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Facile Stereoselective Reduction of Prochiral Ketones using an F₄₂₀-dependent alcohol dehydrogenase

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

Abstract: 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 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 F₄₂₀-dependent alcohol dehydrogenase (ADF) from *Methanoculleus thermophilicus* which was found to reduce various ketones into enantiopure alcohols. The respective S-alcohols were obtained in excellent enantiopurity (>99% e.e.). 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 F₄₂₀ or the unnatural cofactor FOP to achieve full conversions. This study reveals that deazaflavoenzymes complement the biocatalytic toolbox for enantioselective ketone reductions.

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 F₄₂₀-dependent ADH. Such type of ADH had never been explored for biocatalytic reductions of ketones. The deazaflavin cofactor F₄₂₀ 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 F₄₂₀ 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]

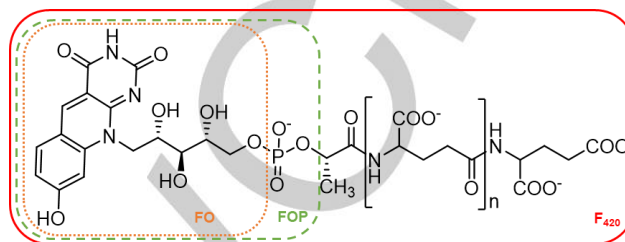


Figure 1. Structural formula of the F₄₂₀ deazaflavin cofactor. Also other deazaflavins are indicated: FO and FOP.

While flavin and nicotinamide cofactors are readily available together with a huge number of enzymes utilizing these cofactors, the biocatalytic exploration of F₄₂₀-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 F₄₂₀-producing microbes, such as *Mycobacterium smegmatis* or a F₄₂₀-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 F₄₂₀-dependent enzymes.^[11] FOP represents an unnatural deazaflavin cofactor for which a relatively easy synthetic procedure has been developed. Another bottleneck for using F₄₂₀-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 F₄₂₀H₂-regenerating enzymes has been satisfied by the recent discovery of several deazaflavo-enzymes. The availability of several F₄₂₀-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] F₄₂₀-dependent enzymes were shown to be involved in the production of antibiotics, and could be used for the asymmetric reduction of prochiral imines and enones.^[16–20] In this work we explored an F₄₂₀-dependent ADH (ADF) for its ability to perform enantioselective reductions of prochiral ketones. The F₄₂₀-dependent alcohol dehydrogenase from *Methanoculleus thermophilicus* was chosen for this. ADF is a relatively small enzyme (37 kDa) which 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 stereoselective manner from the alcohol to the Si-face of the F₄₂₀ cofactor (to the C5 atom).^[22] It was demonstrated that the enzyme is able to oxidize isopropanol to acetone and the

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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: 2-heptanol, 5-amino-1-pentanol, 1-amino-2-propanol, 3-chloro-1,2-propanediol, 1-phenyl-1,2-ethanediol, 1-indanol, 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).

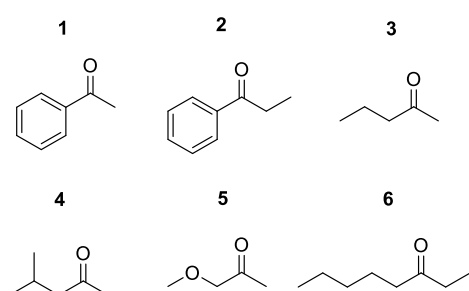


Figure 2. Ketones used for the biocatalytic exploration of ADF.

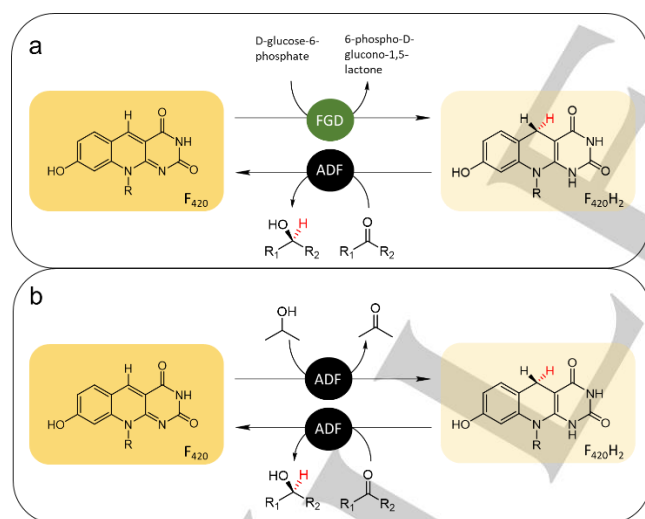


Figure 3. Coupled reactions for the reduction of prochiral ketones by ADF. ADF: F_{420} -dependent alcohol dehydrogenase, FGD: F_{420} -dependent glucose-6-phosphate dehydrogenase. (a) D-glucose-6-phosphate is used by FGD to regenerate $F_{420}H_2$. (b) Self-sufficient use of ADF for ketone reductions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

For the first test conversions, we used the F_{420} -dependent glucose-6-phosphate dehydrogenase (FGD) from *Rhodococcus jostii* RHA1 (FGD) together with ADF (Figure 3a).^[12] Gratifyingly, it was found that the enzyme was able to fully and stereoselectively reduce methyl ketones to the corresponding (S)-

alcohols. For most ketones, excellent enantioselectivity was observed (>99% e.e.), except for substrate 3 (92% e.e.) and substrate 6 (racemic product) (Table 1).

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

Entry	Substrate	Cofactor	Cofactor regeneration ^[b]	Conversion (%)	e.e. (%)
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 μ M ADF, 40 μ M F_{420} or 40 μ M FOP. [b] For cofactor regeneration, 20 mM glucose-6-phosphate with 10 μ M FGD ('FGD') or 200 mM isopropanol ('-') was used. Details are in the Supporting Information. *Conversions were too low for an accurate determination of e.e.

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

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stepwise proton addition from His39 to the ketone oxygen occurs. Hence, the specific orientation of substrate towards the F₄₂₀ 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 accommodates 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-methoxy-2-propanol, as experimentally observed.

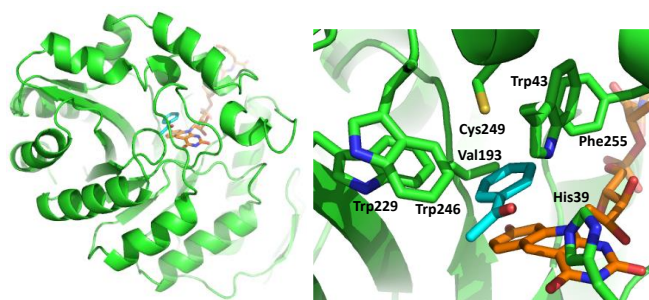


Figure 4 Binding pose of acetophenone (1) in ADF (PDB:1RHC). Left: overall structure of ADF with docked substrate (cyan) and F₄₂₀ (orange) highlighted in sticks. Right: close-up of binding of acetophenone.

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 F₄₂₀H₂, 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 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 F₄₂₀ 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 F₄₂₀-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 F₄₂₀, 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: F₄₂₀-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 homologs 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 (St. Louis, MO, USA) unless indicated otherwise. F₄₂₀ was isolated from *Mycobacterium smegmatis* as described before.^[9] The production strain *M. smegmatis* mc² 4517 was a kind gift from Dr. G. Bashiri from the University of Auckland, New Zealand. Expression and purification of F₄₂₀ 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 F₄₂₀ 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).

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Keywords: biocatalysis • deazaflavin • enantioselective • prochiral ketones • reduction

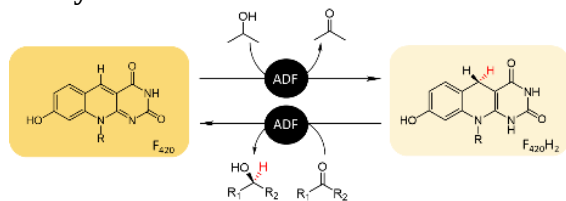
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An F₄₂₀-dependent alcohol dehydrogenase can be used to perform asymmetrical reduction of prochiral ketones. Conveniently, using a cheap sacrificial cosubstrate (isopropanol), regeneration of the deazaflavin cofactor by the same enzyme can be achieved.