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A Novel, Efficient Regenerating Method of NADPH Using a New Formate Dehydrogenase

Karsten Seelbach[1)], Bettina Riebel[2)], Werner Hummel[2)], Maria-Regina Kula[2)], Vladimir I. Tishkov[3)], Alexey M. Egorov[3)], Christian Wandrey[1)], Udo Kragl[*1)]

[1)] **Forschungszentrum Jülich GmbH, Institut für Biotechnologie, D-52425 Jülich, Germany**
[2)] **Institut für Enzymtechnologie, Heinrich Heine Universität, Forschungszentrum Jülich GmbH, Postfach 2050, D-52404 Jülich, Germany**
[3)] **Department of Chemical Enzymology, Chemistry Faculty, Lomonosov Moscow State University, 119899 Moscow, Russia**

Abstract: A NADPH regenerating system using a new, protein engineered formate dehydrogenase (FDH) is investigated. The new enzyme is the first known NAD(P)H dependent FDH. It can be successfully employed in synthesis with other enzymes requiring a NADPH regeneration. All advantages of the known NAD(H) dependent FDH's in these enzyme coupled synthesis can now be transferred to NADP(H) dependent systems.

Many enzymatic synthesis of fine chemicals such as chiral alcohols from prochiral ketones require cofactors like NAD(H) or NADP(H) [1]. Cofactors are too expensive to be used in equimolar amounts for synthesis. Several methods for in-situ regeneration of cofactors have been investigated[2,3]: chemical[4], biological[5], electrochemical[6,7], photochemical and enzymatic regeneration methods. Concerning the enzymatic regeneration[8] the use of glucose-(6-P)-dehydrogenase[9], alcohol dehydrogenase (ADH)[10] and formate dehydrogenase (FDH) are well known. The latter allows an economic regeneration of NADH from NAD^+ with formate[1].

It has been a problem until now that the known FDH's are only working with NAD^+ as cofactor. The consequence is that several known and new NADP(H) dependent alcohol dehydrogenases [11-15] cannot be used economically in synthesis.

The disadvantage of a substrate coupled cofactor regeneration with ADH is that the same ADH used for the desired synthesis is also used for regeneration. As substrate for the regeneration another

alcohol is used such as isopropanol which is oxidized to acetone. The activity of the ADH is distributed between both substrates and part of the activity is lost for the main synthesis. In contrast the use of the enzyme coupled cofactor - regeneration with formate dehydrogenase has several advantages. Formate as substrate for the FDH is one of the cheapest hydrogen sources. The reaction has a favourable equilibrium strongly shifted towards CO_2 and NADH formation[16]. The oxidation product CO_2 can be easily removed from the reaction mixture. Additionally formate normally does not inhibit the other oxidoreductases involved. FDH has a broad pH optimum of activity so that it can be easily implemented in coupled enzymatic synthesis. With a molecular mass of 88,000 Da it can be retained by an appropiate ultrafiltration membrane. Recently it has been shown for NAD(H) dependent FDH from *Candida boidinii* that a technical grade catalyst can be easily obtained in large amounts[17].

We now present data on the application of a new formate dehydrogenase (EC 1.2.1.2.) which was obtained by multipoint site-directed mutagenesis of the gene coding the enzyme from the methylotrophic bacterium *Pseudomonas* sp. 101. Final experiments have shown that the activity of the NADP(H) - specific mutant with $NADP^+$ is about 60% of the activity of wildtype FDH with NAD^+ [18-20].

Stability measurements show no decrease of activity over a period of seven days incubation at a temperature of 25°C. After one year storage at 4°C no loss of activity is detectable.

To show the practicability and stability of the enzyme the reduction of acetophenone in a continuously operated flat membrane reactor[21-24] equipped with an ultrafiltration membrane was investigated (Figure 1).

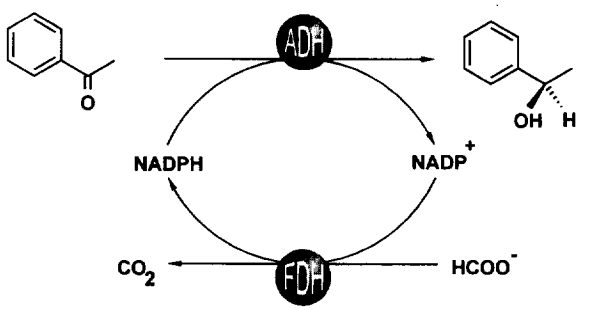

Figure 1: Reaction system for the continuous enzymatic reduction of actophenone using a new NADP(H) dependent formate dehydrogenase

For the reaction a novel NADP(H) dependent ADH (EC 1.1.1.99 [11,12]) from *Lactobacillus* sp. was used which was coupled with the new mutant FDH for cofactor regeneration.

In order to compensate for the deactivation of the ADH[25] every day a small amount of enzyme was added. Reaction conditions[26] and the course of conversion are shown in Figure 2.

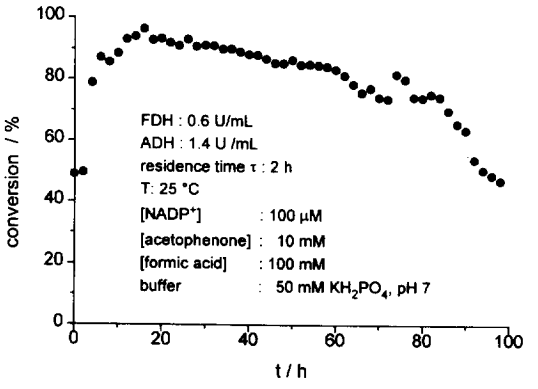

Figure 2: Course of conversion during the continuous enzymatic reduction of acteophenone using a new NADP(H) dependent FDH for cofactor regeneration

In principle conversions[27] higher than 90-95% are possible indicating that the FDH is stable under these conditions. The distinct drop in the conversion after about 90 h was attributed to a microbial contamination after repeated injections of ADH directly into the reactor.

With the experiment shown a total turnover number (ttn)[28] for $NADP^+$ of about 85 was reached under not optimized conditions. A further decrease of the cofactor concentration, which is already below the $K_{M,(NADP+)}$-value of 0.32 mM of the mutant FDH (Figure 3), would affect the activity of the regeneration raction.

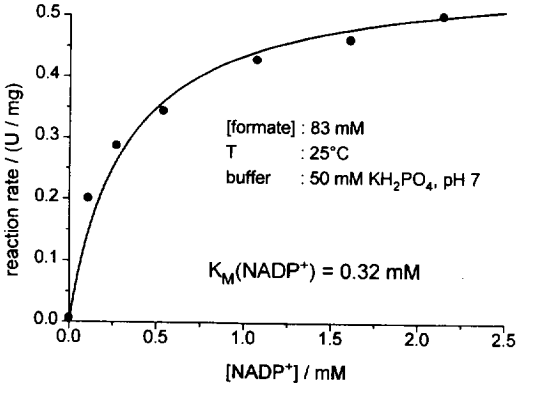

Figure 3: Reaction rate of the new NADP(H) dependent FDH as a function of the $NADP^+$-concentration : measured points and optimized kinetic curve resulting in a K_M for $NADP^+$ of 0.32 mM.

Using other reactor concepts an increase of the ttn can be expected. Often it is possible to extract the not converted substrate or product by means of organic solvents. The cofactor containing solution can then be recycled directly[2]. Another possibility is to replace the ultrafiltration membrane usually used in enzyme membrane reactors by a reverse osmosis membrane[29]. In the latter case bulky cofactors are retained to a certain extent so that the concentration in the feed solution can be decreased. With both reactor concepts the ttn could be increased up to 10 times.

In conclusion our results provide evidence that this new stable NADP(H) dependent FDH can be used for cofactor regeneration in continuous enzymatic synthesis without problems. This new FDH seems to be one of the best alternatives for the problematic $NADP^+$ regeneration to allow new interesting synthesis with NADP(H) dependent enzymes. Other experiments using this FDH for cofactor regeneration in enzymatic synthesis are currently under investigation to show the potential of this new enzyme. Besides, new mutants are engineered with improved kinetic properties.

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25. The stability measurements were determined under following conditions: 25°C, 100 mM phosphate-buffer, pH 7. The residual activity after 3 days of a simple purified crude extract is 72%.
26. The ADH shows only activity if Mg^{2+} - cations are present in the reaction solution. So all solutions contain 1 mM $MgCl_2$.
27. Conversion calculated from peak areas from acetophenone and phenylethanol which were determined on a GC using a chiral stationary phase: Gaschromatograph 438A (Chrompack, The Netherlands) equipped with a column FS-Cyclodextrin ß I/P 50 m×0.32 mm ID (Macherey & Nagel, Düren, Germany), carrier gas H_2, isothermal at 110°C, FID.
28. The total turnover number is defined as mol product per mol cofactor consumed.
29. Seelbach, K.; Kragl, U. , unpublished results.

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