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Synthesis of a Cationic Pyridoxamine Conjugation Reagent and Application to the Mechanistic Analysis of an Artificial Transaminase

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Abstract—An *N*-methylated, cationic pyridoxamine conjugation reagent was synthesized and tethered via a disulfide bond to a cysteine residue inside the cavity of intestinal fatty acid binding protein. The conjugate was characterized and the kinetic parameters compared to its nonmethylated pyridoxamine analogue. Kinetic isotope effects were used for further mechanistic analysis. Taken together, these experiments suggest that a step distinct from deprotonation of the ketimine in the pyridoxamine to pyridoxal reaction is what limits the rate of the artificial transaminase IFABP-Px. However, the internal energetics of reactions catalyzed by the conjugate containing the *N*-methylated cofactor appear to be different suggesting that the MPx reagent will be useful in future experiments designed to alter the catalytic properties of semisynthetic transaminases.

Although native enzymes are versatile and highly efficient catalysts, they are also limited in terms of the reaction types they catalyze and the substrate selectivity that they manifest. In order to design tailor-made biocatalysts, a variety of methods have been developed including RNA aptamers, catalytic antibodies and cyclodextrin-based enzyme mimics; the chemical introduction of a synthetic cofactor into an existing protein has proven to be particularly versatile. Such 'semisynthetic enzymes' use the framework of well-known proteins for binding the substrate in a highly chiral environment and creating an artificial active site.[1–3] Recently, we have expanded this concept by combining chemical and genetic enzyme engineering: a cysteine residue can be introduced by site-directed mutagenesis into a specific position in a protein and used as point of attachment for the chemical tethering of a desired catalytic active group.[4]

The rat intestinal fatty acid binding protein (IFABP) consists of two orthogonal β-sheets which form a large cavity of approximately 600 $Å^3$. The entrance to the protein interior is covered by a flexible α-helical loop, so that the bound substrates (e.g., fatty acids) are completely sequestered within. In earlier work, a cysteine residue was placed in various positions inside the cavity by site-directed mutagenesis and linked to a pyridoxamine-moiety (Px) via a disulfide bond (Fig. 1).[4] The resulting artificial enzymes (IFABP-Px) catalyzed the transamination of α-keto and amino acids similar to native aminotransferases. The reactions were most efficient when the pyridoxamine was linked to Cys 60 which is located in the middle of the first β-sheet element that forms the cavity.[5] Here, we describe the synthesis of a modified artificial pyridoxamine cofactor, its conjugation to IFABP and the implications on the catalytic mechanism of the resulting artificial transaminase. These experiments highlight the versatility of the 'semisynthesis' approach which allows the use of both chemical synthesis and genetic engineering to prepare catalytic systems.

The mechanism of the pyridoxamine-catalyzed transamination of α-keto and amino acids consists of two reversible half reactions (Scheme 1). Early studies of pyridoxal derivatives have shown, that pyridine-4-aldehyde does not catalyze the reaction, whereas 1-methyl-4-formylpyridinium iodide is able to form an aldimine similar to **4** with amino acids, which is converted in a second, slower reaction to a ketimine corresponding with **2**.[6] This difference was attributed to the greater electron withdrawing ability of the quaternized pyridine nitrogen. Comparing the transamination reactions of 3-hydroxy-4-formylpyridine with 1-methyl-3-hydroxy-4-formylpyridinium chloride, the initial rates of reactions promoted by the *N*-methylated species were up to 20-

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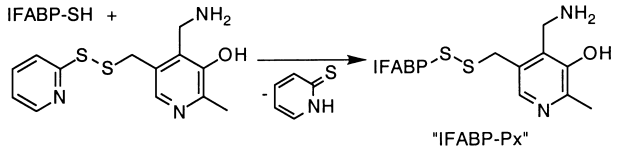


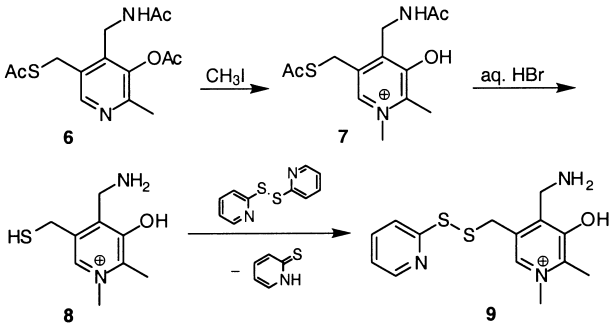
Figure 1. The intestinal fatty acid binding protein mutant Val60Cys (IFABP-SH) was chemically tethered to a pyridoxamine cofactor (Px) forming the artificial transaminase IFABP-Px.

fold increased depending on the pH.[7] However, when the pyridoxal phosphate cofactor present in aspartate aminotransferase was substituted by its *N*-methylated analogue, the enzyme lost >99.8% of its catalytic activity.[8,9] Thus *N*-quaternization may have both positive and negative influence on transamination reactions.

To determine the effect of *N*-methylation on the catalytic properties of IFABP-Px we synthesized a suitable conjugation reagent, 1-methyl-5-(2-pyridyldithio)pyridoxamine **9** (Scheme 2). The triacetylated intermediate **6** was synthesized from the commercially available pyridoxamine dihydrochloride in four steps as described previously.[10] *N*-Methylation was achieved using methyl iodide in benzene[11] followed by acid hydrolysis of the acetyl groups. Finally, the free thiol of **8** was activated for conjugation reactions by conversion to the disulfide **9**.[12] The cationic conjugation reagent was purified by preparative reversed-phase HPLC and an overall yield of 19% (based on **6**) was obtained.

The IFABP-mutant V60C was expressed in *E. coli* cultures and purified to homogenity.[5] After incubating the conjugation reagent **9** (MPx) with the protein for 18 h at room temperature, a disulfide bond with the single cysteine residue in position 60 inside the cavity of IFABP was formed.[10] The excess of **9** was removed from the IFABP-MPx conjugate by gel filtration chromatography. The conjugation efficiency was 92% as determined by titration of the free thiol with 5,5′-dithiobis(2-nitrobenzoic acid) before and after the conjugation reaction.[10] The UV spectrum showed the absorption bands of both the protein (280 nm) and the bound MPx (338 nm), the latter one being red-shifted compared to the nonmethylated IFABP-Px (Fig. 2). A positive band in the CD spectra of IFABP-Px and-MPx indicates the positioning of the cofactors within a chiral environment, i.e., the protein cavity (Fig. 2). Finally, the elution volumes obtained by gel permeation chromatography of IFABP-V60C and both conjugates were identical, suggesting that the overall folding of these chemically modified proteins resembles the native state.[13]

To assess the influence of the cationic pyridoxamine moiety inside IFABP on the protein stability, the properties of the unfolding transition were examined. Measuring tryptophan fluorescence, the guanidine hydrochloride (GdnHCl) induced unfolding of several IFABP mutants including V60C was recently studied.[14] In those experiments, the fluorescence data were fitted to a two state model which suggests that only the native and the fully unfolded forms of the protein are present in significant concentrations at equilibrium.[15] A transition midpoint concentration of 1.18 M GdnHCl was found for IFABP-V60C, indicating a small decrease in protein stability compared to the wild-type IFABP (1.36 M). Using the same experimental conditions, we determined the midpoint concentrations of IFABP-Px and-MPx to be 1.13±0.14 and 1.22±0.13 M GdnHCl, respectively (Fig. 3). These data demonstrate that neither the pyridoxamine group nor the cationic nitrogen tethered



Scheme 2. Synthesis of the cationic pyridoxamine conjugation reagent reagent **9** (MPx).

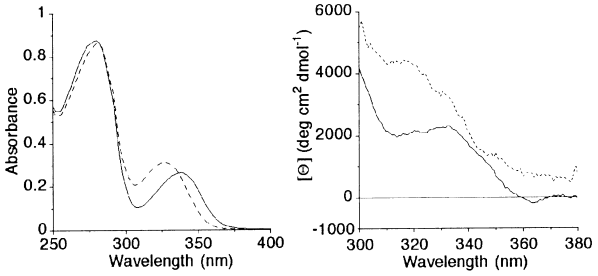
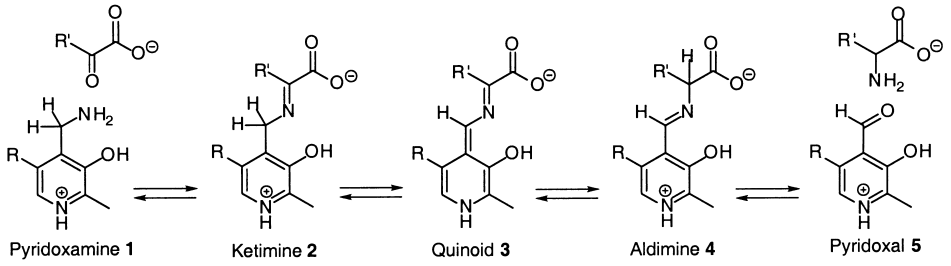


Figure 2. UV and CD spectra of IFABP-MPx (solid) and IFABP-Px (dashed). The spectra were taken in 20 mM HEPES pH 7.5 with 50 μM (UV) or 20 μM (CD) conjugate.



Scheme 1. Reaction mechanism of the pyridoxamine-catalyzed transamination.

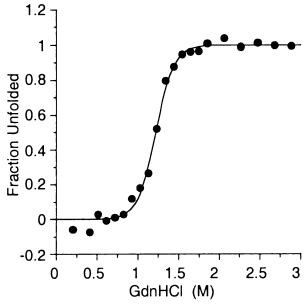


Figure 3. Guanidinium hydrochloride-induced equilibrium unfolding of IFABP-MPx monitored by tryptophan fluorescence (Ex. 280 nm, Em. 338 nm). Samples contained protein (1.0 μM), Tris·HCl (50 mM, pH 7.4), EDTA (0.1 mM) and guanidinium chloride (0–5.0 M). Data were fitted to a two state model, previously used for lipid binding proteins.[14]

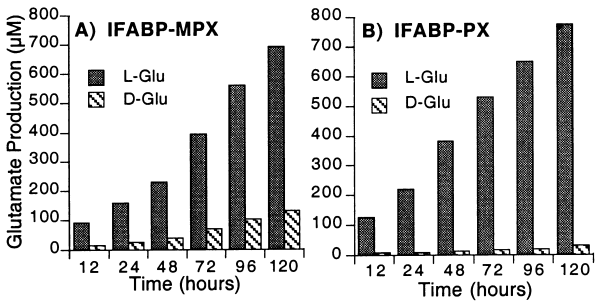


Figure 4. Comparison of glutamic acid production in the catalytic reaction performed by IFABP–MPx (A) or IFABP-Px (B) using tyrosine as amino acid. After 120 h, 16.5 turnovers (70% ee) for the MPx-conjugate and 16 turnovers (92% ee) for the Px-conjugate were achieved. Reactions were performed with 50 μM catalyst, 50 mM α-keto glutarate and 5 mM tyrosine in 0.2 M HEPES pH 7.71 at 37 °C.

inside the hydrophobic protein cavity have a destabilizing effect on the protein.

When IFABP-MPx was incubated at 37 °C with α-ketoglutarate and phenylalanine or tyrosine, respectively, catalytic transamination was observed. Table 1 lists the number of turnovers found and the enantiomeric excess of the L-glutamate produced in 24 h. Inspection of the values given in Table 1 indicates that the overall transamination rates exhibited by IFABP-Px and IFABP-MPx are similar while the enantioselectivity of the latter is significantly lower. Although the above results were obtained after 24 h of incubation, it should be noted that these constructs are active for at least five days under these reaction conditions; after 120 h approximately 16 turnovers occur for each catalyst (Fig. 4). To examine the kinetics of these reactions in more detail, the initial rates were determined by analyzing the amount of glutamate formed after 6 h of reaction (Table 1). After this short time less than one turnover is observed and thus these kinetic parameters reflect events occuring only in the first half reaction (cf. Scheme 1 from left to right). In comparison to the IFABP-Px, the *N*-methylated conjugate showed higher values (1.1- to 3.8-fold) for K_m', whereas the k_{cat}' values were similar for both catalysts.

The greater electron withdrawal of the quaternized pyridine nitrogen was expected to facilitate the deprotonation of ketimine **2** in the benzylic position (**2**→**3**) as well as the deprotonation of aldimine **4** at the C_α of the amino acid (**4**→**3**). However, the kinetic data for the pyridoxamine to pyridoxal half reaction showed that *N*-methylation had no positive effects on the reaction rate. This suggests that the deprotonation of **2** or **4** are not the rate limiting steps in the IFABP-Px catalyzed transaminations. To examine the possibility that proton transfer reactions are rate determining in the reaction catalyzed by IFABP-Px, kinetic isotope effect experiments (KIE) were undertaken (Table 2).

First, it should be noted that the transamination of α-ketoglutarate and phenylalanine to glutamic acid and phenylpyruvate is essentially linear for 100 h at which point at least 10 turnovers have occured. If the second half reaction (pyridoxal to pyridoxamine) was rate determining, the reaction rate would decrease after one turnover. Thus it appears that the pyridoxal to pyridoxamine reaction is not rate determining using these substrates. However, in reactions using α-ketoglutarate and alanine as substrates, the reaction rate is clearly biphasic with a fast phase in the first turnover followed by a slower phase in subsequent turnovers. These latter results illustrate that the pyridoxal to pyridoxamine reaction can be rendered to be rate determining by using a poor substrate.[16] KIE measurements using [$^2H_8$]-phenylalanine give a V_H/V_D of unity. Since an isotope effect would only be observed with this substrate if the pyridoxal to pyridoxamine reaction was rate determining, the absence of a KIE is consistant with the above assertion that the pyridoxamine to pyridoxal reaction is rate determining when α-ketoglutarate and phenylalanine are used as substrates. Interestingly, experiments using [2-^{2}H]-alanine showed no significant KIE, suggesting that although the pyridoxal to pyridoxamine reaction may be rate determining using α-ketoglutarate

Table 1. Kinetic parameters of the transamination reactions catalyzed by IFABP-Px and-MPx[a]

		Turnover/ee[b]	K_m' mM	k_{cat}' h^{-1}	k_{cat}'/K_m' h^{-1} mM^{-1}
IFABP-Px	Phe	3.9/93%	1.8±0.5	0.29±0.02	0.16
	Tyr	4.2/93%	4.2±0.9	0.26±0.02	0.06
IFABP-MPx	Phe	5.8/41%	6.8±1.3	0.23±0.01	0.03
	Tyr	1.7/60%	4.5±1.3	0.17±0.01	0.04

[a]The reactions were performed with 50 μM IFABP conjugate, varying concentrations of α-ketoglutarate and 5 mM amino acid in 0.2 M HEPES pH 7.5 at 37 °C (100 μL total volume). The formation of glutarate was monitored by HPLC.[10] The reactions showed saturation kinetics and the data points were fitted to a standard Michaelis–Menten equation.
[b]The reactions were stopped after 24 h and the production of glutarate analyzed by HPLC. The enantiomeric excess is that of L-glutarate.

Table 2. Primary kintic isotope effects of the transamination reaction catalyzed by IFABP-Px[a]

Substrate/Solvent		IFABP-Px		IFABP-MPx	
		Rate μM/h	V_H/V_D	Rate μM/h	V_H/V_D
Phenylalanine		13.1±1.5		13.6±0.7	
[$^2H_8$]-Phenylalanine		12.8±1.1	1.0±0.1	8.0±1.1	1.7±0.3
Alanine	First phase	5.3±0.5		1.9±0.1	
	Second phase	1.5±0.2		1.3±0.1	
[2-^{2}H]-Alanine	First phase	4.3±0.5	1.2±0.2	1.5±0.2	1.3±0.2
	Second phase	1.6±0.2	0.94±0.2	1.1±0.1	1.2±0.1

[a]The reactions were performed with 50 μM IFABP-Px, 50 mM α-ketoglutarate and 5 mM amino acid in 0.2 M HEPES pH 7.5 at 37 °C (100 μL total volume). The formation of glutarate was monitored by HPLC.[10]

and alanine as substrates, the α-deprotonation step (that is only one of several steps in the conversion between pyridoxal and pyridoxamine) is not the rate determining process in that reaction sequence. For comparison, native aspartate aminotransferse from *E. coli* showed a KIE of ~2 with [2-^{2}H]-aspartate as substrate, suggesting that deprotonation of **4** to **3** is at least partially rate determining.[17,18]

Reactions using α-ketoglutarate and phenylalanine were performed in D_2O to measure the solvent KIE. Since, after several turnovers, deuterium will become incorporated in the methylene position of pyridoxamine, rate measurements in D_2O can provide information about whether deprotonation of the ketimine in the pyridoxamine to pyridoxal direction is rate determining. Analysis of the progress curve for the reaction in D_2O showed biphasic behavior, starting with a fast phase in the first 24–48 h followed by a slower phase in the subsequent 48 h. Comparison of these rates with those measured in H_2O reveal no significant KIE in the first phase ($V_H/V_D = 0.91$) and a modest KIE ($V_H/V_D = 1.7$) in the slower phase; the small magnitude of this value suggests that deprotonation of the ketimine is not substantially rate determining. Taken together, these KIE experiments performed on IFABP-Px suggest that a step distinct from deprotonation of the ketimine in the pyridoxamine to pyridoxal reaction is what limits the rate of the artificial transaminase IFABP-Px. Given these results, it is perhaps not surprising that the preparation of IFABP-MPx did not result in an increase in catalytic efficiency. However, several experiments with IFABP-MPx suggest that the modified MPx cofactor may significantly rearrange the energetics in these transamaination reactions. KIE measurements using [$^2H_8$]-phenylalanine with IFABP-MPx give a V_H/V_D of 1.7. This value is significantly greater than that observed with IFABP-Px and approaches the value observed with aspartate aminotransferase suggesting that the modified cofactor may not be facilitating α-deprotonation of the aldimine as it was designed to do. Moreover, the decrease in enantioselectivity in the IFABP-MPx catalyzed reaction suggests that the modified cofactor is either altering the conformation of the quinoid intermediate (resulting in less facial selectivity) or lowering the barrier for racemization (as might occur if the acidity of the benzylic protons in the ketimine intermediate increased).

In conclusion, a new cationic pyridoxamine conjugation reagent MPx **9** was synthesized and linked to a protein via a cysteine residue previously introduced by site-directed mutagenesis. Although the cofactor contains a permanent positive charge, the protein was not destabilized. The *N*-methylated pyridoxamine moiety offered no catalytic advantage in the transamination reactions of the IFABP-MPx compared to the nonmethylated analogue. This result is consistant with subsequent KIE experiments that indicate that proton transfer steps are not rate determining in the case of this particular artificial enzyme. However, given that proton transfer events are frequently rate determining in natural transaminases,[17,18] it is likely that the MPx reagent will be useful in future experiments as the catalytic efficiency of these semisynthetic enzymes is increased through additional cycles of design.

Acknowledgements

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References and Notes

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11. Triacetylpyridoxamine (100 mg) was dissolved in 20 mL hot benzene and 1 mL methyl iodine added. After 24 h of reflux, the crude product **7** was purified with a Sep-Pak C18 column (42% yield). ^{1}H NMR (300 MHz, D_2O) δ 7.50 (s, 1H), 4.26 (s, 2H), 4.03 (s, 2H), 3.89 (s, 3H), 2.36 (s, 3H), 2.23 (s, 3H), 1.84 (s, 3H).

12. The *N*-methylated compound **7** was hydrolyzed in 40 mL of 48% aq HBr under reflux for 5 h. Excess water and HBr were removed in vacuo. The resulting thiol **8** is instable and was immediately dissolved in 6 mL degassed methanol. 2,2′-Dipyridyl disulfide (Aldrithiol-2; 500 mg in 15 mL methanol) was added in 60 min under a nitrogen atmosphere at room temperature. The solvent was removed, the yellow solid washed with chloroform and dissolved in 2 mL water:acetonitrile (1:1). 1-Methyl-5-(2-pyridyltdithio)pyridoxamine **9** was purified by RP-HPLC on a C18 column (solvent A: water: acetonitrile:TFA 94.8:5:0.2; solvent B: acetonitrile:TFA 99.8: 0.2) with an elution profile of 10 min isocratic 100% A, followed by a 50 min linear gradient to 40% B. Fractions of **9** were collected at 33 min, the solvent removed in vacuo and the final conjugation reagent stored at −80 °C (45% yield). ^{1}H NMR (300 MHz, D_2O) δ 8.14 (d, 1H, $J=5.1$), 7.93 (s, 1H), 7.61 (dt, 1H, $J=7.7$, 1.3), 7.37 (d, 1H, $J=8.1$), 7.13 (dd, 1H, $J=7.5$, 5.1), 4.28 (s, 2H), 4.10 (s, 2H), 3.89 (s, 3H), 2.30 (s, 3H). UV (20 mM HEPES pH 7.5): $\varepsilon_{338}=6210\ M^{-1}\,cm^{-1}$.

13. Gel permeation chromatography was performed on a FPLC system with a Superose 12 HR 30 column. Samples (100 μL) were eluted with 25 mM HEPES pH 7.5 and 150 mM NaCl at a flow rate of 0.5 mL/min. The column was calibrated with bovine lung aprotinin (6.5 kDa), horse heart cytochrome c (12.4 kDa), bovine erythrocyte carbonic anhydrase (29.0 kDa) and bovine serum albumin (66.0 kDa).
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