. Products need to be separable without much effort. Catalysts as well as regeneration reagents should be commercially available or easily producible and the product formation should be thermodynamically as well as kinetically favored. Considering all these factors, enzyme-supported ways have become the methods of choice to regenerate reduced or oxidized nicotinamide coenzymes.

In the following paper different approaches to the regeneration of nicotinamide coenzymes are presented.

2. Principle methods for regeneration of coenzymes

Three different principles to regenerate NADH or NADPH will be presented in the following:

- use of a second enzyme and a second co-substrate,
- use of a second substrate, and
- a reaction-internal (closed-loop) regeneration method without an additional co-substrate.

A general method applicable in any case is the coupling of the primary reaction leading to the desired product with a second reaction that cares for the regeneration only and that enters a second substrate and a second enzyme into the system. A variation of this method only requires a second substrate which is converted by the same enzyme during the primary reaction, yielding in simultaneous product formation and coenzyme regeneration. This regeneration system seems to be an inexpensive one, its applicability depends on the substrate acceptance of the synthesizing enzyme which is responsible for the formation of the desired product as well as for cofactor recycling at the same time. A third method called "closed-loop" (Willetts et al., 1991) or "self-sufficient" redox process utilizes the product of the first reaction, the intermediate, to regenerate the coenzyme at producing the desired end product. By this coupling of an oxidizing and a reducing enzyme reaction neither an additional substrate nor a further regenerating enzyme is required for the recycling reaction. Although this seems to be a very simple and unexpensive straight-forward method which already has been described in the 1990th, only a few examples are published up to now. Selected examples illustrating these different routes focussing in particular on the latter "closed-loop" method will be presented in the following.

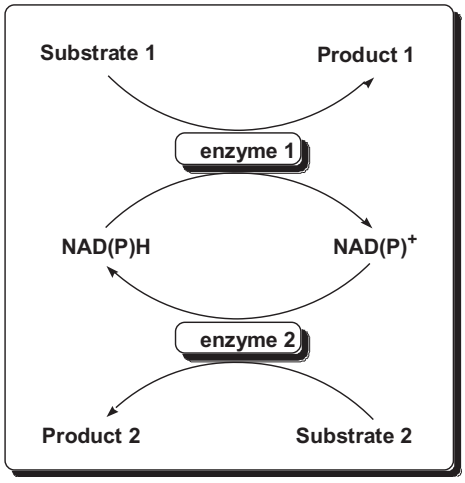
2.1. Use of a second enzyme and a second substrate

Use of a second enzyme and a second regeneration substrate for the regeneration of nicotinamide coenzymes can be considered as the standard method which can be generally applied (Scheme 1). This method is particularly advantageous if the second enzymatic regeneration reaction is irreversible or nearly irreversible. Well-known examples are the formate dehydrogenase and the glucose dehydrogenase reactions.

Coupling the primary reaction with formate and formate dehydrogenase allows reliable regeneration of NADH. Even more useful is the well established reaction with glucose and glucose dehydrogenase which can be used to recycle NADH as well as NADPH.

2.1.1. Formate/formate dehydrogenase

The regeneration of NADH by formate and formate dehydrogenase (FDH) was the first method for the regeneration of a nicotinamide coenzyme to be applied in large scale. It was introduced in 1980 by the group of Whitesides (Shaked and Whitesides, 1980). Later on, it was used industrially for the ton-scale synthesis of *tert.*-leucine (2-amino-3,3-dimethylbutanoic acid) by reductive amination of trimethylpyruvic acid by means of leucine dehydrogenase giving very high yield and excellent optical purity (Gröger et al., 2012a; Scheme 2).

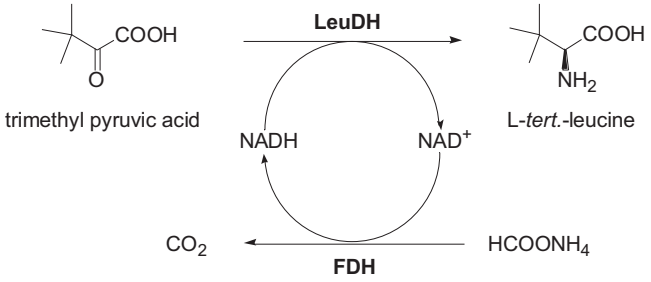


Scheme 1. Regeneration of NADH or NADPH by use of a second (co-) substrate (substrate 2) and a second enzyme (enzyme 2). A (co-) product (product 2) is formed stoichiometrically to product 1.

A major advantage of the formate dehydrogenase catalyzed regeneration reaction is the irreversibility of the reaction. This leads to a high concentration of the reduced coenzyme and a high pressure on the primary reaction leading to nearly 100% conversion of the desired product. Furthermore, the formed carbon dioxide is chemically inert and can be easily removed. Formate is both a very cheap co-substrate and innocuous toward most enzymes, formate dehydrogenase itself is commercially available. The major disadvantage of FDH, however, is its low specific activity (4–6 U/mg) and the limitation to NAD^+ as a substrate.

2.1.2. Glucose/glucose dehydrogenase

Meanwhile, glucose dehydrogenase (GDH; EC 1.1.1.47) and glucose have been used as the regenerator system in many studies for the production of chiral compounds. The resulting lactone is quickly converted into the corresponding acid. Some of the most favorable advantages of the enzyme are its activity with both NAD^+ and $NADP^+$ as cofactors, its high specific activity and high stability; moreover the substrate glucose is inexpensive. However, product isolation may become difficult due to the presence of gluconate. As an example, Scheme 3 demonstrates the formation of actinol ((4*R*,6*R*)-4-hydroxy-2,2,6-trimethylcyclohexanone) from



Scheme 2. Enzyme-catalyzed synthesis of L-*tert.*-leucine as an example for the regeneration of NADH by a second enzyme, formate dehydrogenase, and a second substrate.