Catalytic Enantioselective Reductive Amination in a Host−Guest System Based on a Protein Cavity

Hao Kuang and Mark D. Distefano*

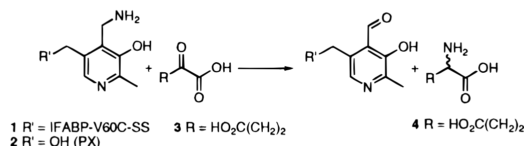
Department of Chemistry, University of Minnesota Minneapolis, Minnesota 55455

Received August 8, 1997

Enzymes are highly efficient catalysts. As a result, many strategies have been employed to design systems that possess similar properties.[1] Proteins are attractive scaffolds for the design of new catalysts because their size allows the formation of a large number of interactions between substrate and catalyst.[2] Additionally, recombinant DNA methods allow protein-based catalysts to be modified in a facile manner either by site-directed mutagenesis or by selection approaches.[3] Fatty acid binding proteins are a structurally unique family of proteins composed of two orthogonal β-sheets and a α-helical region.[4] These secondary structural elements fold into a tertiary structure that forms a 600 Å^3 cavity which completely sequesters the fatty acids bound within. In earlier work, we described the preparation of ALBP-PX, a construct based on adipocyte lipid binding protein that contained a pyridoxamine cofactor covalently attached to a cysteine residue within the cavity.[5] This conjugate reductively aminated several α-keto acids to α-amino acids under single turnover conditions with excellent enantioselectivities in some cases. However, these reactions proceeded at rates comparable to those utilizing free pyridoxamine indicating that the protein scaffold was involved only in controlling reaction selectivity. To improve this system, we decided to attach the pyridoxamine moiety to different positions within the cavity in order to juxtapose the cofactor with arrays of functional groups present elsewhere within the cavity. Here, we describe the properties of IFABP-PX60, a catalyst based on a mutant form of intestinal fatty acid binding protein (IFABP), a protein structurally related to ALBP.[6] This new conjugate reductively aminates α-keto glutarate to glutamic acid with a catalytic efficiency at least 200-fold greater than that of free pyridoxamine. The reaction is catalytic and enantioselective; as many as 50 turnovers with an enantiomeric purity of 95% ee (enantiomeric excess) have been obtained.

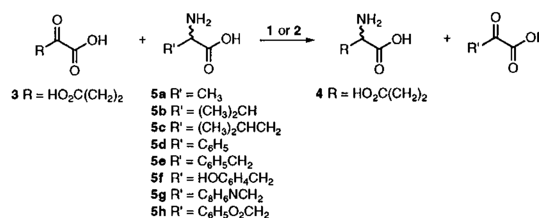
In our earlier work with ALBP-PX, Cys_{117} was used as the site of pyridoxamine attachment. This residue is located near the C-terminus of the protein and is proximal to the region where the carboxylate group of the fatty acid ligand binds. To explore other regions of the protein cavity, we focused on a different fatty acid binding protein, IFABP, that, unlike ALBP, contains no cysteine residues. Thus, new cysteines can be easily introduced into the cavity, and hence, a number of cysteine mutants of IFABP have been prepared.[7] The mutant V60C was particularly interesting to us because it allowed the pyridoxamine moiety to be introduced deeper in the cavity than was possible with ALBP.[8] This region of the protein is rich in aromatic residues including one tryptophan, three tyrosines, and one phenylalanine as well as two arginines.[9] Modeling experiments suggested that these residues might form specific contacts with keto acid or amino acid substrates.

IFABP-PX60 (**1**, a construct incorporating a pyridoxamine moiety at poition 60 of IFABP-V60C) was prepared in a similar manner as previously described for ALBP-PX.[5a] Following characterization of the conjugate, the rate of reductive amination reactions using IFABP-PX60 (50 μM) and α-keto glutarate (**3**, 50 mM) was studied under single turnover conditions at pH 7.5 and 37 °C; this transformation is shown below.[10] Initial results



after 24 h of reaction showed the formation of 1 equiv of glutamic acid (**4**) indicating that the reaction was complete; this was quite different from results obtained in similar experiments performed with ALBP-PX in which only 46% conversion was observed after 24 h. Clearly, the new conjugate reacts at a more rapid rate.[11] The kinetics of this reaction were next analyzed for both the IFABP-PX60 protein as well as for free pyridoxamine (**2**). The results of these experiments are shown in Figure 1. Analysis of these data using a first-order kinetic model gave a k_{obsd} of 0.18 h^{-1} for the protein and 0.0029 h^{-1} for pyridoxamine indicating a 62-fold increase in rate for IFABP-PX60 relative to the free coenzyme under the same conditions. It should be noted that, under these single turnover conditions, IFABP-PX60 produced a 68% ee of L-glutamic acid.

In view of the rapid rate obtained under single turnover conditions, we next examined the ability of the conjugate to perform this reaction under catalytic conditions in which the pyridoxamine cofactor is regenerated by the addition of a second amino acid that serves as an amine source as shown below. The



(1) Schultz, P. G. *Science* **1988**, *240*, 426.
(2) (a) Kaiser, E. T.; Lawrence, D. S. *Science* **1984**, *226*, 505. (b) Levine, H. L.; Nakagawa, Y.; Kaiser, E. T. *Biochem. Biophys. Res. Commun.* **1977**, *76*, 64. (c) Corey, D. R.; Schultz, P. G. *Science* **1987**, *238*, 1401. (d) Polgar, L.; Bender, M. L. *J. Am. Chem. Soc.* **1966**, *88*, 3153. (e) Neet, K. E.; Koshland, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *56*, 1606. (f) Wu, Z. P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513. (g) Planas, A.; Kirsch, J. F. *Biochemistry* **1991**, *30*, 8268. (h) Wuttke, D. S.; Gray, H. B.; Fisher, S. L.; Imperiali, B. *J. Am. Chem. Soc.* **1993**, *115*, 8455. (i) Imperiali, B.; Roy, R. S. *J. Am. Chem. Soc.* **1994**, *116*, 12083. (j) Suckling, C. J.; Zhu, L. M. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 531. (k) Germanas, J. P.; Kaiser, E. T. *Biopolymers* **1990**, *29*, 39. (l) Bell, I. M.; Hilvert, D. *New Biocatalysts via Chemical Modification*; Bell, I. M., Hilvert, D., Eds.; John Wiley & Sons Ltd.: Chichester, 1994; p 73.
(3) (a) Knowles, J. R. *Science* **1987**, *236*, 1252−1256. (b) Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. Science **1990**, *249*, 404−406.
(4) Banaszak, L.; Winter, N.; Xu, Z.; Bernlohr, D. A.; Cowan, S.; Jones, T. A. *Adv. Protein Chem.* **1994**, *45*, 89.
(5) (a) Kuang, H.; Brown, M. L.; Davies, R. R.; Young, E. C.; Distefano, M. D. *J. Am. Chem. Soc.* **1996**, *118*, 10702. (b) Davies, R. R.; Distefano, M. D. *J. Am. Chem. Soc.* **1997**, *119*, 11643.
(6) Lowe, J. B.; Sacchettini, J. C.; Laposata, M.; McQuillan, J. J.; Gordon, J. I. *J. Biol. Chem.* **1987**, *262*, 5931.
(7) Jiang, N.; Frieden, C. *Biochemistry* **1993**, *32*, 11015. (b) Frieden, C.; Jiang, N.; Cistola, D. P. *Biochemistry* **1995**, *34*, 2724.
(8) Kuang, H.; Davies, R. R.; Distefano, M. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2055.
(9) Sacchettini, J. C.; Gordon, J. I.; Banaszak, L. J. *J. Mol. Biol.* **1989**, *208*, 327−339.
(10) For HPLC analysis methods, see: (a) Nimura, N.; Kinoshita, T. *J. Chromatogr.* **1986**, *352*, 169. (b) Buck, R. H.; Krummen, K. *J. Chromatogr.* **1987**, *387*, 255. (c) Koh, J. T.; Breslow, R. *J. Am. Chem. Soc.* **1994**, *116*, 11234.
(11) Incubation of IFABP-V60C under similar conditions produced no glutamate product. Addition of 1.0 mM EDTA has no effect on the rate of reaction with IFABP-PX60 suggesting that rate enhancement via metal ion catalysis is not occurring. Addition of equimolar Cu(II), Ni(II), or Zn(II) actually reduces the rate and ee (although these ions accelerate reactions containing free pyridoxamine).

S0002-7863(97)02771-6 CCC: $15.00 © 1998 American Chemical Society
Published on Web 01/22/1998

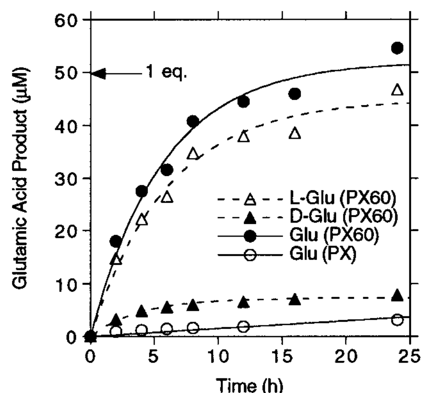


Figure 1. Progress curves for glutamate production promoted by IFABP-PX60 and pyridoxamine under single turnover conditions. Reactions were performed using 50 μM IFABP-PX60 or pyridoxamine and 50 mM α-keto glutarate, in 20 mM HEPES, pH 7.5, at 37 °C. At selected intervals, samples were withdrawn and the amount of glutamate was determined by derivatization and HPLC analysis. Glutamate production from IFABP-PX60 (●) and pyridoxamine (○). Also indicated are the production of L-glutamate (△) and D-glutamate (▲) by IFABP-PX60. Glutamate produced by pyridoxamine is racemic. Curves shown were fit to the equation $[\mathrm{Glu}]_t = [\mathrm{Glu}]_\infty\{1 - \exp(-k_{\mathrm{obsd}}t)\}$.

Table 1. Production of Glutamate in 24 h Catalyzed by IFABP-PX60 in the Presence of Different Amino Acids

amino acid added	turnovers (n)	L-Glu/D-Glu	ee (%)
none	>0.99	5.3 ± 0.03	68 (L)
Ala (**5a**)	1.3 ± 0.05	5.7 ± 0.27	70 (L)
Val (**5b**)	1.1 ± 0.03	8.5 ± 0.14	79 (L)
Leu (**5c**)	1.6 ± 0.11	9.0 ± 0.60	80 (L)
Pgl (**5d**)	1.1 ± 0.01	5.1 ± 0.12	67 (L)
Phe (**5e**)	3.9 ± 0.15	28 ± 0.10	93 (L)
Tyr (**5f**)	4.2 ± 0.07	28 ± 0.11	93 (L)
D-Tyr (**5f**)	0.94 ± 0.04	4.7 ± 0.18	65 (L)
L-Tyr (**5f**)	3.1 ± 0.09	21 ± 0.24	91 (L)
Trp (**5g**)	2.2 ± 0.04	24 ± 0.16	92 (L)
Dopa (**5h**)	4.3 ± 0.12	32 ± 0.09	94 (L)

results of these experiments are summarized in Table 1. Initial experiments with amino acids bearing alkyl side chains including Ala (**5a**), Val (**5b**), and Leu (**5c**) did produce a small amount of turnover. Addition of Leu produced the highest levels of Glu (1.6 turnovers in 24 h). More significant levels of turnover were obtained using amino acids possessing aromatic side chains. The addition of Phe (**5e**), Tyr (**5f**), Trp (**5g**), and Dopa (**5h**) all gave several turnovers (2.2–4.3) in 24 h. This back reaction appears to be stereospecific, since substitution of L-Tyr with D-Tyr results in no conversion beyond what is observed under single turnover conditions. The enantioselectivities obtained in these catalytic reactions are of particular interest. The use of aromatic amino acids as amine sources gives excellent enantioselectivities. Table 1 shows values ranging from 91 to 94% ee. These results are quite different than the lower selectivities obtained under single turnover conditions. A possible explanation for this behavior is that IFABP-PX60 possesses racemase activity. Under single turnover conditions, the amino acid product (Glu, **4**) can remain bound to the protein where racemization can occur. However, under catalytic conditions, the amino acid used as the amine source can displace the bound amino acid product thus preventing racemization. This model is consistent with the correlation we observe between rate and enantioselectivity. With the alkyl-substituted amino acids, low levels of turnover and low values of ee were obtained; with aromatic amino acids, increased turnover and higher enantioselectivity were observed. Finally, longer incubation times with Phe or Tyr result in continued reaction and many turnovers. We have monitored the IFABP-PX60-catalyzed production of Glu for up to 14 days at which time over 50 turnovers and an ee of 95% were obtained. Clearly, this protein-based catalyst is a stable material.

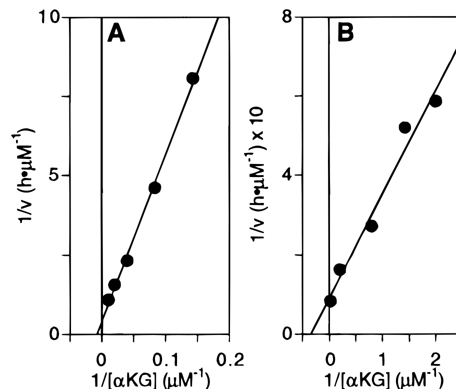


Figure 2. Double reciprocal analysis of the initial rates of glutamate production promoted by IFABP-PX60 and pyridoxamine under catalytic conditions. Reactions were performed using 50 μM IFABP-PX60 or pyridoxamine, 5.0 mM tyrosine, and variable amounts of α-keto glutarate in 200 mM HEPES, pH 7.7, at 37 °C. At selected intervals, samples were withdrawn and the amount of glutamate was determined by derivatization and HPLC analysis. Panel A: Reciprocal of rate versus reciprocal of [α-keto glutarate] for pyridoxamine-catalyzed reaction. Panel B: Reciprocal of rate versus reciprocal of [α-keto glutarate] for IFABP-PX60-catalyzed reaction.

Given the significant increase in rate observed with IFABP-PX60 compared with free PX, we were interested in clarifying the origin of this effect. To accomplish this, the rates of transamination using α-keto glutarate and Tyr were examined at a range of keto acid concentrations and analyzed using a Michaelis–Menten kinetic model. The double reciprocal plots of these data are shown in Figure 2. Analysis of these graphs gave values for K_M and k_{cat} of 150 mM and 0.056 h^{-1} for PX and 2.9 mM and 0.22 h^{-1} for IFABP-PX. Comparison of the k_{cat} values for the protein system versus the free cofactor reveals a modest 3.9-fold increase resulting from performing the reaction in the protein cavity. More interestingly, a similar comparison of the K_M values shows a 52-fold decrease suggesting that the protein conjugate binds α-keto glutarate with much greater affinity than free pyridoxamine.[12] Thus, it appears that the accelerated catalysis observed with IFABP-PX60 occurs primarily due to an increase in substrate binding together with a smaller effect on the maximal rate. Evaluation of the improvement in catalytic efficiency using k_{cat}/K_M as a criterion indicate that IFABP-PX60 is 200-fold more efficient than free PX. The results described here indicate that a host–guest system based on a fatty acid binding protein can be used to generate systems that mimic three key features of enzymatic catalysis: selectivity, rate enhancement, and turnover. Efforts to introduce additional functionality using site-directed mutagenesis to increase the rate of reaction are currently in progress.

Acknowledgment. The authors thank S. Flaim for modeling IFABP-PX60, Dr. C. Frieden for providing *E. coli* MG1655/pIFABP-V60C, M. Brown, C. Lambert, and Dr. R. Davies for assistance with the protein purification, and D. Qi for performing the experiments with metal ions. This work was supported by grants from the National Science Foundation (NSF-CHE-9506793) and the Petroleum Research Fund (PRF#28140-G4).

Supporting Information Available: Descriptions of molecular modeling and the procedures used to prepare, characterize, and study the reactions catalyzed by IFABP-PX60 (7 pages). See any current masthead page for ordering and Internet access instructions.

JA972771H

(12) For pyridoxamine, K_M probably represents the dissociation constant for the ketimine complex. For IFABP-PX60, K_M may include both covalent and noncovalent binding terms.