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EFFECTS OF METAL IONS ON THE RATES AND ENANTIOSELECTIVITIES OF REACTIONS CATALYZED BY A SERIES OF SEMISYNTHETIC TRANSAMINASES CREATED BY 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 and that the rate, enantioselectivity and substrate specificity of these reactions could be altered by site directed mutagenesis. Herein, we show that the chemistry performed by these conjugates can be extended to include catalytic transamination and describe the effects of added metal ions on reaction rate and enantioselectivity. We conclude that metal ions can be used to increase the rate of reactions catalyzed by semisynthetic transaminases; however, the addition of metal ions can also retard the reaction rate. Furthermore, it appears that the presence of metal ions almost always results in an erosion of reaction enantioselectivity. This limits their utility as a practical means of increasing reaction rate. The results reported here, for four independent systems, should be considered in future designs of artificial transaminases.

The design of efficient and highly selective catalysts is important for a range of chemical applications.[1] Semisynthesis, which consist of altering an existing protein by chemical modification, is a powerful approach for creating new catalysts with useful properties.[2] This strategy for catalyst design combines elements of host–guest chemistry with a highly flexible protein scaffold that can be manipulated by both chemical modification and recombinant DNA methods. Fatty acid binding proteins are attractive starting points for catalyst preparation because they contain a large 600 $Å^3$ cavity that effectively sequesters bound molecules within a cavity from bulk solvent.[3] Attachment of catalytic groups to a cysteine residue (Cys117) within the cavity affords a chiral environment for a reaction to occur. In earlier work, we demonstrated that a fatty acid binding protein, ALBP (Adipocyte Lipid Binding Protein, 131 residues), could be chemically modified with catalytic groups to prepare constructs that performed enantioselective reactions.[4] One of these conjugates was equipped with a pyridoxamine cofactor (ALBP-PX) that performed enantioselective reductive amination reactions while a second type of construct, appended with a metal binding ligand (ALBP-Phen), catalyzed enantioselective ester hydrolysis reactions. The structures of both of these semisynthetic enzymes have been solved by X-ray diffraction.[5] Subsequently, site directed mutagenesis was employed to vary the location of the cysteine residue used for cofactor attachment.[6] Using this strategy, a pyridoxamine moiety was positioned at three locations in a closely related protein, IFABP (Intestinal Fatty Acid Binding Protein), including residues 60, 72, and 104 to prepare the conjugates IFABP-PX60, IFABP-PX72, and IFABP-PX104, respectively. Each of these mutant systems manifested a different pattern of reactivity and specificity indicating that the properties of these semisynthetic constructs could be tuned by site directed mutagenesis. Although the above conjugates performed reactions with excellent enantioselectivity in some cases, these results were all obtained under single turnover conditions. One mutant conjugate, IFABP-PX60 was evaluated under catalytic conditions and was found to promote as many as 50 turnovers with a selectivity of 95% *ee*.[7] These results prompted us to evaluate the other mutant constructs under catalytic conditions. We were also interested in studying the effects of added metal ions since the addition

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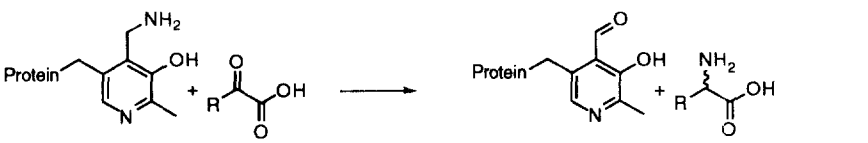
of such agents is known to increase the rate of transamination in many model systems.[8] The results of these experiments are described here.

The constructs used in this study were prepared by expressing the required mutant proteins in *E. coli* and purification by ion exchange and gel filtration chromatography followed by conjugation as previously described.[4a,6,9] As noted above, these conjugates had previously been evaluated in single turnover experiments as shown below in Figure 1. ALBP-PX and IFABP-PX60 promoted reductive amination of α-keto glutarate (**1a**) to Glu (**2a**) in 84% *ee* and 68% *ee*, respectively, while IFABP-PX72 and IFABP-PX104 effected a similar reaction with *p*-hydroxyphenylpyruvate (**1b**) to produce Tyr (**2b**) in 4.0% *ee* and 74% *ee*, respectively.

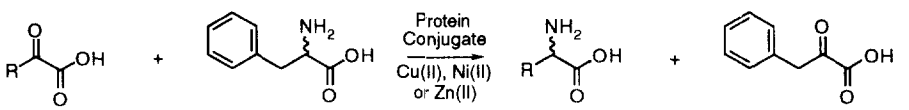


1a R = $HO_2C(CH_2)_2$
1b R = $HOC_6H_4CH_2$

2a R = $HO_2C(CH_2)_2$
2b R = $HOC_6H_4CH_2$

Figure 1. Single turnover reductive amination reactions showing the α-keto acid substrates and α-amino acid products studied with the protein conjugates. Reactions contained 50 μM conjugate, 50 mM α-keto acid (**1a** for ALBP-PX and IFABP-PX60, **1b** for IFABP-PX72 and IFABP-PX104), and 200 mM pH 7.0 buffer (HEPES or imidazole) incubated in an oven at 37 °C. The amount and enantioselectivity of product formation (**2a** and **2b**) was determined by derivatization of an aliquot from the crude reaction mixture with *o*-diphthalaldehyde and *N*-acetyl cysteine to produce diastereomeric isoindole derivatives that were then separated by reversed-phase HPLC and quantitated by fluorescence detection.[10] Single turnover reactions that were monitored by UV/Vis spectroscopy were performed at 25 °C.

The conjugates were then evaluated for their ability to perform catalytic transamination reactions using the same α-keto acid substrates described above. These reactions are shown below in Figure 2.



1a R = $HO_2C(CH_2)_2$
1b R = $HOC_6H_4CH_2$

3

2a R = $HO_2C(CH_2)_2$
2b R = $HOC_6H_4CH_2$

4

Figure 2. Catalytic transamination reactions showing the α-keto acid substrates and α-amino acid products studied with protein conjugates. Catalytic reactions were performed as described for the single turnover reactions except that Phe (**3**) was added at 5.0 mM. The reactions were monitored for 120 h and the rates were generally linear in the first 24–48 h (except for free pyridoxamine in HEPES which gives variable data due to precipitation problems) in this period of time.

The rates and enantioselectivities of these reactions are summarized in Table 1. Inspection of these data reveals several important trends. First, most conjugates were able to catalyze transamination in HEPES buffer with IFABP-PX60 giving the greatest conversion (IFABP-PX60: 17 turnovers in 120 h; IFABP-PX72: 8.3 turnovers in 120 h; ALBP-PX: 5.5 turnovers in 120 h). Second, reactions were always faster in HEPES than in imidazole. These results suggest that buffer molecules may be present in the cavity and participate directly in the reaction perhaps as general acids or bases; it should be noted that fatty acid binding proteins can accomodate a

number of ordered water molecules within the cavity even in the presence of bound fatty acids.[3] Finally, the enantioselectivities of reactions under catalytic conditions are generally higher than under single turnover conditions. The enantioselectivities of all catalytic reactions was greater than 85% *ee* (13:1 selectivity). Thus, it appears that these protein conjugates have some racemase activity in the absence of added amino acid substrate.

Table 1. Rates and Enantioselectivities Obtained with Protein Conjugates under Catalytic Conditions.

	0.20 M HEPES, pH 7.0			0.20 M Imidazole, pH 7.0		
	Rate ($\mu M \cdot h^{-1}$)	L/D Ratio	*ee* (%)	Rate ($\mu M \cdot h^{-1}$)	L/D Ratio	*ee* (%)
	Pyridoxamine			Pyridoxamine		
No Metal	0.40 ± 0.14	-	-	1.0 ± 0.50	-	-
Cu(II)	3.1 ± 0.71	-	-	4.7 ± 0.12	-	-
	ALBP-PX			ALBP-PX		
No Metal	2.3 ± 0.14	15 ± 1.7	87	0.44 ± 0.06	6.1 ± 1.1	72
Cu(II)	2.5 ± 0.26	3.7 ± 0.47	58	0.38 ± 0.06	5.4 ± 0.88	69
Ni(II)	3.5 ± 0.25	6.7 ± 0.74	74	0.35 ± 0.08	4.9 ± 1.5	66
Zn(II)	2.9 ± 0.86	2.7 ± 0.09	46	0.36 ± 0.04	5.5 ± 1.5	69
	IFABP-PX60			IFABP-PX60		
No Metal	14 ± 1.0	90 ± 11	98	4.3 ± 0.54	26 ± 3.9	93
Cu(II)	3.3 ± 0.7	5.2 ± 0.30	68	5.9 ± 1.1	2.9 ± 0.26	49
Ni(II)	6.2 ± 0.5	16 ± 1.8	89	4.7 ± 0.55	6.0 ± 0.27	71
Zn(II)	10 ± 0.3	45 ± 15	97	5.2 ± 0.28	9.4 ± 0.85	81
	IFABP-PX72			IFABP-PX72		
No Metal	9.3 ± 0.5	22 ± 6.2	91	0.38 ± 0.11	15 ± 0.81	88
Cu(II)	6.1 ± 0.3	39 ± 11	95	0.45 ± 0.08	2.6 ± 0.16	44
Ni(II)	2.6 ± 0.2	3.0 ± 0.83	50	0.35 ± 0.05	3.7 ± 1.1	57
Zn(II)	0.53 ± 0.09	3.5 ± 1.0	55	0.53 ± 0.03	5.3 ± 0.73	68
	IFABP-PX104			IFABP-PX104		
No Metal	2.3 ± 0.17	13 ± 0.99	85	0.59 ± 0.21	1.9 ± 0.30	31
Cu(II)	9.5 ± 0.28	3.1 ± 1.26	52	0.66 ± 0.12	4.6 ± 0.21	65
Ni(II)	2.8 ± 0.17	8.8 ± 1.7	80	0.91 ± 0.42	2.1 ± 0.13	35
Zn(II)	3.1 ± 0.17	4.5 ± 0.36	64	1.5 ± 0.51	2.1 ± 0.49	35

Although all of the conjugates performed catalytic transamination reactions at rates that frequently exceeded that of free pyridoxamine, the absolute rates and extents of conversion were still relatively low. In an attempt to increase the reaction rates, we decided to examine the effects of divalent metal ions on reactions catalyzed by the protein conjugates. It is well established that in many pyridoxamine model systems, metal ions can accelerate the reaction rate. A mechanism for the reaction between pyridoxamine and an α-keto acid is shown in Figure 3. Addition of metal ions to such reactions results in the formation of the Schiff base metal complexes shown; rate acceleration occurs either by shifting the position of equilibrium to increase the concentration of the ketimine intermediate (step 1) or by increasing the acidity of the ketimine benzylic protons and thereby facilitating deprotonation (step 2).

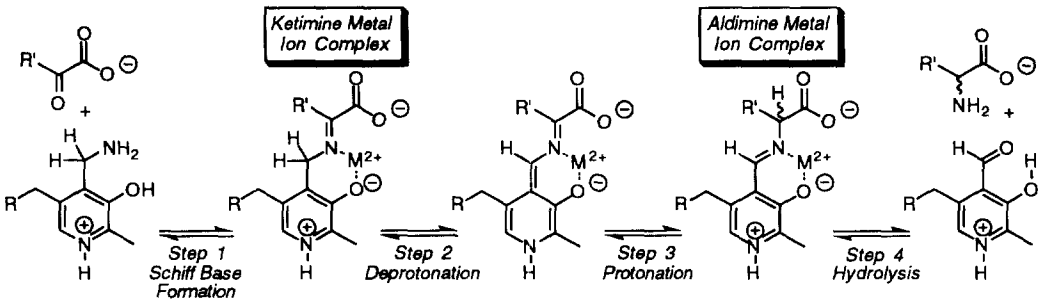


Figure 3. Mechanism for reductive amination reaction between pyridoxamine and an α-keto acid.

Reactions using the protein conjugates were performed under catalytic conditions (HEPES or imidazole) in the presence of 50 μM $CuSO_4$, $NiSO_4$ or $ZnSO_4$; the rates and enantioselectivities for these reactions are tabulated in Table 1. Interestingly, a range of effects were observed. Addition of Cu(II), Ni(II) or Zn(II) to reactions containing IFABP-PX60 or IFABP-PX72 resulted in a decrease in reaction rate when compared to reactions performed in the absence of metal ions. IFABP-PX60 reacted the slowest in the presence of Cu(II) (4.4–fold less than in the absence of metal ion) while IFABP-PX72 reacted the slowest in the presence of Zn(II) (18–fold less). In contrast, IFABP-PX104 reacted faster in the presence of all metal ions. Of particular note, this protein reacted 4.7-fold faster in the presence of Cu(II). Metal ions had little effect on reactions catalyzed by ALBP-PX; the presence of Ni(II) caused only a 1.5-fold increase in reaction rate. In general, the addition of metal ions caused a decrease in the reaction enantioselectivity. For IFABP-PX60, the selectivity dropped from 98% *ee* to 68% *ee* in the presence of Cu(II) while for IFABP-PX72, the selectivity in the presence of Zn(II) dropped from 91% *ee* to 55 % *ee*. Although catalysis by IFABP-PX104 was accelerated by the addition of Cu(II), the enantioselectivty decreased from 85% *ee* to 52% *ee*. Even in the case of ALBP-PX, where little effect on the rate was observed in the presence of metal ions, a decrease in enantioselectivity was obtained; addition of Zn(II) to ALBP-PX decreased the reaction enantioselectivity from 87% *ee* to 46% *ee*. Reactions performed in imidazole buffer showed fewer effects in the presence of Cu(II), Ni(II) or Zn(II). Addition of metal ions to IFABP-PX60 or IFABP-PX72 resulted in little effect on the reaction rate but a general decrease in enantioselectivity. Reactions with IFABP-PX104 gave more interesting results. Addition of Cu(II) to IFABP-PX104 had little effect on the reaction rate but caused a significant increase in enantioselectivity from 31% *ee* to 65% *ee*. In contrast, the addition of Zn(II) to this protein resulted a 2.6-fold increase in reaction rate but little change in enantioselectivity. No significant effects were observed with ALBP-PX.

Given that the above experiments only measured the effect of added metal ions on reaction rate, we felt that it was important to confirm that the metal ions were actually binding to the pyridoxamine cofactor and not to some other position on the protein. Although none of the conjugates contain any His or free Cys residues, it was still possible that the added metal ions were binding to other residues resulting in the observed changes in catalytic properties. To examine the binding of metal ions to the pyridoxamine moiety attached to the different conjugates, difference UV/vis spectroscopy was employed. The spectroscopic properties of pyridoxamine, pyridoxal and the various Schiff base complexes between these cofactors and α-keto acids and amino acids have been extensively characterized in model studies.[11] The aldimine-metal ion complex is particularly useful for spectroscopic studies

because the absorption maximum of this species is typically centered near 400 nm which is in a region devoid of other complicating features. Thus the detection of absorbance near 400 nm provides good evidence for the existence of an aldimine-metal ion complex. Figure 4A shows UV/vis difference spectra obtained at various times from a reaction performed with free pyridoxamine and **1b** in the presence of Cu(II); the aldimine-metal ion complex is prominent in these spectra. Figure 4B shows UV/vis difference spectra obtained for the reaction between IFABP-PX104 and **1b** in the presence of Cu(II). These spectra clearly indicate the formation of an aldimine-metal ion complex. It should also be noted that that the absolute magnitude of absorbance at 400 nm in these two cases is comparable. Thus, the increase in rate observed with IFABP-PX104 in the presence of Cu(II) discussed above is likely to be due to the formation of cofactor-metal ion complexes of the type shown in Figure 3. In contrast, Figure 4C shows UV/vis difference spectra obtained under similar conditions using **1b** as a substrate for IFABP-PX72. No evidence for an aldimine-metal ion complex can be seen in these spectra. This is consistent with the fact that neither the rate nor the enantioselectivity of reactions catalyzed by this conjugate are significantly perturbed by the addition of Cu(II). Figure 4D shows UV/vis difference spectra obtained from reactions performed with free pyridoxamine and **1a** in the absence and presence of Cu(II). Figures 4E and 4F show UV/vis difference spectra obtained under similar conditions with ALBP-PX and IFABP-PX60, respectively. These spectra all indicate that at least some aldimine-metal ion complex is formed suggesting that the effects observed on the reaction rates and enantioselectivities with these constructs resulted from the formation of cofactor-metal ion complexes. Finally, it is interesting to note that variation of the reaction buffer can alter a conjugate's metal binding properties. UV/vis difference spectra of IFABP-PX104 in the presence of Cu(II) obtained in imidazole buffer show no aldimine-metal ion complex is present (data not shown).

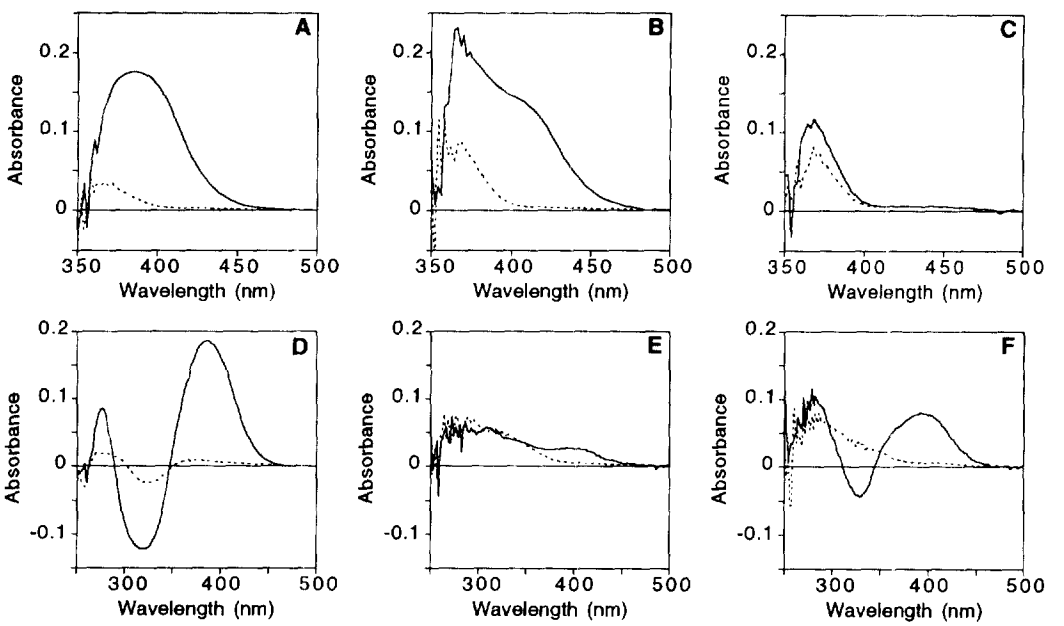


Figure 4. UV/vis difference spectra from reductive amination reactions performed under single turnover conditions. Spectra were obtained after 60 min of reaction in the presence (——) and absence (• • •) of Cu(II). (A) Pyridoxamine and **1b**. (B) IFABP-PX104 and **1b**. (C) IFABP-PX72 and **1b**. (D) Pyridoxamine and **1a**. (E) ALBP-PX and **1a**. (F) IFABP-PX60 and **1a**.

From these studies, we conclude that metal ions can be used to increase the rate of reactions catalyzed by semisynthetic transaminases; a 4.7-fold increase was observed with IFABP-PX104 in the presence of Cu(II). However, the addition of metal ions can also retard the reaction rate. A 18-fold decrease was observed with IFABP-PX72 in the presence of Zn(II). A 2.5-fold rate decrease upon the addition of Cu(II) for a protein/pyridoxamine system was also noted by Imperiali and Roy.[21] Perhaps this lower rate of reaction occurs as the result of overstabilization of the aldimine intermediate and a net deceleration of hydrolysis of this species. Furthermore, it appears that the presence of metal ions almost always results in an erosion of reaction enantioselectivity. This limits their utility as a practical means of increasing reaction rate. The results reported here, for four independent systems, illustrate some of the problems of extrapolating trends obtained in simple model systems to more complex protein-based constructs and should be considered in future designs of artificial transaminases.

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