New approaches for flavoenzyme applications

Krzek, Marzena

CHAPTER 2

COVALENT IMMOBILIZATION OF A FLAVOPROTEIN MONOOXYGENASE VIA ITS FLAVIN COFACTOR

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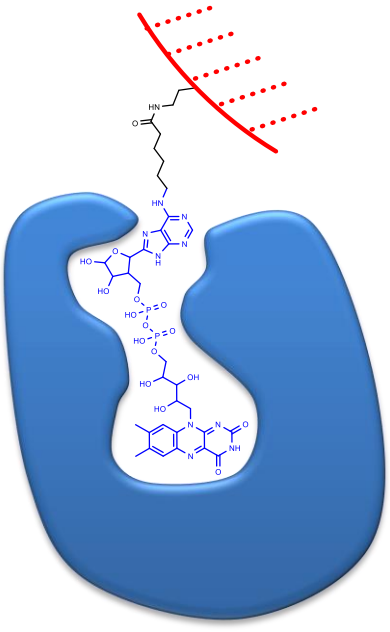
ABSTRACT

A generic approach for flavoenzyme immobilization was developed in which the flavin cofactor is used for anchoring enzymes onto the carrier. It exploits the tight binding of flavin cofactors to their target apo proteins. The method was tested for phenylacetone monooxygenase (PAMO) which is a well-studied and industrially interesting biocatalyst. Also a fusion protein was tested: PAMO fused to phosphite dehydrogenase (PTDH-PAMO). The employed flavin cofactor derivative, N^6-(6-carboxyhexyl)-FAD succinimidylester (FAD*), was covalently anchored to agarose beads and served for apo enzyme immobilization by their reconstitution into holo enzymes. The thus immobilized enzymes retained their activity and remained active after several rounds of catalysis. For both tested enzymes, the generated agarose beads contained 3 U per g of dry resin. Notably, FAD-immobilized PAMO was found to be more thermostable (40% activity after 1 h at 60 °C) when compared to PAMO in solution (no activity detected after 1 h at 60 °C). The FAD-decorated agarose material could be easily recycled allowing multiple rounds of immobilization. This method allows an efficient and selective immobilization of flavoproteins via the FAD flavin cofactor onto a recyclable carrier.

Keywords: Flavoenzyme, FAD, immobilization, apo enzyme, monooxygenase



cofactor-mediated flavoenzyme immobiization

1. INTRODUCTION

Enzyme immobilization methods aim at generating a stable and reusable biocatalyst and is driven by diverse enzyme-based applications in industry, analytics and medicine. For detailed information on the various known method of enzyme immobilization, we refer to some recent reviews (Rodríguez *et al.*, 2009; Franssen *et al.*, 2013; Guzik, Hupert-Kocurek and Wojcieszynska, 2014; Barbosa *et al.*, 2015). Typical immobilization methods result in uncontrolled enzyme-carrier orientations which may affect the enzyme performance due to mass transfer obstruction. Apart from that, covalent coupling to the carrier often involves chemicals and conditions that are harmful for the target enzyme, resulting in a decreased activity and/or stability of the biocatalyst (Sheldon, 2007). On the other hand, non-covalent adsorption of enzymes usually involves mild conditions but often results in labile systems (Sheldon, 2007). Moreover, most available methods tend to require either an excess or high purity of the enzyme to be immobilized, which is not favorable for economic purposes. Therefore, it is desirable to develop efficient immobilization methods for creating robust immobilized enzymes with high catalytic performance.

In the field of redox biocatalysis, flavin-containing enzymes are regarded as particularly useful. Flavoprotein reductases, oxidases and monooxygenases convert substrates with high stereo-, enantio- and regioselectivity into valuable products such as functionalized building blocks for further use in polymer, pharmacological, food and fine-chemical industry (van Berkel, Kamerbeek and Fraaije, 2006; Torres Pazmiño, Dudek and Fraaije, 2010; Winter and Fraaije, 2012). In the last decade, the toolbox of flavoenzymes has significantly expanded thanks to enzyme engineering and discovery efforts (Fraaije *et al.*, 2005; Torres Pazmiño, Dudek and Fraaije, 2010; van Beek, Gonzalo and Fraaije, 2012). The majority of flavoenzymes contain a tightly and non-covalently bound FAD cofactor. Various methods have been developed to prepare flavoproteins devoid of their native flavin cofactor (Hefti, Vervoort and Van Berkel, 2003). Once prepared, such apo proteins are typically readily reconstituted with FAD or FAD analogues (Fruk *et al.*, 2009). A large number of synthetic FAD derivatives have been explored before, mostly with the aim to elucidate mechanistic features of FAD-containing enzymes (Massey, 2000). For these studies, the isoalloxazine is typically modified to probe the effects on catalysis. For changing the binding properties of a FAD cofactor without affecting the redox properties, derivatization of the adenine moiety is a better candidate as it is distant from the redox active moiety, the isoalloxazine ring (Figure 2, Chapter 1). In fact, in many flavoproteins the adenine part of FAD is close to the protein surface or even partially exposed to the solvent. Adenine N^6-FAD-derivatives have been shown to be more efficient over adenine N^1-FAD in reconstituting apo forms of flavoprotein oxidases (Zappelli *et al.*, 1978). Using N^6-(2-aminoethyl)-FAD, Willner et al. have shown that it is feasible to anchor glucose oxidase on electrodes (Willner *et al.*, 1996). With this system, a glucose-dependent electric current could be monitored. Moreover, D-amino acid oxidase and

L-aspartate oxidase were shown to bind N^6-adenine modified FAD yielding artificial covalent flavoprotein oxidases (Stocker, Hecht and Bückmann, 1996; Willner *et al.*, 1996). Nevertheless, it resulted in a significant decrease in oxidase activity. In this paper we demonstrate that N^6-hexyl-FAD-decorated agarose beads could be used for efficient immobilization of a flavoprotein monooxygenase.

For this study we have chosen phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* as a model flavoenzyme. It has been demonstrated by several groups that this bacterial monomeric FAD-containing monooxygenase can be used for a number of industrially interesting oxygenation reactions, e.g., enantioselective sulfoxidations and Baeyer–Villiger oxidation (van Berkel, Kamerbeek and Fraaije, 2006; de Gonzalo, Mihovilovic and Fraaije, 2010). In addition, its crystal structure has been solved (Malito *et al.*, 2004), the catalytic performance and substrate scope has been well described, the expression in Escherichia coli is efficient (van Bloois *et al.*, 2012), and the enzyme displays remarkable stability against elevated temperatures and a wide range of organic solvents (Rodríguez *et al.*, 2009; Secundo *et al.*, 2011). This renders it as a convenient model flavoenzyme. PAMO belongs to the class of Baeyer–Villiger monooxygenases (BVMOs) which require the coenzyme NADPH for catalysis (Figure 1). A number of research efforts have been directed toward efficient regeneration of the nicotinamide coenzyme (Wichmann and Vasic-Racki, 2005). One approach for efficient NADPH recycling is represented by the production of Baeyer–Villiger monooxygenases fused to a thermostable phosphite dehydrogenase (PTDH) rendering the monooxygenase a self-sufficient biocatalyst. PTDH is able to regenerate NADPH at the expense of relatively cheap phosphite. The fused PTDH-BVMO biocatalysts were shown to display a high catalytic performance, moreover the expression of BVMOs fused to PTDH was found to boost protein expression (Torres Pazmiño *et al.*, 2009).

In this contribution, we present an approach for immobilizing flavoenzymes via their FAD cofactor which offers a mild and controllable enzyme loading on the target carrier material.

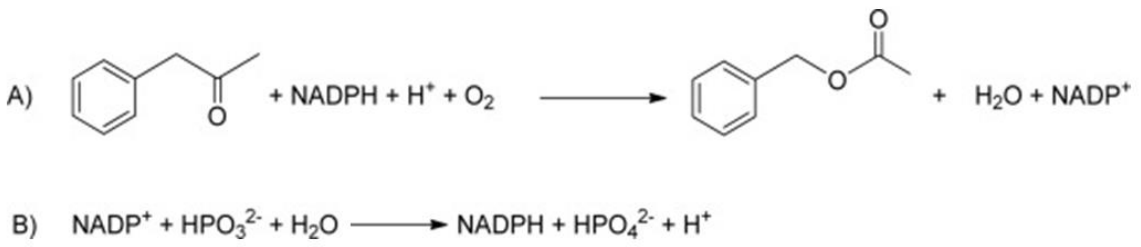


Figure 1. *(A) The Baeyer–Villiger oxidation of phenylacetone into benzyl acetate catalyzed by PAMO. The enzyme uses NADPH as electron donor and molecular oxygen asoxygen donor. (B) Phosphite oxidation catalyzed by PTDH which results in formation of NADPH.*

2. MATERIALS AND METHODS

2.1. MATERIALS

Low density aminoethyl 6 Rapid Run™ agarose beads (spherical beads of 50–150 μm, with 15–25 μmol/mL of gel) were obtained from Agarose Beads Technology. The amino groups have been introduced as described in literature (Fernandez-Lafuente *et al.*, 1993). N^6-(6-carboxyhexyl)-FAD succinimidylester (FAD*) (Figure 6, chapter 1) was synthesized by BioLog. Nickel-Sepharose HP (GE Healthcare) and DG-10 EconoPac desalting columns (BioRad) were used for protein purification and preparation of the apo forms of enzymes. All other chemicals were purchased from Sigma–Aldrich, Merck or ACROS Organics.

2.2. ENZYME EXPRESSION, PURIFICATION, AND ASSAY

His-tagged PAMO (referred to as PAMO) and His-tagged PTDH-PAMO were overexpressed in E. coli TOP10. The enzymes were purified as previously described (Torres Pazmiño *et al.*, 2009; Dudek *et al.*, 2010). The activity was verified by monitoring NADPH absorbance depletion at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1} in 50 mM Tris/HCl pH 7.5) with 0.10 mM NADPH and 0.80 mM of phenylacetone as substrate (Torres Pazmiño *et al.*, 2008) while using an atmospheric dioxygen concentration (0.24 mM).

2.3. FAD-CARRIER PREPARATION

FAD-decorated agarose was prepared by incubating FAD* with aminoethyl agarose beads (0.31 μmol FAD* per 0.25 mg of dry beads, which equals 0.09 μmol amine groups, in a total volume of 2.25 mL) in 50 mM phosphate pH 8.0 at room temperature for 3 h (Fig. 1). Subsequently, the resin was transferred to an empty column and washed with 0.5 M NaCl, 50 mM Tris/HCl (pH 7.5) in order to remove non-covalently bound FAD. After this procedure the agarose beads displayed an intense yellow color due to the coupled FAD.

2.4. APO ENZYMES PREPARATION AND RECONSTITUTION

Different combinations of urea and KBr were tested for release of the FAD cofactor from PAMO when bound to the nickel-loaded Sepharose column (10 mg protein per 1 mL of column material). The non-covalently bound FAD was washed from the protein immobilized on the column until the yellow color had disappeared. The retained apo enzyme was subsequently washed with 50 mM Tris/HCl (pH

7.5) and finally released from the column with 200 mM imidazole with 20% glycerol in 50 mM Tris/HCl (pH 7.5). The eluted apo protein solution was immediately applied on the desalting column and desalted using 50 mM Tris/HCl (pH 7.5). The amount of obtained apo enzyme was calculated from the absorbance at 280 nm using the following absorption coefficients: 157 mM^{-1} cm^{-1} and 210 mM^{-1} cm^{-1} for PAMO and PTDH-PAMO, respectively.

To test the reconstitutability of the obtained apo enzymes, 5.0 μM apo protein in 50 mM Tris/HCl (pH 7.5) was incubated with 100 μM FAD at room temperature for 2 h. Activity of the obtained holo enzymes was measured as described in Section 2.2. The apo enzymes were also tested for storage stability by freezing samples at −20 °C. For that, their reconstitutability with FAD has been measured before and after freezing. Additionally, UV–vis spectra of reconstituted protein, after using a desalting column for removing free FAD, were collected. The reconstitution of apo PAMO with FAD* was performed and evaluated as done for FAD. Additionally, ESI–MS and SDS-PAGE analysis for detecting covalent FAD* was performed as described previously (Jin *et al.*, 2007). PyMOL was used for manual docking of FAD* and visualization of the PAMO structure (PDB ID: 1W4X).

2.5. IMMOBILIZATION OF APO ENZYMES ON FAD*-AGAROSE

Typically, FAD-decorated agarose beads (prepared from 8 mg of dry aminoethyl agarose resin) were incubated with 2.5 mg apo enzyme in 8.0 mL 50 mM Tris/HCl (pH 7.5) on a rotating platform at room temperature for 3 h. Subsequently, the resin was washed with 10 times agarose resin volume using the same buffer. As a control experiment, the agarose beads without covalently attached FAD were incubated with apo enzyme using the same conditions. Some apo PTDH-PAMO tended to form protein aggregates upon mixing with agarose beads. Protein aggregates were removed by centrifugation before testing the material.

2.5.1. ENZYME LOADING DETERMINATION

To determine the amount of enzyme immobilized on the agarose, the bound protein was released from the agarose in gravity flow columns using the deflavinylation solution until complete removal of protein (monitored by 280 nm absorbance detection). The amount of retrieved protein was calculated from its absorbance at 280 nm. Enzyme loading was also determined for beads after repetitive use and for beads after high-temperature incubation.

2.5.2. CONVERSIONS USING IMMOBILIZED ENZYME

For conversions, agarose with immobilized enzyme (151 mg dry carrier in 7 mL) was incubated in a reaction mixture containing 8.0 mM phenylacetone, 100 μM NADPH, 16 mM phosphite, 50 mM Tris/Cl (pH 7.5). In case of immobilized PAMO, 1.0 μM PTDH was added to the reaction mixture to

assure regeneration of NADPH. The reaction mixture in a glass vial was placed on a rotating platform. Samples were withdrawn at various time points and extracted with ethyl acetate containing 0.1% mesitylene. The samples were dried over $MgCl_2$ and subsequently analyzed by GC (AT5 column, 30 m × 0.25 mm × 0.25 μm, Grace) using the following temperature program: 2 min at 100 °C, 5 °C/min up to 120 °C, 20 °C/min up to 160 °C. Retention times of phenylacetone and benzyl acetate were 6.96 min and 7.45 min, respectively. Two negative controls were performed: (1) FAD-decorated beads that had not been incubated with the apo enzyme, and (2) aminoethyl agarose beads (without FAD) incubated with the apo enzyme.

2.5.3. Immobilized enzymes reusability

The reusability of the enzymes immobilized on FAD-decorated agarose beads was tested by determining the activity after repeated use for biocatalytic conversions. After each conversion test, the beads were washed three times with 50 mM Tris/HCl (pH 7.5) by subsequent centrifugation and resuspension, and stored at 4 °C before the next conversion.

2.5.4. Thermostability and reusability of immobilized PAMO

To determine thermostability, PAMO immobilized on the FAD-functionalized agarose and PAMO free in solution (both in 50 mM Tris/HCl, pH 7.5) were incubated at room temperature, 50 °C, and 60 °C. Samples were taken at the indicated time intervals, placed on ice, and subsequently tested for monooxygenase activity by measuring time-dependent oxygen consumption using a Firesting O2 Fiber-optic Oxygen Meter (PyroScience). The oxygen sensor was calibrated for 100% oxygen content with room-temperature air-equilibrated Milli-Q. Activity was measured using 50 mM Tris/HCl pH 7.5) with 0.10 mM NADPH and 0.80 mM of phenylacetone as substrates. The linear decrease in oxygen concentration versus time during the first two minutes after substrate addition was used to determine enzymatic activity.

The FAD-agarose with immobilized enzymes was recycled by protein removal using the deflavinylation solution (4.0 M urea and 2.0 M KBr in 50 mM Tris, pH 7.5). The obtained material could be reused for another round of enzyme immobilization.

3. RESULTS AND DISCUSSION

3.1. PREPARATION AND RECONSTITUTION OF APO PAMO AND APO PTDH-PAMO

To date, a variety of methods to prepare apo forms of flavoproteins have been developed (Hefti, Vervoort and Van Berkel, 2003; Fruk *et al.*, 2009). A relatively recent development is the deflavinylation of His-tagged flavoproteins while bound on nickel-charged resin (Hefti, Vervoort and Van Berkel, 2003). Using this approach, we could successfully prepare the apo forms of PAMO and PTDH-PAMO. It was found that a solution of 4.0 M urea and 2.0 M KBr in 50 mM Tris, pH 7.5 (deflavinylation solution) was needed to induce (partial) unfolding which results in release of the FAD from PAMO and PTDH-PAMO when bound to nickel-Sepharose. After eluting the FAD-free proteins from the nickel-Sepharose with imidazole, the colorless protein solution was applied on a desalting column to exchange the buffer into 50 mM Tris/HCl, pH 7.5. The typical yield of apo protein obtained by this procedure was 70–80%. The resulting apo enzyme did not show any activity and did not show absorbance at 450 nm which confirms that the protein is devoid of the flavin cofactor. Moreover, it was found that apo PAMO (50 mM Tris, pH 7.5) could be stored for three weeks at −20 °C without affecting its reconstitution properties. This shows that apo PAMO, even without its prosthetic group, is a rather robust protein. The apo form of PTDH-PAMO was found to be less stable as extensive shaking or centrifugation caused partial enzyme precipitation.

3.2. RECONSTITUTION OF APO PAMO AND APO PAMO-PTDH

Addition of FAD to apo PAMO led to complete incorporation of the flavin cofactor as evidenced by the complete recovery of activity. The same was found for apo PAMO-PTDH. For another verification of formation of holo PAMO, UV/vis absorbance spectra confirmed its binding to the native protein. The spectral features of the flavin absorbance spectrum and the A280/A440 ratio (indicative for the protein/cofactor content) after FAD reconstitution of apo PAMO were the same when compared with the native holo enzyme (Fraaije *et al.*, 2005; Dudek *et al.*, 2011). This confirms that the microenvironment around the flavin in the reconstituted protein is identical to the native holo protein.
Due to the presence of the succinimidyl-ester moiety, binding of FAD* to apo PAMO can potentially lead to covalent coupling of the flavin cofactor to lysine residues. To test whether FAD* is able to bind and perhaps form a covalent protein-bound FAD, the FAD*-reconstituted enzyme was prepared and analyzed by SDS-PAGE and subsequent in-gel flavin fluorescence detection. This revealed that the FAD*-reconstituted protein indeed contained covalently bound FAD as evidenced by flavin fluorescence of the protein in the gel (Fig. S1). Moreover, it was found that the FAD*-reconstituted

enzyme displayed a similar K_M towards phenylacetone ($K_{M,phenylacetone}$ = 69 μM for FAD*-bound PAMO vs. 66 μM for native holo PAMO), while the apparent kcat had been decreased up to 50% of the value of native PAMO. The FAD*-reconstituted enzyme also did not show an increased uncoupling rate when compared with the native enzyme (consumption of NADPH in the absence of NADPH). The minor effects on the kinetic parameters is in line with the fact that the modification on the flavin (located on the adenine part) is far from the redox-active isoalloxazine moiety. The activity decreased when the incubation with FAD* was prolonged. While many covalent flavoprotein oxidases have been reported in literature, this is the first example of a flavoprotein monooxygenase containing a covalently anchored flavin cofactor. Inspection of the crystal structure of PAMO disclosed that there is one plausible candidate for such covalent FAD* tethering: the NH_2 group of Lys398 is only 7.6 Å away from the N^6-adenine of the FAD while all other lysines are at a distance of >10 Å. MS analysis of a tryptic digest of FAD*-bound PAMO did not reveal any FAD-containing peptides, possibly due to FAD-peptides being too large or displaying poor ionization behavior. Yet, comparison of the tryptic digest MS peptide patterns of PAMO reconstituted with FAD or FAD* showed that the peptide containing Lys398 decreased significantly when analyzing FAD*-reconstituted PAMO. ESI–MS analysis of unfolded FAD*-bound PAMO revealed several protein species: a species that corresponds to merely the protein (25% of the total intensity of PAMO peaks), a species with the mass of the protein and an additional mass of an FAD* molecule (40%), a species with the mass of the protein and two attached FAD* molecules (20%) and some other higher mass protein species with multiple FAD* molecules (Fig. S2).

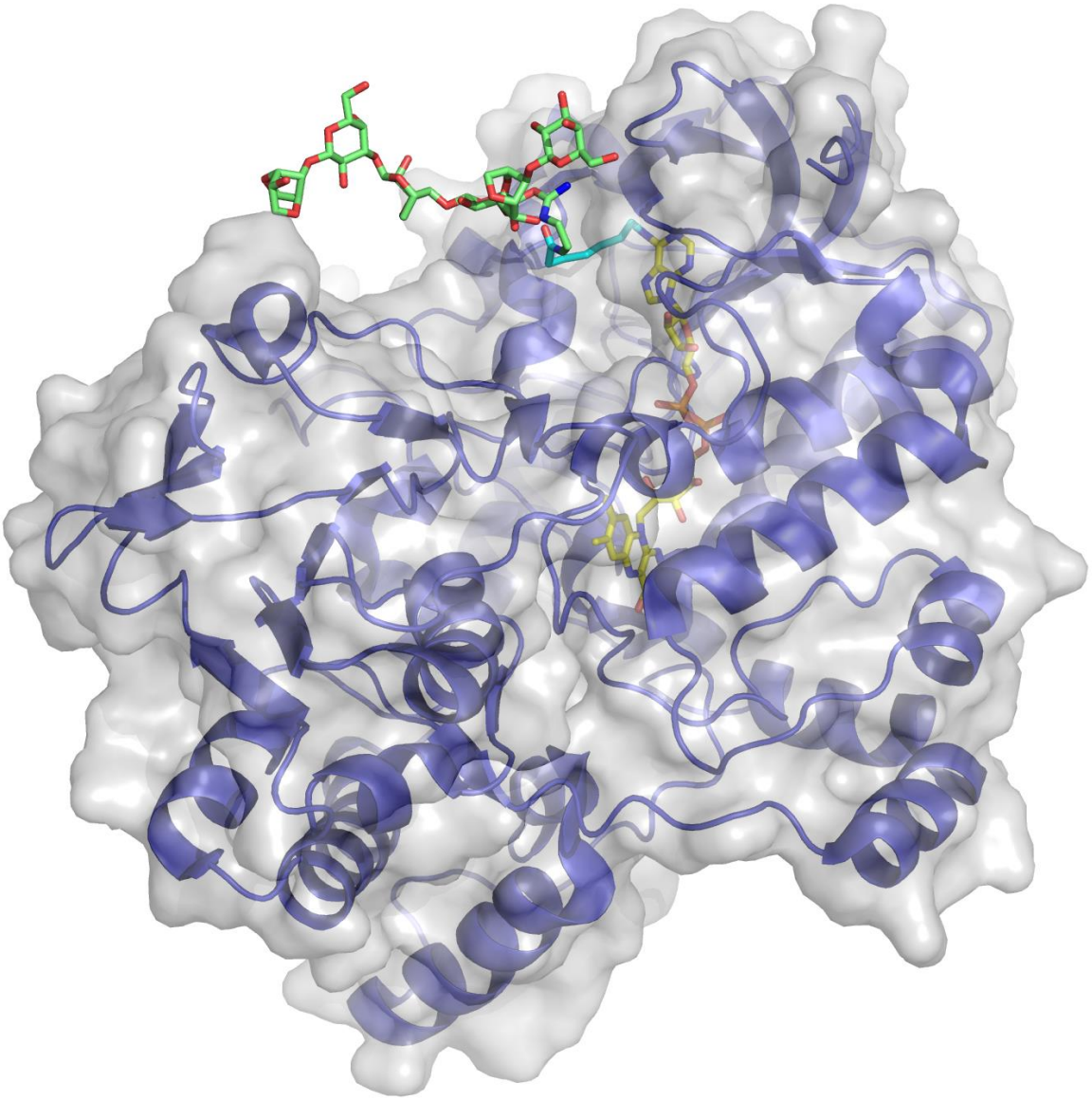


Figure. 2. *PAMO reconstituted with N^6-(6-carboxyhexyl)-FAD succinimidylester on the aminoethyl agarose beads. Binding of FAD* was modeled in the structure of PAMO (PDB ID: 1W4X) by manually adding to the N^6-adenine atom of FAD (FAD carbons in yellow) the hexyl linker part (carbons in cyan) coupled to an ethyl-N-agarose moiety (carbons in green).*

This revealed that except for PAMO with one covalently attached FAD*, some protein does not contain any covalently attached FAD while other protein molecules have two or more covalently attached FAD* molecules. The heterogeneous FAD* binding modes may well explain the lower activity observed for the FAD*-reconstituted PAMO. Overall, the results show that apo PAMO is able to accommodate FAD* in a catalytically competent conformation.

3.3. PREPARATION OF FAD-FUNCTIONALIZED AGAROSE

After having established that PAMO can be reconstituted with a N^6-adenine-FAD derivative into a fully active biocatalyst, we explored the possibility to exploit this promiscuous cofactor binding property to anchor the enzyme to a carrier via its cofactor. For the envisaged method of enzyme immobilization, FAD-functionalized carrier had to be prepared. Binding of apo protein to such material would allow immobilization of any target apo flavoprotein while it would also eliminate the binding of multiple flavin cofactors to single apo protein as observed above. For this, aminoethyl agarose was coupled to the FAD* via the succinimidyl ester moiety (Fig. 6 in Chapter 1). As a result, a stable amide bond was formed between the agarose-based carrier and the flavin derivative resulting in a brightly yellow agarose material. The generated FAD-agarose was stable upon extensive washing with buffer, water and 1.0 M sodium chloride as it retained its yellow color. Moreover, a similar incubation with normal FAD (negative control) yielded colorless beads.

3.3.1. ENZYME IMMOBILIZATION

The FAD-agarose was tested as carrier for enzyme immobilization using two apo flavoproteins, apo PAMO and apo PTDH-PAMO. Inspection of the PAMO crystal structure suggested that the designed FAD*-agarose would allow binding to the apo protein: the hexyl linker between the FAD and agarose carrier seems compatible with the protein structure (Figure. 2). Incubation with both apo proteins resulted in agarose material that indeed displayed monooxygenase activity (Figure. 3, Table S1). The FAD-agarose beads incubated without apo proteins did not show any activity. Enzyme loading on the FAD-agarose was 3.9 mg (0.060 μmol) and 3.5 mg (0.035 μmol) of protein per gram dry beads for PAMO and PTDH-PAMO respectively. The specific activity was 2.9 and 3.0 U per gram of dry beads (Table S1). These results show that the immobilized enzymes display activities that are similar when compared with their free forms. It indicates that the porous beads do not impose diffusion limitations that translate into lower rate of conversion. By varying the concentration of reactive amino groups on the carrier and optimizing the incubation conditions higher enzyme loading may be achieved but such optimization was not pursued in this study. Recently, it was shown that PAMO can also be covalently immobilized on polyphosphazenes yielding 8 U per gram of carrier after multiparametrical optimization (Cuetos *et al.*, 2012). However, in that case, a significant decrease of enzymatic performance was observed upon immobilization.

3.3.2. STABILITY OF PREPARED BIOCATALYSTS AND CARRIER

A study on the reusability of the immobilized enzymes was performed. Figure. 3 summarizes the catalytic performance of PAMO and PTDH-PAMO immobilized on FAD-agarose. Each enzyme

variant was immobilized on agarose beads and the immobilized enzyme was used in three subsequent conversions. The data show that PAMO-containing beads lose 13% of their activity after the first conversion. This may be caused by dissociation of some apo enzyme molecules during recycling of the immobilized biocatalyst. The loss of activity for PTDH-PAMO (44%) is more severe and may be related to higher mechanical stress experienced by the fusion protein and/or by a lower cofactor affinity. Nonetheless, the data show that FAD-immobilized PAMO and PTDH-PAMO can be reused. Furthermore, for PAMO we observed that the immobilized biocatalyst can be stored for over 5 months at 4 °C while retaining 68% of its activity. For generating a more stable immobilized biocatalyst, the use of heterofunctional support could be considered as this would allow, after FAD-mediated immobilization of the enzyme, to strengthen immobilization by a subsequent alternative covalent immobilization approach (Dos Santos *et al.*, 2015). This would prevent enzyme release while it still affords control on how the enzyme is positioned on the support.

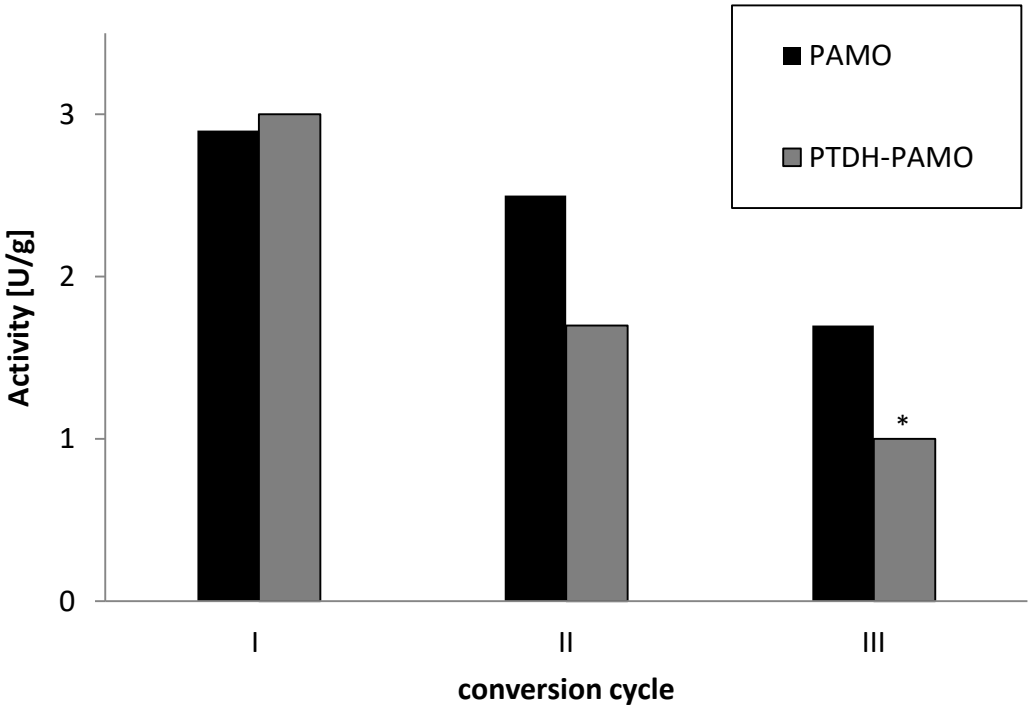


Figure . 3. *Activity of immobilized enzymes after several round of conversion.*

To probe the thermostability of immobilized PAMO, the time dependent inactivation at elevated temperatures (50 and 60 °C) was monitored. This revealed that the immobilization has a beneficial effect on enzyme stability (Figure. 4). After an incubation for 1 h at 60 °C, the agarose beads with immobilized PAMO retained 40% activity, while the free enzyme displayed no significant activity anymore and started to precipitate. The latter is in line with the previously determined apparent melting temperature of native PAMO: T_m = 60 °C (Cuetos *et al.*, 2012). The activity decrease for immobilized enzyme at elevated temperatures was accompanied by release of apo protein from the carrier.

By using the deflavinylation solution, PAMO-agarose could be regenerated into FAD-agarose by stripping of the protein. The resulting FAD-agarose lost all enzyme activity confirming release of the protein. The recycled FAD-agarose was reused for another round of immobilization. The resulting immobilized PAMO again displayed catalytic activity identical to the freshly immobilized enzymes. This shows that the method is flexible in the sense that the generated FAD-agarose can be reused for other flavoprotein immobilizations.

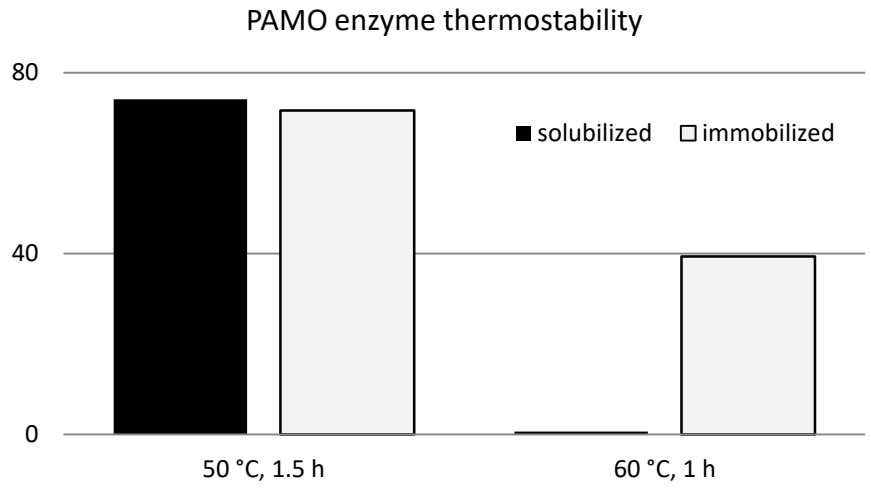


Figure 4. *PAMO activity after incubation for 90 min at 50 °C and 60 min at 60 °C. Enzyme activity was measured by monitoring oxygen depletion.*

4. CONCLUSIONS

We developed a new method for FAD-mediated flavoenzyme immobilization. It yielded fully active and stable immobilized biocatalysts. For this method, agarose was covalently functionalized with a synthetic FAD cofactor which could be used for an efficient and quick reconstitution and immobilization of apo PAMO and apo PTDH-PAMO. The immobilized enzymes restored full activity, could be reused, and displayed a higher thermostability than the enzyme in solution. The FAD-agarose was shown to be a robust carrier, recyclable for multiple immobilizations. The presented method opens up new avenues for the tunable and gentle immobilization of other flavoenzymes.

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SUPPLEMENTARY DATA

Appendix A.

Table S1. *Activity and conversion results for immobilized PAMO and PTDH-PAMO in comparison to both enzymes when free in solution.*

	cycle	Enzyme activity based on benzyl acetate formation per mass of dry resin [U/g]	Referred to theoretical activity calculated for amount of enzyme on the agarose [d] [%]
PAMO	I [a]	2.9	72
	II	2.5	
	III [b]	1.7	
PTDH-PAMO	I [c]	3	94
	II	1.7	
	III	1	

a: the enzyme loading of the agarose was 3.8 mg/g dry agarose; b: the protein-stripped FAD-agarose was stored for 23 weeks at 4 °C before reuse; c: the enzyme loading of the agarose was 3.5 mg/g dry agarose; compared with the known k_{cat} values of PAMO and PTDH-PAMO, respectively.

SUPPORTING INFORMATION FIG. S1

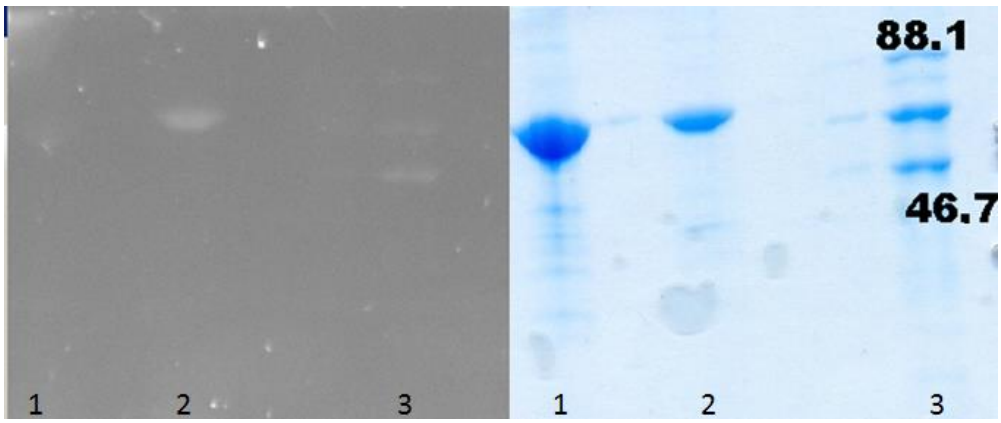

Fig. S1. *SDS PAGE analysis: left, UV-fluorescence for detecting protein-bound flavins in gel; right, Coomassie blue protein staining. Lane 1, protein reconstituted with FAD; lane 2, protein reconstituted with FAD*; lane 3, fluorescent proteins markers.*

SUPPORTING INFORMATION FIG. S2

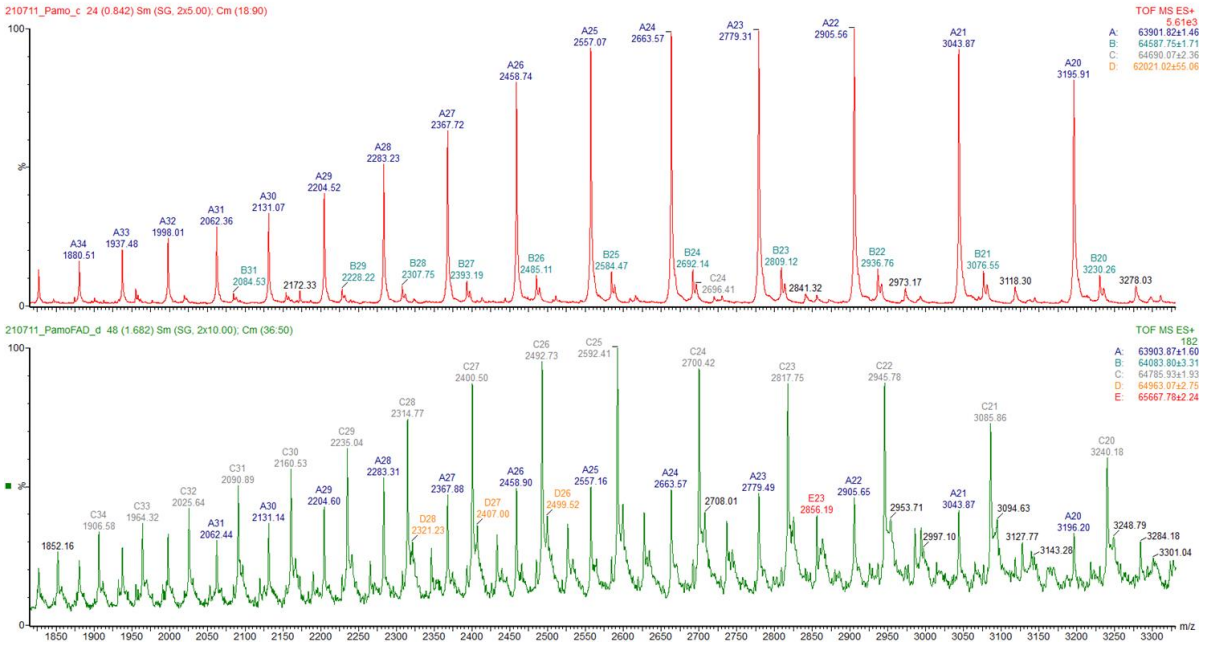

Fig S2. ESI-MS analysis of denatured native PAMO (upper panel) and denatured FAD*-reconstituted PAMO (lower panel). Species A represents the protein without any cofactor bound and is most dominant in native PAMO, while in FAD*-reconstituted sample species C (additional mass of FAD*, ~882 Da) is dominant.