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Converting a fatty acid binding protein to an artificial transaminase: novel catalysts by chemical and genetic modification of a protein cavity

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Abstract

Despite the widespread use of enzymes in synthetic applications, their "native" characteristics are often insufficient for many chemical transformations. To meet this challenge we have used protein cavities for the design of new biocatalysts. A pyridoxamine derivative (PX) was chemically tethered within the spacious cavity of intestinal fatty acid binding protein (IFABP). The cysteine residue, which anchors the cofactor of the artificial transaminase IFABP-PX, can be placed in different regions by site-directed mutagenesis. Catalytic reactions with high enantioselectivities (up to 94% ee) and varying substrate specificity of the transamination of α-keto and amino acids were achieved. IFABP-PX mutants were further optimized by introducing lysine residues in order to mimic the active site of native transaminases.

Keywords: Semisynthetic enzyme; Transamination; Fatty acid binding protein; Pyridoxamine

1. Introduction

Native enzymes are extremely efficient and selective catalysts. However, their application in organic synthesis is restricted by the inherent specificity regarding the reaction type or substrate selectivity. To circumvent these limitations the development of artificial enzymes has received considerable attention. One approach for the tailored design of such biocatalysts is to modify a known protein at a defined site with a catalytic active group. In these semisynthetic enzymes, the established selectivity of enzymes is combined with the desired catalytic activity of the chosen cofactor [1,2].

Recently, the potential of semisynthetic enzymes for biocatalytic synthesis was demonstrated by means of the semisynthetic peroxidase seleno-subtilisin. By introducing a selenium into the active site of the protease subtilisin, the resulting seleno-subtilisin catalyzed the enantioselective reduction of racemic hydroperoxides [3]. A straightforward gram-scale synthesis of the catalyst was developed [4] and it was used for preparative stereoselective biotransformations [5]. The versatility of this chemical engineering approach was underlined by the preparation of cross-linked crystals of seleno-subtilisin: not only was the active site chemically altered, but also the protein framework [6].

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Despite these recent advances in the area, further challenges remain to be met. The point of attachment of the new catalytic group should be variable at will. Once the cofactor is tethered to a protein, the microenvironment should be amenable to further modification. For example, certain amino acids might assist in more efficient catalysis by steric or electronic effects. To reach these goals, the chemical engineering of proteins has to be combined with genetic engineering. Established methods of protein mutation in combination with computational modeling open the path to further optimization of semisynthetic enzymes. We summarize here our recent efforts towards such "chemogenetic" enzymes exemplified by an artificial transaminase.

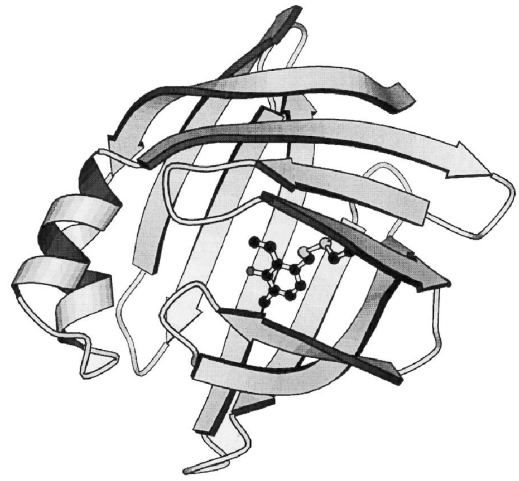

Fig. 2. ALBP containing a pyridoxamine cofactor attached to a unique cysteine within the protein cavity (adapted from the crystal structure described by Ory et al. [8]).

2. Results and discussion

In our earlier work, we used the fatty acid binding protein adipocyte lipid binding protein (ALBP) as a protein template for the construction of a semisynthetic transaminase [7]. ALBP forms a large cavity of about 600 $Å^3$ that completely encloses bound ligands. A pyridoxamine-derivative was linked via a disulfide bond to a single cysteine residue in the interior of the protein host (Fig. 1) as determined by its crystal structure [8] (Fig. 2). This construct catalyzed the transamination of α-keto acids to amino acids with rates similar to the free pyridoxamine, yet with enantioselectivities of up to 94% ee [7].

Based on these promising results, we used the structurally related but cysteine-free intestinal fatty acid binding protein IFABP for further studies. A single cysteine was placed in different positions within the protein cavity by site-specific mutation. Thus, the pyridoxamine could be inserted into varying environments resulting in different catalytic rates and substrate (stereo-) selectivities (Table 1) [9]. Best results were obtained when using the IFABP-Val60Cys mutant as the protein framework. The reactions of this artificial transaminase called IFABP-PX60 were studied in more detail (Table 2) [10]. Kinetic analysis of the IFABP-PX60 catalyzed transamination of phenylalanine and α-ketoglutaric acid yielded values for K_M and k_{cat} of 2.9 mM and 0.22 h^{-1}, respectively. Attempts to further improve the catalytic efficiency by addition of metal ions (e.g. Cu(II), Ni(II), Zn(II) are known to increase the rates of the free pyridoxamine-catalyzed transamination) were not successful [11].

The most valuable archetypes for the design of artificial catalysts are native enzymes. Their high catalytic efficiency and selectivity depend not only

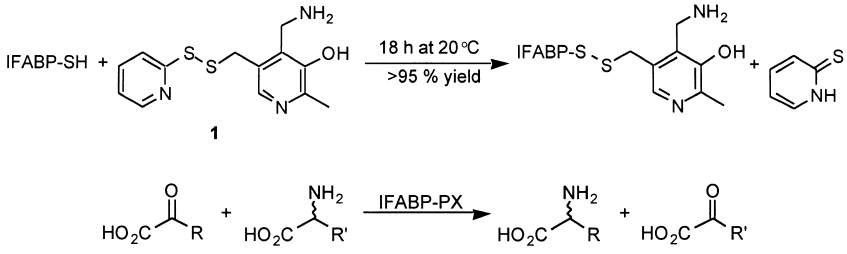

Fig. 1. Synthesis of the semisynthetic transaminase IFABP-PX (top) and catalytic transamination reaction (below).

Table 1
Amino acid conversions and enantioselectivities of the transamination depend on the positioning of the pyridoxamine within the IFABP cavity (the position of the cysteine mutation in the IFABP-PX mutants is V60C, L72C and A104C)

Amino acid	IFABP-PX60	IFABP-PX72	IFABP-PX104
	Conversion[a] (%)/ee (%)		
Ala	62/27 (D)	50/16 (D)	5/87 (L)
Leu	56/26 (L)	65/28 (D)	< 0.1/–[b]
Tyr	90/47 (L)	54/4 (L)	12/74 (L)
Glu	99/68 (L)	14/31 (L)	< 0.1/–[b]

[a]Conversions were measured after 24 h under single turnover conditions.
[b]No ee observed.

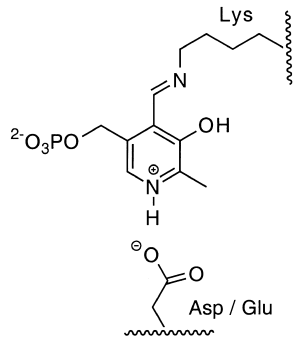

Fig. 3. In native transaminases, a strictly conserved lysine forms an internal imine with the pyridoxalphosphate. The carboxylic acid group of Asp or Glu interact with the pyridine-nitrogen in order to stabilize its postive charge.

on cofactors but also on an optimized arrangement of assisting amino acids in the active site. In native transaminases, strictly conserved lysine and aspartate/glutamate residues are located in the active site. The ε-amino group of lysine forms an internal imine with the aldehyde group of pyridoxalphosphate and serves as an internal acid or base. The β- or γ-carboxylic acid group of Asp or Glu, respectively, stabilizes the positive charge at the pyridine-nitrogen (Fig. 3). The substitution of one of these amino acids in mutant transaminases decreases the catalytic activity between 10^5 and 10^6-fold [12,13].

In view of this dramatic effect, we designed IFABP-PX mutants, which contain a lysine residue in close proximity to the aldehyde group of PX. Guided by the structural motifs of various native aminotransferases, molecular modeling experiments suggested introduction of lysine at position 38 and 51 in IFABP. The point mutations Leu38Lys or Glu51Lys, respectively, were introduced into pMON-IFABP recombinant plasmids and the mutant proteins were expressed in *E. coli* JM105. Using the scheme outlined in Fig. 4, the IFABP-mutants were purified to homogenity and conjugated yielding the semisynthetic transaminases IFABP-PX38 and IFABP-PX51 (the number indicates the position of the lysine). Both IFABP-PX mutants were able to catalyze the transamination of α-ketoglutaric acid and amino acids. For example, after 24 h the turnovers using phenylalanine or tyrosine were in the range of 5.5 and 6.9. L-Glutamic acid was produced with good enantioselectivities of 84–94% ee.

Efforts to prepare IFABP-mutants containing both lysine and glutamic acid active site residues have been unsuccessful to date. The stability of such mutants was dramatically decreased after the introduction of glutamic acid and we were not able to purify sufficient protein for further studies. However,

Table 2
Production of glutamate in 24 h catalyzed by IFABP-PX 60 in the presence of different amino acids

Amino acid	Turnovers[a] (*n*)	ee (%) of L-Glu
Ala	1.3	70
Phe	3.9	93
Tyr	4.2	93
D-Tyr	0.9	65
L-Tyr	3.1	91
Dopa	4.3	94

[a]Turnovers were measured after 24 h. Reactions were run in 0.1 ml of 50 mM α-ketoglutaric acid, 5 mM amino acid, 50 μM IFABP-PX and 0.2 M HEPES (pH 7.5).

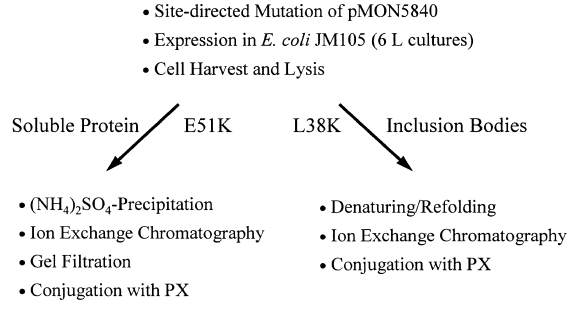

Fig. 4. Purification scheme for IFABP-L38K and IFABP-E51K.

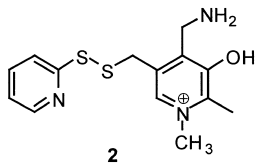

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Fig. 5. An *N*-methylated pyridoxamine mimic with a permanent positive charge.

facing problems with genetic engineering of the protein framework, the goal of stabilizing a positive charge at the pyridine nitrogen of the pyridoxamine was addressed by chemical engineering techniques.

To this end, an *N*-methylated pyridoxamine mimic (MPX **2**, Fig. 5) with a permanent positive charge was designed and synthesized in six steps starting from pyridoxamine (18% overall yield). This new cofactor was tethered within the protein cavities of both IFABP-L38K and -E51K mutants yielding the conjugates called IFABP-MPX38 and IFABP-MPX51. Preliminary studies indicate that these conjugates are able to catalyze the transamination of α-ketoglutaric acid and various amino acids. The turnovers after 24 h are approximately 2–3 fold higher in comparison to the IFABP-PX, whereas the enantioselectivity decreased to values around 42–80% ee for the production of L-glutamic acid.

3. Conclusions

The objective of the work described here is to develop novel methods for the design of stereoselective biocatalysts. The catalytic efficiency of artificial enzymes like the presented IFABP-PX conjugates is still several orders of magnitude lower than their natural archetypes. Nevertheless, our pioneering work demonstrates important features of a novel approach towards developing future catalysts: The chemical introduction of a catalytic active group into a well-known protein yields enzyme mimics with desired catalytic activities and substrate selectivities. In combination with genetic engineering techniques, this approach of catalyst design embraces now the variation of the point of attachment or the introduction of assisting amino acids.

Acknowledgements

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