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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, N6-(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.

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 [1–4]. 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 [5]. On the other hand, non-covalent adsorption of enzymes usually involves mild conditions but often results in labile systems [5]. 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 [6–8]. In the last decade, the toolbox of flavoenzymes has significantly expanded thanks to enzyme engineering and discovery efforts [8–10]. 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 [11]. Once prepared, such apo proteins are typically readily reconstituted with FAD or FAD analogues [12]. A large number of synthetic FAD derivatives have been explored before, mostly with the aim to elucidate mechanistic features of FAD-containing enzymes [13]. 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

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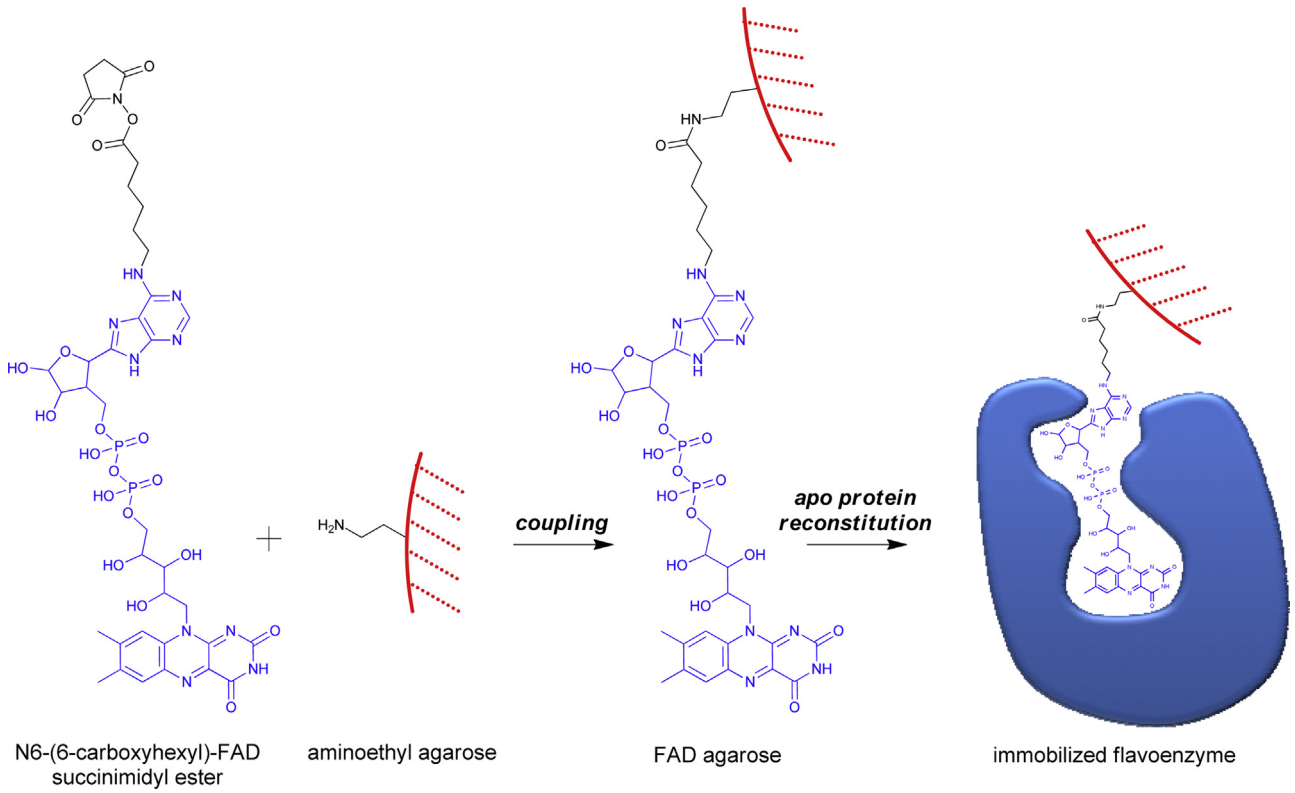

Fig. 1. Structural formula of the employed FAD analogue N6-(6-carboxyhexyl)-FAD succinimidylester and the schematic approach of the FAD-mediated immobilization method. The FAD part of the structural formula is in blue and the agarose support in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isoalloxazine ring (Fig. 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 N6-FAD-derivatives have been shown to be more efficient over adenine N1-FAD in reconstituting apo forms of flavoprotein oxidases [14]. Using N6-(2-aminoethyl)-FAD, Willner et al. have shown that it is feasible to anchor glucose oxidase on electrodes [15]. With this system, a glucose-dependent electric current could be monitored. Moreover, D-amino acid oxidase and L-aspartate oxidase were shown to bind N6-adenine modified FAD yielding artificial covalent flavoprotein oxidases [16,17]. Nevertheless, it resulted in a significant decrease in oxidase activity. In this paper we demonstrate that N6-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 oxidations [18,19]. In addition, its crystal structure has been solved [20], the catalytic performance and substrate scope has been well described, the expression in *Escherichia coli* is efficient [21], and the enzyme displays remarkable stability against elevated temperatures and a wide range of organic solvents [22,23]. 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 (Fig. 2). A number of research efforts have been directed toward efficient regeneration of the nicotinamide coenzyme [24]. 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 [25].

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.

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 [26]. N6-(6-carboxyhexyl)-FAD succinimidylester (FAD*) (Fig. 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 [25,27]. 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 substrates [28] while using an atmospheric dioxygen concentration (0.24 mM).