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Exploring deazaflavoenzymes as biocatalysts

Kumar, Hemant

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Summary

Hemant Kumar and Marco W. Fraaije

Flavin-dependent enzymes form an important class of enzymes capable of catalyzing a wide range of reactions. As biocatalysts, they are especially known for their ability to catalyze oxidations and reductions in an enantio- and regioselective manner. Enzyme discovery and engineering tools such as genome mining, computational design, directed evolution and structural biology altogether have contributed significantly towards improving the overall performance of flavoenzymes for industrial applications and are continuing to do so. However, there are still a lot of enzymes hidden in the genomic databases which are potentially promising. Except for flavin-dependent enzymes, there are also other enzyme classes that may be sources for development of redox biocatalysts. Cofactor F_{420}-dependent enzymes belong to such class of enzymes which have hardly been explored. Cofactor F_{420} is a natural deazaflavin analogue of flavin cofactors which has more reducing power than the conventional flavins due to its lower redox potential. Research described in this thesis focused on unveiling the biocatalytic potential of F_{420}-dependent enzymes, deazaflavoenzymes.

Chapter 1 provides a general introduction about cofactor F_{420}: its chemical properties and biosynthetic pathway in archaea and bacteria, and details about F_{420}-dependent enzymes which are promising from a biocatalytic point of view. F_{420}-dependent enzymes can only catalyze two electron (hydride) transfer reactions which limits their applicability. Yet, because of the redox properties of the cofactor, (in theory) they can catalyze more demanding hydride transfer reaction when compared with enzymes that employ a flavin or nicotinamide cofactor. $F_{420}H_2$-dependent reductases have been shown to degrade recalcitrant aflatoxins, activate pro-drugs for treating tuberculosis (such as PA-824) and perform enantioselective biotransformations (chapter 5), advocating their potential as biocatalysts. Therefore, it is attractive to use genome mining, proteomics, structural biology, and enzyme engineering tools to identify, characterize and engineer F_{420}-dependent enzymes. Such novel enzymes will complement the current set of available redox biocatalysts, especially complementing the biocatalyst toolbox used for selective reductions.

We have combined a newly developed proteomics approach, involving a cofactor affinity chromatography step, to identify novel F_{420}-binding proteins (chapter 2). The method was validated using cell extract of *Mycobacterium smegmatis*. Covalently immobilized cofactor F_{420} on one hand fished out F_{420}-dependent proteins from the cell free extract of *M. smegmatis* and by mass spectrometry the protein sequences were

determined. For this method, the deazaflavin cofactor was covalently immobilized on a series of commercially available column materials of varying spacer lengths. The free carboxylic groups of F_{420} reacted with the amine-activated column materials. While the polyglutamyl tail is covalently tethered to the carrier material, the part of the cofactor essential for binding to the apo proteins remains available for binding. It was found that the type of column material played an important role in non-specific binding of proteins. Hydroxyl groups of agarose were found to be responsible for such non-specific binding. Other column material types, such as polymethacrylate, gave better results. Mass spectrometry results confirmed the specific binding of known and predicted F_{420}-dependent proteins to the F_{420}-polymethacrylate-based affinity column material. It also resulted in identifying proteins with no known function; they may represent truly novel deazaflavoenzymes with unexplored catalytic properties.

The fact that cofactor F_{420} is not commercially available and only very low amounts (1 µmole F_{420} /L) can be isolated from a conventional host (*M. smegmatis*) poses one of the challenges to study and utilize F_{420}-dependent enzymes. In order to perform biocatalysis using $F_{420}H_2$–dependent reductases without using a significant amount of F_{420}, $F_{420}H_2$ recycling enzymes are desperately needed. We have identified a thermostable F_{420}:NADPH oxidoreductase (FNO) (chapter 3) by genome mining of the mesophilic bacterium *Thermobifida fusca.* To best of our knowledge, Tfu-FNO is the first bacterial FNO for which a crystal structure was solved (1.8 Å resolution). Tfu-FNO was heterologously expressed in *E. coli* Top10 cells with high yield (200 mg/L). As NADPH, the natural substrate of FNO, is ten times costlier than NADH, we performed engineering of the NADPH binding site to make it accepting NADH. Residues interacting with the 2'-phosphate moiety of $NADP^+$ (T28, S50, R51 and R55) were mutated to other amino acids. Results showed that these residues are important in discriminating $NADP^+$ from NAD^+. Mutant S50E was able to lower the K_m almost three times as compared to wild type FNO. Wild type FNO and mutant S50E can be used for $F_{420}H_2$ recycling at the expense of NADPH and NADH, respectively.

In chapter 4, we investigated evolutionary aspects of F_{420}-dependent enzymes belonging to the one particular enzyme family. Cofactor F_{420} was first of all discovered in methanogens and is also found in archaea where it plays an important role in metabolism. This gives the impression that the cofactor F_{420}-dependent enzymes might be primitive or even the ancestors of the flavoenzymes. Through a detailed

phylogenetic analysis, we found out that the previous statement is not true. F_{420}-dependent enzymes have actually emerged from a common FMN-dependent ancestor. During phylogenetic analysis of F_{420}-dependent dehydrogenases, we identified a novel subgroup of dehydrogenases which also accept substrates other than glucose-6-phosphate, hence called sugar-6-phosphate dehydrogenases (FSD). The resurrected ancestor of FSD and FGD was thermotolerant and flexible in terms of substrate acceptance.

The reducing power of cofactor F_{420} is utilized by $F_{420}H_2$-dependent reductases. In chapter 5, we report for the first time on the ability of F_{420}-dependent enantio- and regioselective *ene*-reductions using $F_{420}H_2$-dependent reductases (FDRs). These relatively small deazaflavoenzymes can convert α,β-unsaturated aldehydes and ketones with excellent enantioselectivity (often with >99% *ee*). Interestingly, the enantioselectivity obtained using these enzymes is opposite to that observed for the well-known and explored flavin-dependent *ene*-reductases. Unfortunately, the rate at which the reductions are catalyzed are rather low. These newly studied $F_{420}H_2$-dependent enzymes are a good starting point for future enzyme engineering attempts to obtain more efficient biocatalysts.

Flavin-containing Baeyer-Villiger monoxygenases (BVMOs) are examples of well-studied flavoenzymes used for biocatalytic oxidations. In chapter 6, we report on the ability of BVMOs to act on furanoid aldehydes, a class of compounds that had not been tested with BVMOs before. Surprisingly, most of the tested BVMOs were found to accept furfural as substrate. Furfural and several other furanoid aldehydes were converted to the acid instead of being converted into the expected formate esters via a typical Baeyer-Villiger oxidation. This affords a new biocatalytic route towards producing an attracttive polymer precursor, 2,5-furandicarboxylic acid (FDCA).