ChemBioChem

Communications
doi.org/10.1002/cbic.202000651

Chemistry Europe
European Chemical
Societies Publishing

Facile Stereoselective Reduction of Prochiral Ketones by using an F_{420}-dependent Alcohol Dehydrogenase

Caterina Martin,[a] Gwen Tjallinks,[a] Milos Trajkovic,[a] and Marco W. Fraaije*[a]

Effective procedures for the synthesis of optically pure alcohols are highly valuable. A commonly employed method involves the biocatalytic reduction of prochiral ketones. This is typically achieved by using nicotinamide cofactor-dependent reductases. In this work, we demonstrate that a rather unexplored class of enzymes can also be used for this. We used an F_{420}-dependent alcohol dehydrogenase (ADF) from *Methanoculleus thermophilicus* that was found to reduce various ketones to enantiopure alcohols. The respective (*S*) alcohols were obtained in excellent enantiopurity ($>99\%$ *ee*). Furthermore, we discovered that the deazaflavoenzyme can be used as a self-sufficient system by merely using a sacrificial cosubstrate (isopropanol) and a catalytic amount of cofactor F_{420} or the unnatural cofactor FOP to achieve full conversion. This study reveals that deazaflavoenzymes complement the biocatalytic toolbox for enantioselective ketone reductions.

Figure 1. Structural formula of the F_{420} deazaflavin cofactor. The deazaflavins FO and FOP are also indicated.

The biocatalytic production of chiral alcohols is feasible by employing different classes of enzymes: oxidoreductases (EC 1), hydrolases (EC 3), and lyases (EC 4).[1] Several methods allow for the production of chiral alcohols and an effective method involves the enantioselective reduction of prochiral ketones by alcohol dehydrogenases (ADHs). ADHs have gained great interest due to their enantioselectivity, allowing for the development of many biotechnological applications.[2] The applicability of ADHs is somewhat limited due to their cofactor dependence: most ADHs rely on the nicotinamide cofactors NADH or NADPH for performing reductions.[3] Often, cofactor regeneration is achieved by using an enzyme-coupled process.[4] We explored another type of ADH: an F_{420}-dependent ADH. Such type of ADH had never been explored for biocatalytic reductions of ketones. The deazaflavin cofactor F_{420} was discovered in 1972, first in methanogens and later also in actinobacteria and other bacteria.[5–7] The redox active part of the cofactor resembles the isoalloxazine ring of the canonical flavin cofactors (Figure 1). The differences are the lack of the
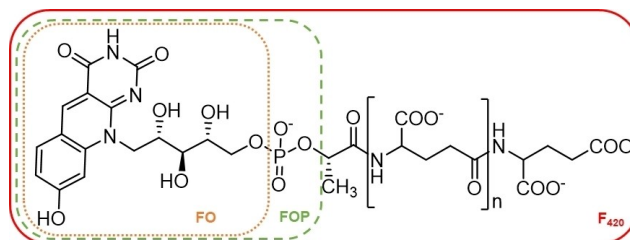
N5, different groups on the benzylic moiety, and the poly-γ-glutamate lactyl tail instead of the adenine part of FAD. The absence of N5 (a carbon instead) dictates that the cofactor is an obligatory hydride transfer agent, similar to the nicotinamide cofactors. The F_{420} cofactor displays an absorption maximum at 420 nm, hence its name. The redox potential of this redox cofactor is exceptionally low: -380 mV [(220 mV for FAD and -320 mV NAD(P)].[5,8]

While flavin and nicotinamide cofactors are readily available together with a huge number of enzymes utilizing these cofactors, the biocatalytic exploration of F_{420}-dependent enzymes is lagging behind. This is partly due to the fact that the deazaflavin cofactor was difficult to obtain. The deazaflavin cofactor can be isolated from F_{420}-producing microbes, such as *Mycobacterium smegmatis* or a F_{420}-producing recombinant microorganism.[9,10] Although it involves a laborious process and yields small amounts, it is nowadays available for biocatalytic studies. Furthermore, we have recently demonstrated that a truncated version of the cofactor, lacking the poly-γ-glutamate lactyl tail (FOP, see Figure 1), is also accepted as cofactor by F_{420}-dependent enzymes.[11] FOP represents an unnatural deazaflavin cofactor for which a relatively easy synthetic procedure has been developed. Another bottleneck for using F_{420}-dependent enzymes has been the lack of knowledge of and access to such biocatalysts. Particularly, these redox enzymes seem promising for performing selective reductions by virtue of the unique low redox potential deazaflavin cofactor. This would require regeneration of the reduced deazaflavin cofactor. This demand for $F_{420}H_2$-regenerating enzymes has been satisfied by the recent discovery of several deazaflavoenzymes. The availability of several F_{420}-dependent glucose-6-phosphate dehydrogenases is especially attractive for this.[12]

In the last decade the interest in exploring deazaflavoenzymes for biocatalysis has increased.[8,13–15] F_{420}-dependent enzymes were shown to be involved in the production of antibiotics, and could be used for the asymmetric reduction of

[a] C. Martin, G. Tjallinks, Dr. M. Trajkovic, Prof. Dr. M. W. Fraaije
Molecular Enzymology Group, University of Groningen
Nijenborgh 4, Groningen (The Netherlands)
E-mail: m.w.fraaije@rug.nl

ChemBioChem

Communications
doi.org/10.1002/cbic.202000651

Chemistry
Europe
European Chemical
Societies Publishing

prochiral imines and enones.[16–20] In this work we explored an F_{420}-dependent ADH (ADF) for its ability to perform enantiose-
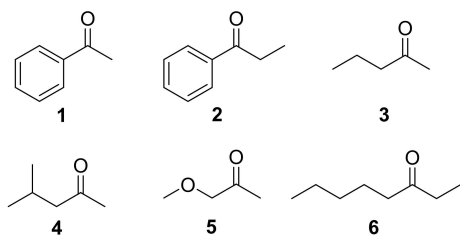

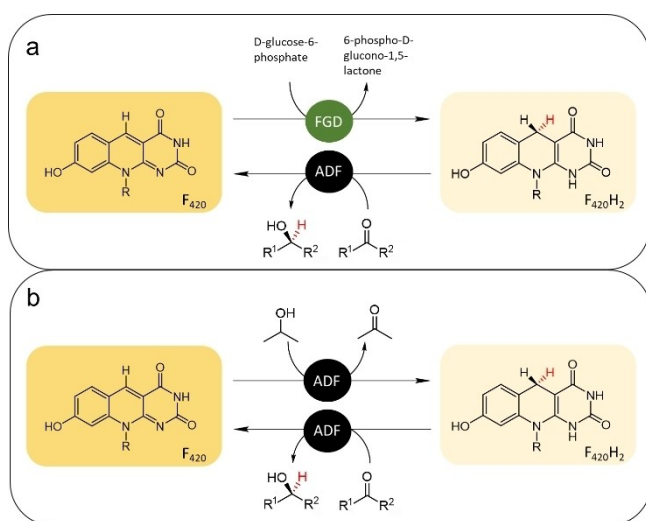**Figure 2.** Ketones used for the biocatalytic exploration of ADF.

Figure 3. Coupled reactions for the reduction of prochiral ketones by ADF. ADF: F_{420}-dependent alcohol dehydrogenase, FGD: F_{420}-dependent glucose-6-phosphate dehydrogenase. a) D-glucose-6-phosphate is used by FGD to regenerate $F_{420}H_2$. b) Self-sufficient use of ADF for ketone reductions with isopropanol as cosubstrate to regenerate $F_{420}H_2$.

lective reductions of prochiral ketones. The F_{420}-dependent alcohol dehydrogenase from *Methanoculleus thermophilicus* was chosen for this. ADF is a relatively small enzyme (37 kDa) that was first described in 1989. It can be recombinantly produced in *Escherichia coli*, and its crystal structure has been elucidated.[17,21] Previously it was shown that ADF converts small aliphatic secondary alcohols into ketones. It was shown that during this oxidation step a hydride is transferred in a stereo-selective manner from the alcohol to the *si* face of the F_{420} cofactor (to the C5 atom).[22] It was demonstrated that the enzyme is able to oxidize isopropanol to acetone and the reverse reaction.[17] This was a clear indication that the enzyme may act as an enantioselective ketone reductase through an enantioselective hydride transfer.[21]

First, we explored the substrate scope of ADF to find out whether it is able to accept alcohols different from isopropanol. Alcohol oxidation activity could be easily monitored by measuring the decrease of absorbance of F_{420} (the reduced form does not absorb at 420 nm). ADF did not convert the following alcohols: heptan-2-ol, 5-aminopentan-1-ol, 1-amino-propan-2-ol, 3-chloropropane-1,2-diol, 1-phenylethane-1,2-diol, indan-ol, acetoin, carveol, cyclohexylmethanol, tetrahydropyran-2-methanol, 3-butyn-2-ol. Nevertheless, we could identify a set of aromatic and aliphatic alcohols that were not reported before as substrate of ADF. The corresponding ketones were investigated as prochiral substrates for ADF (Figure 2).

For the first test conversions, we used the F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD) from *Rhodoccocus jostii* RHA1 (FGD) together with ADF (Figure 3a).[12] Gratifyingly, it was found that the enzyme was able to fully and stereo-selectively reduce methyl ketones to the corresponding (S) alcohols. For most ketones, excellent enantioselectivity was observed (>99% *ee*), except for substrate 3 (92% *ee*) and substrate 6 (racemic product; Table 1).

In silico docking studies were performed to understand the molecular basis for the (S) stereoselectivity of ADF. For

Table 1. Reduction of prochiral ketones by ADF.[a]

	Substrate	Cofactor	Cofactor regeneration[b]	Conversion [%]	*ee* [%]
1EF$_{420}$	1	F$_{420}$	FGD	96	>99 (S)
1IF$_{420}$	1	F$_{420}$	–	95	>99 (S)
1IFop	1	FOP	–	5	n.d.
2EF$_{420}$	2	F$_{420}$	FGD	77	>99 (S)
2IF$_{420}$	2	F$_{420}$	–	80	>99 (S)
2IFop	2	FOP	–	3	n.d.
3EF$_{420}$	3	F$_{420}$	FGD	>99	92 (S)
3IF$_{420}$	3	F$_{420}$	–	>99	72 (S)
3IFop	3	FOP	–	94	>99 (S)
4EF$_{420}$	4	F$_{420}$	FGD	87	>99 (S)
4IF$_{420}$	4	F$_{420}$	–	93	>99 (S)
4IFop	4	FOP	–	2	n.d.
5EF$_{420}$	5	F$_{420}$	FGD	>99	>99 (S)
5IF$_{420}$	5	F$_{420}$	–	>99	>99 (S)
5IFop	5	FOP	–	>99	>99 (S)
6EF$_{420}$	6	F$_{420}$	FGD	>99	0
6IF$_{420}$	6	F$_{420}$	–	>99	0
6IFop	6	FOP	–	>99	0

[a] Values obtained using 250 mM sodium phosphate pH 7.0, 10% glycerol, 1% DMSO, 2.0 mM substrate, 2.0 mM ethyl benzene (internal standard), 20 μM ADF, 40 μM F420 or 40 μM FOP. [b] For cofactor regeneration, 20 mM glucose-6-phosphate with 10 μM FGD ("FGD") or 200 mM isopropanol ("–") was used. Details are given in the Supporting Information. n.d.: Conversions were too low for an accurate determination of *ee*.

acetophenone (1) and methoxyacetone (5) docking suggested that the structurally favorable product is in both cases indeed the (S) enantiomer. This can be explained by examining the role and position of the active-site residues. Previous studies postulated that His39, Trp43, Glu12 and Glu108 are crucial for substrate binding and catalysis.[21] In the reduction reaction, a hydride transfer from the C5 atom of $F_{420}H_2$ to the ketone carbon with a simultaneous or stepwise proton addition from His39 to the ketone oxygen occurs. Hence, the specific orientation of substrate towards the F_{420} cofactor and His39 is decisive in the enantioselective outcome of the reaction. Docking of acetophenone revealed that Trp43 together with Val193, Trp229, Trp246, Cys249 and Phe255 form a hydrophobic pocket which snugly accomodates the aromatic ring of the substrate (Figure 4). As a result, acetophenone binds in the active site in such a way that only the (S) enantiomer can be formed: the hydride can only be transferred to the re face of the substrate. Positioning of acetophenone necessary to form the R-enantiomeric alcohol is prevented because its aromatic ring cannot be accommodated in the active site in any other conformation. Docking of methoxyacetone resulted in an analogous optimal binding pose in which the apolar pocket next to the deazaflavin cofactor plays a crucial role in positioning the substrate such that hydride attack will occur on the re face of the substrate, assisted by proton transfer by His39. This will, again, only allow formation of (S)-1-methoxypropan-2-ol, as experimentally observed.

The second aim of this work was to establish an efficient cofactor regeneration system. While the used glucose-6-phosphate dehydrogenase is effective in recycling $F_{420}H_2$, such cofactor regeneration system still requires an addition enzyme and a relatively expensive cosubstrate. Therefore, we explored whether ADF can be used as reductase and dehydrogenase in one pot, using a sacrificial and cheap alcohol as cosubstrate. This would eliminate the need for another enzyme for cofactor recycling. For this, we tested the use of isopropanol as it had been reported to be a good ADF substrate. In order to determine the highest concentration of isopropanol tolerated in conversions, we first assessed the thermostability of ADF in the presence of different amounts of isopropanol and its kinetic parameters with isopropanol. ADF was found to be relatively tolerant towards isopropanol with only a slight change in

apparent melting temperature up to 200 mM isopropanol (T_m went from 57.5 to 56.0 °C, see Supporting Information). The steady-state kinetic analysis confirmed that isopropanol is an effective substrate with a K_M value of 1.3 mM and a k_{cat} of 1.7 s^{-1} (see the Supporting Information). A concentration of 200 mM of isopropanol was selected to probe it as cosubstrate for the ADF-catalyzed reduction of prochiral ketones. Remarkably, the use of isopropanol as cosubstrate worked extremely well. In fact, there was no difference between the use of glucose-6-phosphate dehydrogenase and glucose-6-phosphate or merely isopropanol for cofactor regeneration (Table 1). This shows that the use of isopropanol as coupled substrate is a valid and simple alternative to use ADF as enantioselective ketone reductase.

Finally, we also explored whether F_{420} can be replaced by an unnatural deazaflavin cofactor: FOP. We have recently shown that FOP can be prepared in a relatively easy manner and is often accepted by F_{420}-dependent enzymes.[11] The results obtained using FOP as alternative cofactor with ADF and isopropanol as cosubstrate revealed that ADF can also operate with this alternative deazaflavin (Table 1). Yet, the use of FOP resulted in lower conversions for some of the ketones tested, while the enantioselectivity was largely retained. The inferior performance, when compared with F_{420}, probably reflects a poor recognition of FOP by ADF.

In conclusion, the finding that ADF can reduce various prochiral ketones in a highly S-stereoselective manner unveils a new biocatalytically relevant class of enzymes: F_{420}-dependent ketone reductases. They can be regarded as alternatives to nicotinamide cofactor-dependent enzymes.[23] Moreover, we demonstrate that isopropanol can be used as cheap cosubstrate for cofactor recycling, rendering ADF self-sufficient. With the crystal structure of ADF available and many genes encoding for ADF homologues in the genome sequence database, it will be exciting to explore other variants for more demanding selective reductions.

Experimental Section

Reagents and chemicals were purchased from Sigma-Aldrich unless indicated otherwise. F_{420} was isolated from *Mycobacterium smegmatis* as described before.[9] The production strain *M. smegmatis* mc^2 4517 was a kind gift from Dr. G. Bashiri (University of Auckland, New Zealand). The expression and purification of F_{420} dependent enzymes are described in the Supporting Information.

For ketone reductions, reaction mixtures contained 200 μL of 250 mM sodium phosphate pH 7.0, 10% glycerol, 1% DMSO, 2.0 mM substrate, 2.0 mM ethyl benzene (internal standard), 20 μM ADF, 40 μM F_{420} or 40 μM FOP, and 200 mM isopropanol or 20 mM glucose-6-phosphate with 10 μM FGD. The reaction was performed in a 1.5 mL Eppendorf tube in an Eppendorf Thermomixer at 25 °C and 500 rpm for 24 h. The reaction was extracted twice using 200 μL of ethyl acetate. The reactions passed over anhydrous magnesium sulfate and finally analyzed using GC (details in the Supporting Information).
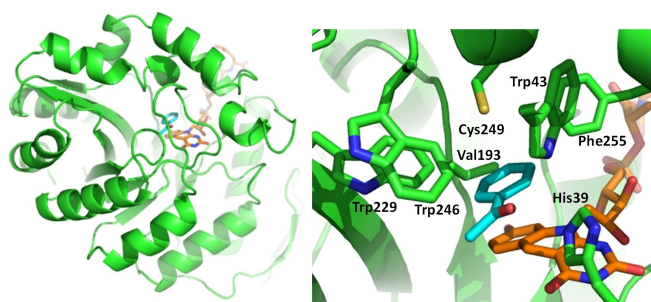

Figure 4. Binding pose of acetophenone (1) in ADF (PDB ID: 1RHC). Left: overall structure of ADF with docked substrate (cyan) and F_{420} (orange) highlighted in sticks. Right: close-up of acetophenone binding.

ChemBioChem

Communications
doi.org/10.1002/cbic.202000651

Chemistry
Europe
European Chemical
Societies Publishing

[1] K. Goldberg, K. Schroer, S. Lütz, A. Liese, *Appl. Microbiol. Biotechnol.* **2007**, *76*, 237–248.
[2] Y. G. Zheng, H. H. Yin, D. F. Yu, X. Chen, X. L. Tang, X. J. Zhang, Y. P. Xue, Y. J. Wang, Z. Q. Liu, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 987–1001.
[3] E. Tassano, M. Hall, *Chem. Soc. Rev.* **2019**, *48*, 5596–5615.
[4] W. Hummel, M. R. Kula, *Eur. J. Biochem.* **1989**, *184*, 1–13.
[5] R. S. Cheeseman, P. Toms-Wood, A. Wolfe, *J. Bacteriol.* **1972**, *112*, 1887–1891.
[6] L. Daniels, N. Bakhiet, K. Harmon, *Syst. Appl. Microbiol.* **1985**, *6*, 12–17.
[7] B. Ney, F. H. Ahmed, C. R. Carere, A. Biswas, A. C. Warden, S. E. Morales, G. Pandey, S. J. Watt, J. G. Oakeshott, M. C. Taylor, et al., *ISME J.* **2017**, *11*, 125–137.
[8] M. Taylor, C. Scott, G. Grogan, *Trends Biotechnol.* **2013**, *31*, 63–64.
[9] D. Isabelle, D. R. Simpson, L. Daniels, *Appl. Environ. Microbiol.* **2002**, *68*, 5750–5755.
[10] G. Bashiri, J. Antoney, E. N. M. Jirgis, M. V. Shah, B. Ney, J. Copp, S. M. Stuteley, S. Sreebhavan, B. Palmer, M. Middleditch, et al., *Nat. Commun.* **2019**, *10*, 1558.
[11] J. Drenth, M. Trajkovic, M. W. Fraaije, *ACS Catal.* **2019**, *9*, 6435–6443.
[12] Q. T. Nguyen, G. Trinco, C. Binda, A. Mattevi, M. W. Fraaije, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 2831–2842.
[13] M. L. Mascotti, H. Kumar, Q. T. Nguyen, M. J. Ayub, M. W. Fraaije, *Sci. Rep.* **2018**, *8*, 1–10.
[14] C. Greening, F. H. Ahmed, A. E. Mohamed, B. M. Lee, G. Pandey, A. C. Warden, C. Scott, J. G. Oakeshott, M. C. Taylor, J. Jackson, *Microbiol. Mol. Biol. Rev.* **2016**, *80*, 451–493.
[15] M. V Shah, J. Antoney, S. W. Kang, A. C. Warden, C. J. Hartley, H. Nazem-Bokaee, C. J. Jackson, C. Scott, *Catalysts* **2019**, *9*, 868.
[16] H. Ichikawa, G. Bashiri, W. L. Kelly, *J. Am. Chem. Soc.* **2018**, *140*, 10749–10756.
[17] F. Widdel, R. S. Wolfe, *Arch. Microbiol.* **1989**, *152*, 322–328.
[18] W. Li, S. Chou, A. Khullar, B. Gerratana, *Appl. Environ. Microbiol.* **2009**, *75*, 2958–2963.
[19] M. C. Taylor, C. J. Jackson, D. B. Tattersall, N. French, T. S. Peat, J. Newman, L. J. Briggs, G. V. Lapalikar, P. M. Campbell, C. Scott, et al., *Mol. Microbiol.* **2010**, *78*, 561–575.
[20] S. Mathew, M. Trajkovic, H. Kumar, Q. T. Nguyen, M. W. Fraaije, *Chem. Commun.* **2018**, *54*, 11208–11211.
[21] S. W. Aufhammer, E. Warkentin, H. Berk, S. Shima, R. K. Thauer, U. Ermler, *Structure* **2004**, *12*, 361–370.
[22] R. Klein, H. Berk, E. Purwantini, L. Daniels, R. K. Thauer, *Eur. J. Biochem.* **1996**, *239*, 93–97.
[23] K. Bleicher, J. Winter, *Eur. J. Biochem.* **1991**, *51*, 43–51.