Exploring deazaflavoenzymes as biocatalysts

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Identifying novel F_{420}-dependent proteins through a proteomic approach

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Abstract

Cofactor F_{420} serves as a natural deazaflavin cofactor in methanogenic and non-methanogenic archaea, and in various bacteria. Although the role of cofactor F_{420} in methane metabolism is well known, its role is still unclear in F_{420}-containing bacteria such as Mycobacterium tuberculosis. Using computational approaches, these organisms have been predicted to be rich in F_{420}-binding proteins. In this study, we used a newly developed proteomic approach to identify F_{420}-binding proteins by making use of their affinity towards F_{420} that was covalently tethered to column material. The free carboxylic groups of the F_{420} cofactor were chemically coupled to amine-functionalized column material. The initial experiments revealed that coupling of F_{420} to polymethacrylate beads, that contain two carbons long linkers, resulted in the best affinity chromatography material. The bound proteins from extracts of Mycobacterium smegmatis could be eluted using F_{420} and analyzed by mass spectrometry. Of the identified proteins, a large portion indeed were predicted to be F_{420}-dependent enzymes while also some aspecific binding was observed. Intriguingly, also proteins were identified for which no function is known. These proteins may well be F_{420}-dependent enzymes for the function still has to be uncovered.

2.1. Introduction

Flavins serve as cofactor for various classes of enzymes and equip them with unique functionalities (Romero et al. 2018). Most of the known flavoenzymes rely on FAD or FMN as cofactor. FAD- and FMN-dependent enzymes are the most extensively studied group among cofactor dependent enzymes. The majority of these enzymes contain the flavin cofactor as a tightly bound prosthetic group. In fact, in a significant number of flavoenzymes, the flavin cofactor is covalently tethered to the protein (Heuts et al. 2008). Except for FAD and FMN, also some other flavin cofactors are used by enzymes. Several derivatives of FAD or FMN have been discovered to act as cofactor. For example, it was found that 8-formyl FAD is the native cofactor in formate oxidase (Robbins et al. 2017). This FAD derivative seems to be formed from FAD bound in the enzyme and the oxidized FAD variant is better in supporting catalysis by the oxidase. Another recently discovered alternative FAD-based cofactor was identified in an enzyme involved in the synthesis of enterocin (Teufel et al. 2015). The respective redox enzyme was found to contain FAD in which the N5 was oxygenated. This over-oxidized FAD cofactor allows the enzyme to perform two subsequent oxidations of its substrate. An even more astonishing discovery was made in 2016 when a prenylated form of FMN was encountered in (de)carboxylases (Payne et al. 2015).

All flavoenzymes mentioned above contain a riboflavin molecule as core moiety. In fact, biosynthesis of FMN and FAD and their derivatives involve the incorporation of riboflavin. FMN is produced by phosphorylation of riboflavin and FAD can be regarded as FMN decorated with an AMP moiety. Yet, there is another natural flavin cofactor that is not built out of riboflavin. Already a few decades ago a chromophore was isolated from methanogenic bacteria which displayed a particular feature: high absorbance at 420 nm (Cheesman et al. 1972) therefore, it was called cofactor F_{420}. Elucidation of its structure revealed that it shows some resemblance with the commonly known flavin cofactors. However, a fundamental difference is the fact that the flavin N5 atom is replaced by a carbon atom (Figure 1). Hence F_{420} is also referred to as a deazaflavin cofactor. Furthermore, it does not carry the typical methyl groups in the phenyl part of the isoalloxazine ring, but only a hydroxyl group at the 8'-position. These features result in significantly different spectral properties of the F_{420} cofactor when compared with FMN and FAD. Another important difference is in the modification of the ribityl moiety. Except for a phosphate group, which is common for flavin cofactors, it has also an unusual lactyl-polyglutamyl extension in which the number of glutamyl moieties varies between different species.

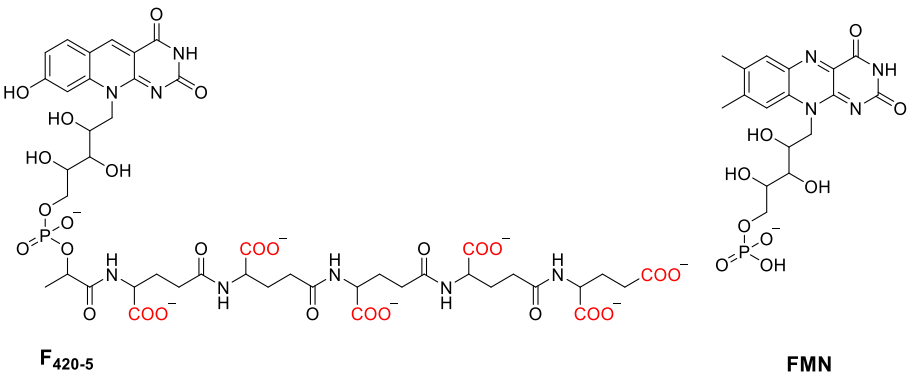


Figure 1. Structure of FMN and F_{420} with 5 glutamate residues.

While the F_{420} cofactor was first isolated almost 50 years ago, knowledge on F_{420}-dependent enzymes is still lagging behind when compared with other flavoenzymes. In the last few decades only a small number of F_{420}-dependent enzymes have been isolated and studied (Greening et al. 2016). While it was first thought that this deazaflavin was rather an aberrant cofactor only used in a restricted number of microorganisms, e.g. methanogens, recent studies have revealed that the F_{420} cofactor is much more widespread in nature. Genome analysis of F_{420} biosynthetic genes suggest that it is present in various bacterial and archaeal taxa. Biochemical studies have confirmed that F_{420}-dependent enzymes play a crucial role in methane metabolism in methanogens. It has also been demonstrated that they fulfil various roles in metabolism of actinobacteria. Comparative genomic studies on using sequences of known F_{420}-dependent enzymes, revealed that there are more than 20 probable F_{420}-dependent proteins in *Mycobacterium tuberculosis.* Yet, only of a few of them their function is known. It is even more extreme when analyzing the predicted proteome of *Rhodococcus jostii* RHA1: it is predicted to contain >100 F_{420}-dependent enzymes with unknown function. Clearly, there is a huge gap in knowledge on F_{420}-dependent enzymes.

While the above-mentioned studies predict that many microbes contain many unexplored F_{420}-dependent enzymes, these predictions are all based on analyzing genomes/proteomes for homologs of enzymes that have been isolated in the past. In this study we aimed at developing an experimental approach to identify F_{420}-binding proteins in an unbiased manner. All known F_{420}-dependent enzymes described so far utilized the deazaflavin cofactor as a coenzyme. Similar to most NAD-dependent enzymes, they only temporarily bind the oxidized or reduced deazaflavin cofactor in order to catalyze a hydride transfer. The elucidated structures of F_{420}-dependent enzymes also confirm that the polyglutamyl tail of the

cofactor is always solvent accessible. Based on these observations, we set out to develop a F_{420}-based affinity chromatography method that would allow isolating and identifying F_{420}-binding proteins by attaching the deazaflavin cofactor to column material via their polyglutamyl tail. After preparing polymethacrylate-based carrier material decorated with F_{420}, extracts of *Mycobacterium smegmatis* were used to isolate F_{420}-binding proteins (Figure 2).

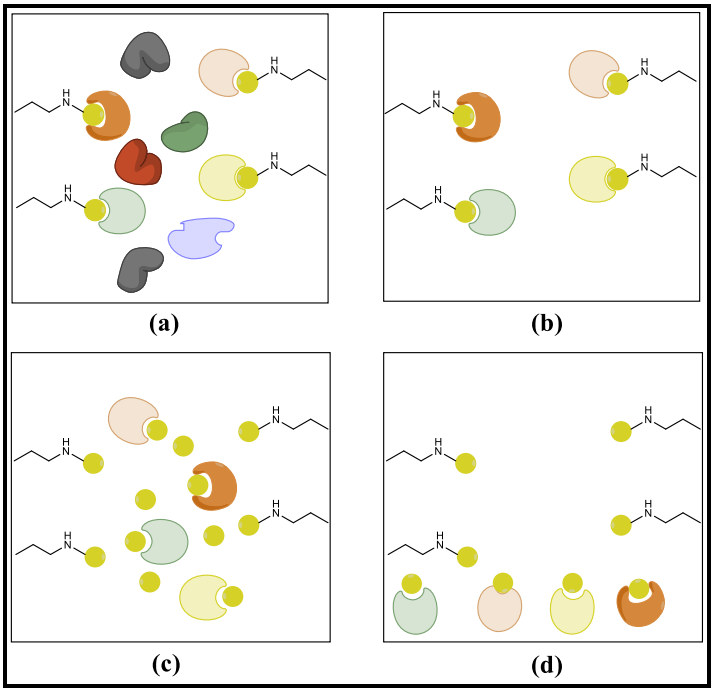


Figure 2. Schematic representation of the F_{420}-binding protein identification method using a F_{420}-immobilized column. a) deazaflavoproteins present in the cell extract will bind to the F_{420} immobilized column. FMN and other flavin binding proteins may also bind, but with lower affinity. b) unbound or loosely bound proteins will be removed during the washing step. c) bound proteins can then be eluted using F_{420} and/or high salt and identified using mass spectrometry.

2.2. Experimental section

2.2.1. Materials

Low density aminoethyl functionalized agarose beads were purchased from Agarose Bead Technologies (ABT), Madrid, Spain. Amine functionalized polyvinyl alcohol magnetic beads (M-PVA N12) were purchased from PerkinElmer, Germany. These superparamagnetic beads consist of a matrix of polyvinyl alcohol, which is subsequently aminated using an eight-atom spacer. Hexamethylenamino- and ethylenediamino-functionalized polymethacrylate beads were purchased from ReliZyme™. All other chemicals, unless mentioned, were purchased from Sigma Aldrich.

2.2.2. Purification of F_{420} and F_{420}-binding proteins

F_{420} was isolated using Mycobacterium smegmatis (kindly provided by Dr. G. Bashiri) cells. A protocol for F_{420} purification was based on a previously described method (Isabelle et al. 2002). As reference proteins, F_{420}-dependent glucose-6-phosphate dehydrogenase from Rhodoccous jostii RHA1 (Nguyen et al. 2017), F_{420}:NADPH oxidoreductase (Kumar et al. 2017) and F_{420} dependent ene-reductase from Mycobacterium hassiacum (chapter 5) were purified using the described methods.

2.2.3. Preparation of the F_{420}-immobilized column

The isolated F_{420} cofactor was crossed-linked to the functionalized beads/cross-linked polymers through a coupling reaction catalyzed by EDC (N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide). This results in an amide linkage between free carboxyl groups of F_{420} and amine groups from the beads or matrix. The immobilization protocol was based on previously described literature (Haase et al. 1992). Amine-functionalized beads (0.5 g) were first washed with 25 mL of 1.0 M NaCl followed by wash with 25 mL of 1.0 mM NaCl (pH 4.5). Then, the washed beads were mixed with 5 mL of 1 mM NaCl solution (pH 4.5) containing 70-100 µM of F_{420} and 100 mM (95 mg) EDC. The beads were then incubated at 4° C in a rocking shaker for three hours in dark. After incubation, the beads were poured into a column and the solution was drained. The next step was to block the unreacted free amino groups present in the column material. To do so, the beads were further incubated with 5 mL solution containing 25 mM sodium acetate (pH 4.8) and 100 mM EDC (3 h, 4° C). After that, the beads were washed with 25 mL of 25 mM sodium acetate solution (pH 4.0) followed by 25 mL 50 mM Tris/HCl buffer (pH 8.0) containing 0.5 M NaCl. As a control, column material was also treated without F_{420}: all free amino groups were blocked by using 25 mM sodium acetate instead of F_{420} and following a similar procedure. The different types of

functionalized beads used in this study are shown in table 1. The column names mentioned in the table will be used hereafter. The column material without the F_{420} bound will be referred to as control column while the one with bound F_{420} will be called test column.

Prepared column material	Spacer length (carbons)	Original column material	Coupled F_{420}
C_{EA2}	2	Agarose	no
F_{EA2}	2	Agarose	yes
C_{PM2}	2	Poly methacrylate	no
F_{PM2}	2	Poly methacrylate	yes
C_{PM6}	6	Poly methacrylate	no
F_{PM6}	6	Poly methacrylate	yes
C_{PV8}	8	Poly vinyl alcohol (magnetic)	no
F_{PV8}	8	Poly vinyl alcohol (magnetic)	yes

Table 1. Column materials used in this study.

2.2.4. Affinity chromatography using F_{420}-decorated column

M. smegmatis $mc^2$4517 cells were grown in 250 mL baffled flasks containing 50 mL medium. Medium contained (in grams per liter) soluble starch (25), glucose (5), yeast extract (5), soy peptone (10), ammonium sulfate (2), and KH_2PO_4 (0.3), as reported before (Isabelle et al. 2002). Cells were grown at 30 °C for 72 hours under shaking condition (200 rpm). Cells were harvested by centrifugation (6000 rpm) and resuspended in 10 mL 50 mM Tris-HCl (pH 8.0) containing 20 % glycerol, 1.0 mM DTT, 0.01% Triton X-100, and 0.1 mM PMSF (polymethyl sulfonyl fluoride). Cells were disrupted at 4 °C using a VCX130 Vibra-Cell sonicator (Sonics&Materials, Inc., Newtown, USA) for 10 mins (10 sec on, 15 sec off cycle). Cell debris was removed by centrifuging at 40,000 × g for 45 mins, 4 °C and discarding the pellet. The supernatant was filtered using 0.45 µm syringe filters to obtain a cleared cell extract (CCE). 5 ml of CCE was incubated for 3 h with 2 ml of test column (F_{420}-coupled column) and control column (column without coupled F_{420}) which were pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 20 % glycerol, 1.0 mM DTT and 0.01% Triton X-100. The unbound proteins were removed by washing with buffer using gravity flow. Proteins were eluted using either 50 µM F_{420}, or 50 mM Tris-HCl buffer (pH 8.0) containing different concentrations of the NaCl (50, 100, 500 and 1000 mM). Fractions from each elution were concentrated using 10 kDa cutoff filters and used for SDS-PAGE analysis and subsequent LC-MS/MS analysis.

2.2.5. In-solution and in-gel trypsin digestion

Protein concentrations of the samples were determined using the Bradford assay. For in solution trypsin digestion, the protein samples were denatured, followed by alkylation. Protein denaturation was started by mixing protein samples with urea to make a total volume of 40 µL (1.6 M urea and 10-100 µg protein). Concentrated samples were diluted using 100 mM ammonium bicarbonate. 1.0 µL of 0.5 M TCEP (TRIS(2-carboxyethyl)phosphine) was added to the mixture and vortexed, and incubated at 37 °C for 1 h. Samples were alkylated in the dark upon addition of 1.0 µL iodoacetamide (0.4 M) at 25 °C for 30 minutes at 500 rpm. 1.0 µL of trypsin (1.0 µg/µL) was added to the solution after checking the pH of the sample which should be around pH 8-9. Ammonium bicarbonate (1 M) was used to adjust the pH if needed. The mixture was incubated at 37 °C overnight. Trypsin was inactivated by adding 8.0 µL of 5 % TFA (1% final concentration) followed by centrifugation (13,000 × g) at 4 °C. The supernatant was transferred to fresh tubes and used for solid phase extraction. In this step, the peptide samples were reconstituted with 1% TFA and cleaned with Pierce® C18 tips (87784; Thermo) according to the instruction manual. The eluted fractions were dried under vacuum and reconstituted with 20 µL 2% ACN, 0.1% formic acid (FA).

2.2.6. Liquid chromatography coupled to tandem mass spectrometry

Peptide separation was performed with 2 µL peptide samples using a nano-flow chromatography system (EASY nLC II; Thermo) equipped with a reversed phase HPLC column (75 µm, 15 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 3 µm resin; Dr. Maisch) using a linear gradient from 95% solvent A (0.1% FA, 2% acetonitrile) and 5% solvent B (99.9% acetonitrile, 0.1% FA) to 28% solvent B over 45 min at a flow rate of 200 nL/min. The peptide and peptide fragment masses were determined by an electrospray ionization mass spectrometer (LTQ-Orbi-trap XL; Thermo)

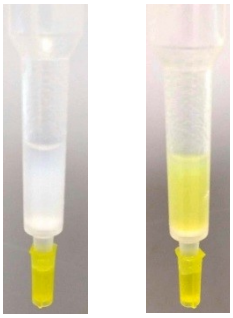
2.2.7. Data analyses

Raw files were imported into the Peaks Studio software (Bioinformatics Solutions) analyzed against forward and reverse peptide sequences of the predicted *M. smegmatis* proteome. The search criteria were set as follows: one end tryptic specificity was required (cleavage after lysine or arginine residues but not when followed by a proline); three missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) and deamination (NQ) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.5 Da for fragment ions.

2.3. Results and discussion

2.3.1. F_{420} binds to the amino-functionalized column

Cofactor F_{420} isolated from *M. smegmatis* MC^24715 was successfully immobilized on amino-functionalized beads composed of agarose (F_{EA2} column), polyvinyl alcohol (F_{PV8} column), polymethacrylate (F_{PM2} & F_{PM6} column) and different linker lengths. After the immobilization procedure, the modified column material retained the characteristic yellow color of F_{420} in all cases, except for column F_{PV8}. Due to its intense brown color, the decoration with F_{420} could not be verified by eye. A similar treatment of resin materials with FMN did not show any significant immobilization of the flavin cofactor as evidenced by visual inspection. This was supported by the observation that the amount of eluted FMN after all washing steps was equal to the applied amount. However, in case of F_{420} treatment, only 15-20% of the initial amount was recovered after washing in all cases, meaning that most of the F_{420} was utilized for immobilization. The covalent attachment was dependent on the free carboxyl groups of cofactor F_{420}. Once F_{420} was covalently coupled, the remaining free amino groups were blocked using 25 mM sodium acetate. Based on eluent absorption at 400 nm, we were able to estimate the amount of coupled F_{420}: 4.5 µmoles/g of the column material. To check the functionality of the column, known F_{420}-binding proteins were used to test their binding to the column. The following purified F_{420}-dependent enzymes were tested: F_{420}-dependent glucose-6-phosphate dehydrogenase from *Rhodococcus jostii* RHA1 (Nguyen et al. 2017), F_{420}:NADPH oxidoreductase from *Thermobifida fusca* (Kumar et al. 2017) and F_{420}-dependent reductases (chapter 5). In case of F_{420}-bound agarose column material, the control column, C_{EA2}, showed non-specific binding of the F_{420}-dependent proteins at low salt concentration. This was probably due to the column material polymer because we did not observe this with the polymethacrylate control column, C_{PMA2}. Purified F_{420}-dependent proteins did bind to the polymethacrylate F_{420} columns (F_{PM2} & F_{PM6}) and could be eluted using F_{420} or high salt concentration (o1 M NaCl). In case of column F_{PV8}, we observed a very low binding efficiency which might be due to the longer spacer arm. The use of the C_{PV8} & F_{PV8} columns was abandoned thereafter. Among all the columns tested, polymethacrylate column F_{PM2} showed the best binding to the proteins while C_{PM2} bound to the least number of proteins. This column material could be used multiple times without any significant loss in efficiency.



(A) **(B)**

Figure 3. Ethylamine-functionalized agarose beads without (A) and with F_{420} bound (B). The F_{420}-immobilized column retains a yellowish color indicative of covalently attached F_{420}.

2.3.2. SDS-PAGE gel analysis of proteins with affinity towards the F_{420}-decorated column material using M. smegmatis cell free extract

Cell free extract of *M. smegmatis* $mc^2$4517 was used for exploring the use of the generated F_{420}-modified column material to isolate F_{420}-binding proteins. SDS-PAGE analysis of proteins eluted from both the F_{420}-decorated column material as well as proteins eluted from a similarly treated column material, but without F_{420} exposure (in essence, material with only blocked amino groups), was done to confirm selective binding of proteins. SDS-PAGE analysis of samples obtained using agarose as carrier material clearly shows that the control column also binds to a significant number of proteins (Figure 4). Yet, clearly there are quite a number of proteins specifically enriched by using the F_{420}-bound column material (Figure 4, lane 5). Upon MS analysis of some gel spots from lane 2, 5 and 6 (Figure 4), we found that most of the proteins that bound to the column are ribosomal binding proteins. Although ribosomal binding proteins were frequently identified, also some F_{420}-binding protein homologues were found. This means that the size of ribosomal binding proteins and F_{420}-binding proteins was similar and hence they both appeared in the results. Due to their intracellular abundance and their affinity towards RNA, the binding of these proteins appears to be caused by aspecific binding. Nonetheless, two out of 11 proteins analyzed were clearly putative luciferase-like monooxygenases (MSMEG_5715 and MSMEG_3380) which are in fact predicted to be F_{420}-binding proteins. This shows that the method works to some extent but suffers from aspecific binding of proteins using this specific activated agarose as carrier. To investigate the obtained protein samples in more detail, we also performed MS analysis of whole elution fractions and compared the results of the columns F_{EA2} and C_{EA2}. Using whole fraction comparative analysis, we were able to pinpoint those

proteins which were only bound by the F_{420}-decorated column (F_{EA2}). To rule out the hypothesis that F_{420} actually binds to ribosomal binding proteins, we switched to another column material (polymethacrylate), and repeated similar experiments. SDS-PAGE gels from Figure 5A (lane 5) and 5B (lane 3 & 5) clearly show that the control column displays minimal non-specific binding and MS analysis revealed that the background noise was significantly lower. We observed that elution with 50 µM F_{420} resulted into specific elution of a number of proteins as shown in Figure 5A (lane 6). Similarly, elution with buffer containing 500 mM NaCl and 1 M NaCl also resulted into elution of specific proteins (Figure 5B, lane 4 & 6). It is worth noticing that according to gel pictures, the proteins eluted using F_{420} and NaCl are not similar.

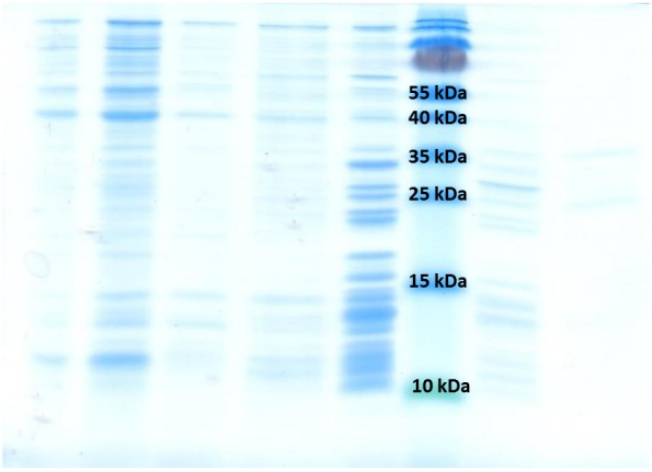
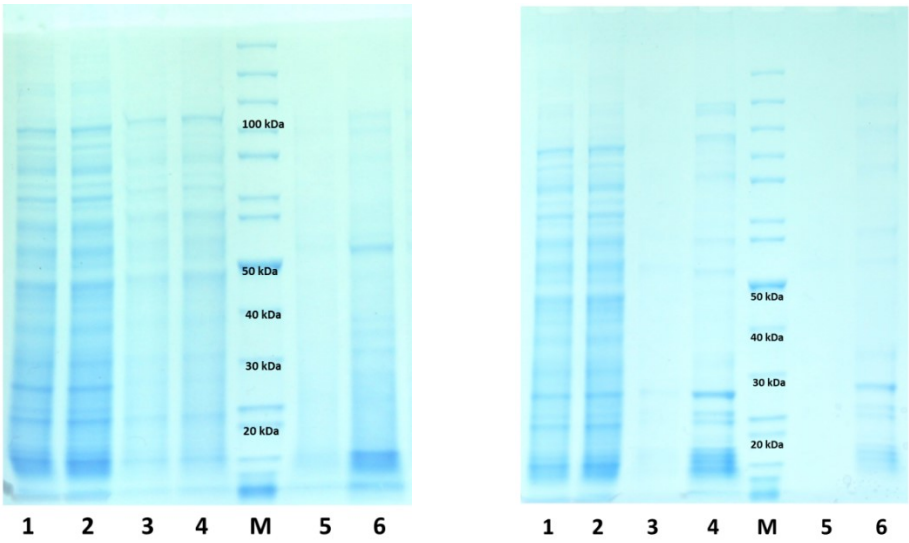


Figure 4. A SDS nobilized agarose column (F_{EA2}) al ne 1 corresponds to the buffer wash fraction of control column. Lane 2, 3 and 4 are fractions from control column eluted using 300 mM, 600 mM and 1 M of NaCl in the buffer respectively. Lane 5, 6 and 7 correspond to fractions eluted from F_{420}-coupled column material.



(A) **(B)**

Figure 5. SDS-PAGE gel (12%) pictures of proteins eluted using control and F_{420}-bound polymethacrylate columns (C_{PM2} & F_{PM2}). Bound proteins were eluted using 50 µM F_{420} (A) and different concentrations of NaCl (B). In gel A, lane 1, 3 and 5 are flow through, wash fraction and elution fraction using control column. Lane 2,4 and 6 are flow through, wash fraction and elution fraction using F_{420}-bound column. In gel B, lane 1 and 2, represent flow through fractions. Lane 3 and 4 represent elution fractions using 500 mM of NaCl in the buffer. Lane 5 and 6 shows proteins eluted using 1 M of NaCl in buffer. Lane 1, 3 and 5 are from control column while lane 2, 4 and 6 are from F_{420}-immobilized column.

Sr. No.	Name	Uniprot ID	Predicted family	Elution using F_{420}	Elutes with NaCl
1	Putative oxidoreductase MSMEG_2516	A0QVB6_MYCS2	Luciferase like domain	yes	yes
2	Cold shock protein A MSMEG_0559	A0QPY2_MYCS2		yes	yes
3	Uncharacterized protein MSMEG_5592	A0R3T9_MYCS2	Luciferase like domain	yes	yes
4	Pyridoxamine 5'-phosphate oxidase family protein MSMEG_0048	A0QNH8_MYCS2	Pyridoxamine 5'-phosphate oxidase family	yes	yes
5	Uncharacterized protein MSMEG_3977	A0QZC7_MYCS2	Luciferase like domain	yes	yes
6	Uncharacterized protein MSMEG_4321	A0R0A8_MYCS2	DUF3052 superfamily	yes	yes
7	FeS assembly protein SufD MSMEG_3123	A0QX00_MYCS2	FHA domain, Fe-S cluster assembly	yes	yes
8	Ribosome-binding factor A MSMEG_2629	RBFA_MYCS2	Ribosome-binding factor family	yes	no

Table 2. List of the proteins bound to F_{420}-affinity column material, F_{PM2}, and that were specifically eluted with the 50 µM of F_{420} or NaCl.

2.3.3. Identification of the proteins bound to F_{420} column

Proteins bound to F_{420}-bound ethylene polymethacrylate column material (F_{PM2}) and eluted using 50 µM of F_{420} were analyzed in more detail. There were 100-110 proteins which selectively bound to the F_{420}-bound column and could be identified by MS analysis. Based on the known and predicted F_{420}-dependent proteins, we confirmed the selective binding of F_{420}-dependent proteins to the column as shown in table 2. Among the top 5 most abundant proteins (based on spectrum count), four of them are known or predicted to be F_{420}-binding proteins in the literature (Selengut and Haft 2010). This clearly shows that the F_{PM2} column works very well. Besides these proteins, we also observed a number of proteins which have never been reported or predicted to be F_{420}-dependent. For example, MSMEG_0559 and MSMEG_4321 show selective binding to the column $F_{PM2.}$ MSMEG_0559 is a very small protein (monomer size - 6 kDa) containing a lot of acidic residues (pI = 4.1). MSMEG_4321 has a domain of unknown function (DUF3053) and is highly conserved among actinobacteria which possess F_{420}-synthesizing machinery. We found that MSMEG_4321 contains sequence features very similar to the fingerprint sequence for F_{420}-dependent proteins (unpublished data). Future research on heterologously expressed protein will tell more about the function of such proteins. Other proteins (MSMEG_3123 and MSMEG_2629) also have lower pI values due to abundant acidic residues, so the binding based on charge cannot be excluded. The spectrum count of almost most of the proteins not mentioned in the table was very low (1-3) indicating they were present in relatively low amounts. Among these low abundant proteins were several known or predicted F_{420}-dependent enzymes (MSMEG_2027, MSMEG_3609, MSMEG_5170, MSMEG_1027, MSMEG_1566, MSMEG_3863). Among the proteins that were eluted in both the test (F_{PM2}) and control (C_{PM2}) column, we noticed significant enrichment of some proteins on column F_{PM2}. We observed that F_{420}-dependent glucose-6-phosphate dehydrogenase in F_{PM2} was enriched more than three times (based on the spectrum count) as compared to C_{PM2}. This can also be due to the higher intracellular levels of such enzymes and the washing may not be enough for removing them fully from the column C_{PM2}. When we compared the F_{420}-eluted proteins to that of the NaCl-eluted proteins, we observed similar results when considering the known F_{420}-dependent proteins

2.4. Conclusion

F_{420} is a comparatively little studied but interesting deazaflavin cofactor and has a unique distribution among organisms. It shares significant structural similarity with the riboflavin based cofactors but it is different in chemical properties. Unlike flavins, it serves as dissociable coenzyme for F_{420}-dependent enzymes and catalyzes hydride transfer reactions. However, riboflavin-based cofactors typically are tightly bound to enzyme in order to function properly. In some flavoenzymes, the flavin cofactor is even mono- or bicovalently bound. We exploited the dissociable binding of F_{420} as coenzyme in a newly developed and generic affinity chromatography method to identify novel deazaflavoenzymes. We show that a F_{420}-decorated affinity column can be used to selectively fish out F_{420}-dependent proteins from the cell free extract of *M. smegmatis*. The carboxylic groups of the F_{420} polyglutamyl tail can be used to covalently immobilize F_{420} on column material. Dependent on the type of column material, there is a risk for aspecific protein binding. Hydroxyl groups of agarose tend to bind proteins non-specifically. This non-specific binding can be significantly lowered by using polymethacrylate as column material, as observed in this study. Another variable in coupling a ligand to column material is the linker length between the carrier and the activated group. Based on the binding efficiency, a two carbons long spacer was found to result in better binding efficiency. Upon incubating the cell free extract of *M. smegmatis* with the best performing F_{420}-decorated column material and eluting with 50 µM F_{420}, mass spectrometric analysis of whole elution fractions were performed. It was found that the column does have specificity towards F_{420}-dependent proteins as known proteins were found to bind selectively. As hypothesized, F_{420}-bound column also bound selectively to certain proteins which were not known to be F_{420}-dependent. Among many others, one of the interesting candidates to look into is MSMEG_4321. Interestingly, there has not been any biochemical studies performed on this protein. Future binding and structural studies on heterologously expressed candidate deazaflavin-binding proteins will reveal their role in *M. smegmatis*.

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