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MODULATION OF THE RATE, ENANTIOSELECTIVITY, AND SUBSTRATE SPECIFICITY OF SEMISYNTHETIC TRANSAMINASES BASED ON LIPID BINDING PROTEINS USING SITE DIRECTED MUTAGENESIS

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Abstract: Fatty acid binding proteins are a class of small 15 kDa proteins with a simple architecture that forms a large solvent sequestered cavity. In previous work, we demonstrated that reductive amination reactions could be performed in this cavity by covalent attachment of a pyridoxamine cofactor to the protein. Here, we report the results of experiments in which the position of pyridoxamine attachment has been varied by site directed mutagenesis. The conjugate IFABP-PX60 reacts at least 9.4-fold more rapidly than our original conjugate ALBP-PX, while IFABP-PX72 inverts the enantioselectivity of reactions (compared to ALBP-PX) and IFABP-PX104 displays very selective substrate specificty. These results indicate that site-directed mutagenesis can be used to tune the rate, enantioselectivity, and substrate specificity of semisynthetic transaminases based on fatty acid binding proteins. © 1997 Elsevier Science Ltd.

Naturally occuring enzymes are extremely efficient catalysts. For this reason, a variety of approaches have been employed to construct systems that can perform chemical transformations with comparable selectivities and rates.[1] To meet this challenge, one strategy of catalyst design involves the preparation of "semisynthetic enzymes" in which an enzyme is altered by covalent modification; such constructs can result in enzymes with new catalytic functions or specificities.[2] Recently, we described the preparation of adipocyte lipid binding protein (ALBP) appended with a pyridoxamine cofactor and termed the resulting construct ALBP-PX.[3] This protein was chosen as a scaffold because it possess a large 600 $Å^3$ cavity that is sequestered from bulk solvent such that fatty acid ligands that are bound in this cavity are completely encapsulated within the protein.[4] It was reasoned that such a structure would maximize the number of interactions between the protein and the catalyst positioned within. In our earlier work, we demonstrated that ALBP-PX reductively aminated a variety of α-keto acids to α-amino acids in an enantioselective manner; selectivities as high as 94% *ee* were obtained.[3] Since the reactivity of the pyridoxamine moiety is likely to be influenced by its microenvironment, we were interested in positioning the cofactor in different regions within the protein cavity and in studying the effects of such modifications on the selectivity and rate of reactions performed therein. Here we describe the preparation of three fatty acid binding protein-pyridoxamine conjugates using a protein related to ALBP called intestinal fatty acid binding protein (IFABP).[5] These three conjugates differ only in the location of the unique cysteine residue (varied by site directed mutagenesis) used to attach the pyridoxamine cofactor. The results of reductive amination reactions between these constructs and several α-keto acids are also reported.

Three mutant forms of IFABP were chosen for study including V60C, L72C and A104C.[6] As shown in Figure 1, these mutations all involve residues in the IFABP cavity in which the side chains point into the protein interior and each mutation positions the unique cysteine residue in a distinct region of the protein cavity. Position 117 is also noted in Figure 1; this was the site of attachment used to prepare ALBP-PX. The desired IFABP mutant proteins were obtained by expressing the corresponding mutant genes in *E. coli* MG1655 under control of

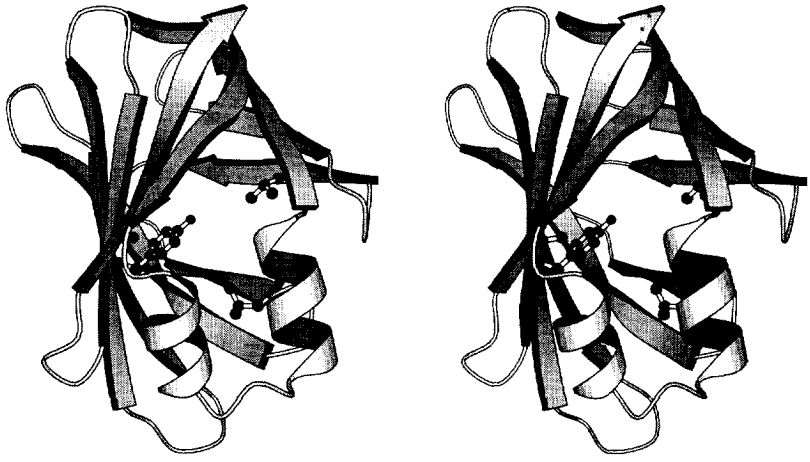


Figure 1. Stereo view from the structure of IFABP showing the side chain locations of residues V60, L72, Y117 and A104 in ball and stick representations (listed clockwise from upper right).[7]

the RecA promoter. Growth of the bacteria in LB media followed by induction with nalixic acid and further growth at 37 °C yielded approximately 30 g of cell paste per 6 L of liquid culture. The proteins were purified by ammonium sulfate precipitation followed by anion exchange chromatography (QAE Sephadex) and gel filtration chromatography (Sephadex G-75 superfine); approximately 100 mg of each protein was obtained using this procedure.[8] The protein-pyridoxamine conjugates (IFABP-PX) were prepared via the procedure shown in Figure 2 in which a given IFABP mutant possessing a unique cysteine residue was reacted with the reagent thiopyridine-PX (T-PX)[3] under native conditions for 24 h at room temperature followed by purification via size exclusion chromatography (Bio-Rad P-6 DG). Conjugation efficiencies, tabulated in Table 1, were determined by thiol titration of the proteins with DTNB[9] before and after reaction with T-PX and were typically greater than 80%. Reaction between the A104C mutant and T-PX was found to be slow ($t_{1/2}$ > 24 h); consequently, this conjugate was prepared under denaturing conditions (4.8 M guanidine•HCl) and refolded by dialysis. The native molecular masses for the mutant proteins and their conjugates (see Table 1) were determined by gel filtration chromatography and were found to be quite similar indicating that attachment of the pyridoxamine cofactor does not disrupt the overall IFABP structure.

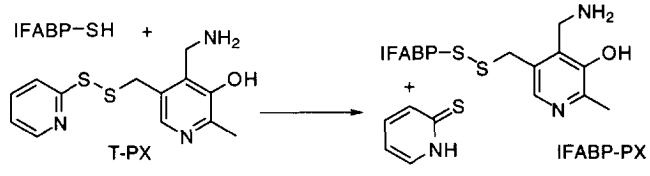


Figure 2. Reaction used for the preparation of IFABP-PX conjugates.

UV/vis spectroscopy of the IFABP-PX conjugates, shown in Figure 3, revealed the presence of a new chromophore centered at 325 nm characteristic of a pyridoxamine moiety and consistant with attachment of the cofactor to the protein. This new absorbance, measured under denaturing conditions to remove perturbing effects

due to the protein, allowed the conjugation efficiencies for the various mutants to be determined in an independant manner. Using this method, values similar to those determined by thiol titration were obtained (see Table 1). Comparison of the IFABP-PX spectra obtained under native and denaturing conditions (Figure 3) reveal different results for the various mutant proteins. For the PX72 and PX104 conjugates, a ca. twofold increase in extinction of the pyridoxamine chromophore occurs while very little change is observed with the PX60 protein. Fluorescence measurements of the IFABP-PX constructs also reveal interesting differences in the mutants. Attachment of the pyridoxamine moiety results in substantial quenching of the protein fluorescence. Quenching is also observed in measurements of the pyridoxamine fluorescence; in this case the extent of quenching is different for each mutant with the PX60 conjugate having the highest fluorescence and the PX104 protein having the lowest. These spectroscopic results clearly show that the cofactor environment can be controlled by varying its position within the cavity.

Table 1. Physical and Spectroscopic Properties of IFABP-PX Conjugates.

Protein	Conj. Eff. by DTNB[a] (%)	Conj. Eff. by UV[b] (%)	Molecular Mass[c] (kDa)	ε_{328} Native[d] ($M^{-1}cm^{-1}$)	Protein Fluor.[e] (%)	PX Fluor.[f] (%)
PX60	96	99	18.7	6.5	25	26
PX72	91	82	18.7	2.9	31	9.0
PX104	81	79	18.7	2.3	35	2.6

[a]Conjugation efficiencies determined by thiol titration of mutant IFABP proteins with DTNB before and after reaction with T-PX. [b]Conjugation efficiencies obtained by spectroscopic measurement (UV/vis) of pyridoxamine content after denaturation in 4.8 M guanidine•HCl. [c]Molecular mass determined by gel filtration chromatography employing a Superose 12 column (Pharmacia). The molecular mass of IFABP under these conditions was found to be 18.6 kDa. [d]Obtained from UV/vis spectroscopy in 20 mM HEPES, pH 7.5. [e]Protein fluorescence (excitation at 280 nm, emission at 340 nm) of conjugate relative to unmodified protein both measured with 0.5 μM protein (determined by Bradford protein assay) in 20 mM HEPES, pH 7.5, at 25 °C. [f]Pyridoxamine fluorescence (excitation at 325 nm, emission at 390 nm) of conjugate relative to free pyridoxamine measured with 2.0 μM pyridoxamine (determined by UV/vis) in 20 mM HEPES, pH 7.5, at 25 °C.

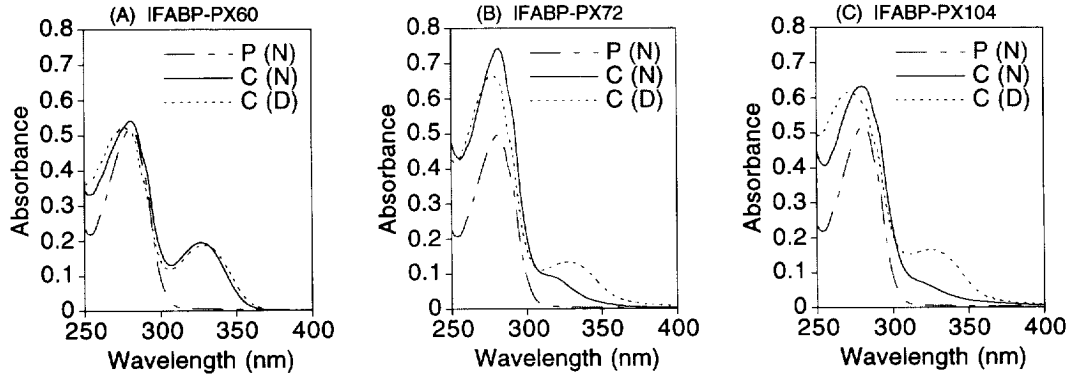
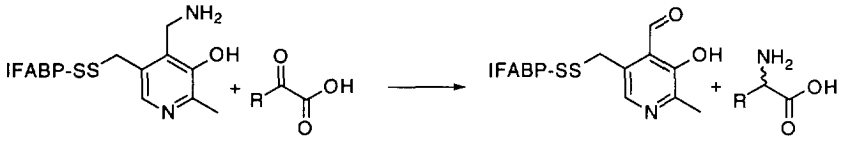


Figure 3. UV/vis spectra of mutant IFABP-PX conjugates. All spectra were obtained using 30 μM conjugate (determined by Bradford protein assay). P (N): spectrum of unmodified protein obtained under native conditions (20 mM HEPES, pH 7.5). C (N): spectrum of conjugate obtained under native conditions (20 mM HEPES, pH 7.5). C (D): spectrum of conjugate obtained under denaturing conditions (4.8 M guanidine•HCl).

The IFABP-PX conjugates were next evaluated for their ability to perform the reductive amination reactions shown below in Figure 4; the types of keto acid substrates (**1a-1e**) studied include alkyl, carboxyl, and phenyl substituted α-keto acids. The reactions were carried out under pseudo first order, single turnover conditions to facilitate analysis. Reactions contained IFABP-PX conjugate (50 μM) and α-keto acid substrate (50 mM) in HEPES buffer (20 mM, pH 7.5) and were performed at 37 °C for 24 h. Amino acid production was determined by derivatization of the crude reaction mixture with *o*-diphthalaldehyde and *N*-acetyl cysteine or *N*-BOC cysteine to produce diastereomeric isoindole derivatives that were then seperated by reversed-phase HPLC and quantitated by fluorescence detection.[3,10]



1a R = CH_3
1b R = $(CH_3)_2CH$
1c R = $(CH_3)_2CHCH_2$
1d R = $HOC_6H_4CH_2$
1e R = $HO_2C(CH_2)_2$

2a R = CH_3
2b R = $(CH_3)_2CH$
2c R = $(CH_3)_2CHCH_2$
2d R = $HOC_6H_4CH_2$
2e R = $HO_2C(CH_2)_2$

Figure 4. Reductive amination reactions showing α-keto acid substrates and α-amino acid products studied with mutant IFABP-PX conjugates.

Analysis of reductive amination reactions with the IFABP-PX conjugates gave the results listed in Table 2; the results obtained previously with ALBP-PX are included for comparison. Reactions with the IFABP-PX60 protein are without exception faster than the corresponding reactions with ALBP-PX. For the alkyl substituted α-keto acids, the conversion after 24 h of reaction increases 1.4-fold for leucine, 1.8-fold for valine, and 3.4-fold for alanine when compared with ALBP-PX. Tyrosine and glutamate are particularly interesting given the high extents of conversion observed for these amino acids. Since these reactions were performed under pseudo first order conditions, direct comparison of the extent of product formation at high levels of conversion does not accurately represent the relative rates. Preliminary experiments that measure the production of glutamate at shorter times (36% conversion after 2 h) indicate that the IFABP-PX60 protein accelerates the reductive amination of α-keto glutarate relative to ALBP-PX by at least 9.4-fold. A similar comparison between IFABP-PX60 and free pyridoxamine reacting with α-keto glutarate (3.0% conversion after 24 h) reveals a 17-fold acceleration for the protein conjugate. The more rapid rates obtained here are not due to adventitious metal contamination (metal ions are known to accelerate pyridoxamine-promoted reductive amination reactions) since the rates are insensitive to the presence of 1 mM EDTA in the reaction mixtures. Although a thorough examination of the kinetics of this reaction will be necessary to determine the magnitude of the rate acceleration, *the high levels of conversion observed with tyrosine and glutamate suggest that it should be possible to render these reactions catalytic* by the addition of a second amino acid to convert the protein-bound cofactor from the aldehyde form to the amino form.

The results of reductive amination reactions with the IFABP-PX72 and IFABP-PX104 conjugates are also summarized in Table 2. The IFABP-PX72 protein displays very different substrate specificity and

enantioselectivity when compared to ALBP-PX or the other IFABP constructs. The production of alanine using the PX72 protein is 2.8-fold higher while the production of glutamate is 3.8-fold lower when compared to reactions containing ALBP-PX. However, the most dramatic results using the IFABP-PX72 protein concern the enantioselectivites obtained in the production of valine and leucine. Using ALBP-PX, the L-enantiomer was the predominant isomer formed when valine and leucine were studied; L-valine was produced in 94% *ee* (32.3 L/D ratio) while L-leucine was generated in 54% *ee* (3.4 L/D ratio). With the IFABP-PX72 protein, *the enantioselectivity for these two amino acids was inverted.* The D isomers of valine and leucine were produced in 63% *ee* (4.41 D/L ratio) and 28% *ee* (1.78 D/L ratio), respectively. Thus, these results employing the IFABP-PX72 mutant demonstrate that, at least in some cases, the enantioselectivity of these semisynthetic transaminases can be tuned by site directed mutagenesis.

Table 2. Amino Acid Conversions and Enantioselectivities Obtained with IFABP-PX Conjugates.

Amino Acid	Conversion After 24 h (%)	*ee* (%)	Conversion After 24 h (%)	*ee* (%)
	ALBP-PX		IFABP-PX60	
Ala (**2a**)	18 ± 3.6	42 (D) ± 5.1	62 ± 2.0	27 (D) ± 0.4
Val (**2b**)	28 ± 1.9	94 (L) ± 0.2	49 ± 1.4	26 (L) ± 2.9
Leu (**2c**)	39 ± 1.2	54 (L) ± 0.8	56 ± 0.9	26 (L) ± 2.7
Tyr (**2d**)	42 ± 1.5	67 (L) ± 3.3	90 ± 1.7	47 (L) ± 0.9
Glu (**2e**)	46 ± 5.6	84 (L) ± 2.2	>99 ± 2.3	68 (L) ± 1.7
	IFABP-PX72		IFABP-PX104	
Ala (**2a**)	50 ± 6.5	16 (D) ± 0.3	5.2 ± 2.1	87 (L) ± 0.1
Val (**2b**)	26 ± 2.6	63 (D) ± 5.1	< 0.1	-
Leu (**2c**)	65 ± 2.9	28 (D) ± 0.4	< 0.1	-
Tyr (**2d**)	54 ± 1.8	4.0 (L) ± 0.4	12 ± 1.5	74 (L) ± 1.1
Glu (**2e**)	14 ± 0.9	31 (L) ± 1.6	< 0.1	-

Reactions with IFABP-PX104 also gave interesting results. This protein is much more selective regarding the type of α-keto acid substrate that it will react with. *In contrast to the other conjugates, which will process most α-keto acids, IFABP-PX104 will only react with pyruvate and hydroxyphenylpyruvate to produce alanine and tyrosine, respectively;* no production of valine, leucine or glutamate was observed. The enantioselectivites obtained for alanine (87% *ee*) and tyrosine (74% *ee*) were also good in comparison to those obtained for the same amino acids produced by ALBP-PX and the other IFABP-PX conjugates. It is interesting to note that the conjugation reaction involving the A104C mutant was particularly slow. Inspection of the IFABP crystal structure shows that this position is deeply burried within the cavity. Therefore, the low reaction rate for conjugation and the high substrate specificity for reductive amination observed with this mutant may simply be a result of stringent steric requirements in this region of the protein cavity.

The results described in this paper demonstrate that reductive amination reactions can be performed in the sequestered cavity of IFABP similar to results reported earlier with a different fatty acid binding protein, ALBP. Using site directed mutagensis, the position of pyridoxamine attachment within the cavity can be varied; three

mutant proteins were studied. Spectroscopic measurements suggest that the cofactor environment is different depending on its location within the cavity. Reductive amination reactions using pyridoxamine conjugates, prepared from three mutant proteins, together with a series of α-keto acids, produced products at different rates with different selectivities depending on the mutant protein employed. These results indicate that site-directed mutagenesis can be used to tune the rate, enantioselectivity, and substrate specificity of semisynthetic transaminases based on fatty acid binding proteins.

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