ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 1, pp. 36-40. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 1, pp. 47-53.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-059, December 14, 2008.

Enantioselective Reductive Amination of α-Keto Acids by Papain-Based Semisynthetic Enzyme

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Received February 19, 2008
Revision received July 29, 2008

Abstract—Alkylation of a cysteine residue in papain with a pyridoxamine (PX) cofactor was carried out. The resulting semisynthetic enzyme (papain–PX) has no detectable protease activity but has the ability to catalyze enantioselective reductive amination of α-keto acids. The conjugate was characterized by ion-exchange chromatography, and the optimal reaction conditions were found. We report that papain–PX reductively aminates the alkyl side chain of functionalized α-keto acids to give the respective α-amino acids with high enantioselectivities, greater than 70%. Based on these studies, we propose a new model for the catalytic activity of the semisynthetic enzyme with Interchem software. The results of the study demonstrate the effectiveness of the modified enzyme and its potential for engineering new catalytic specificity.

DOI: 10.1134/S0006297909010052

Key words: semisynthetic enzyme, papain, pyridoxamine, chemical modification

Native enzymes are highly efficient catalysts, and the importance of these proteins in chemical synthesis is widely recognized. However, limitation can occur in catalytic specificity and substrate selectivity. A major challenge for bioorganic chemistry is discovering new protein-based catalysts with predetermined catalytic activities. A variety of methods have been developed to design tailor-made biocatalysts [1]. One method for altering enzyme catalytic properties makes use of chemical modification of the enzyme.

Native proteins are increasingly used as scaffolds for the designing of new catalysts. A great deal of information is available for selecting suitable proteins based on their physical and chemical properties. For example, the protein papain is of a particular interest. The extended groove in papain is approximately 25 Å long in the vicinity of the active site residue Cys25 [2].

In earlier works, Kaiser et al. [3-11] described the preparation of flavopapain, a construct that can function as a redox catalyst, in which the flavin cofactor was covalently attached to a cysteine residue within the cavity. Suckling et al. [12] introduced thiazolium and pyridinium moieties into papain through alkylation of the same Cys25 residue, which resulted in semisynthetic enzymes. Similarly, Distefano et al. [13-19] prepared ALBP (adipocyte lipid binding protein)–PX (pyridoxamine) and IFABP (intestinal fatty acid binding protein)–PX, semisynthetic enzymes based on adipocyte lipid binding protein or intestinal fatty acid binding protein, respectively, in which a pyridoxamine cofactor was introduced into the protein cavity through a disulfide bond.

Based on the outcomes of the above studies, we attempted the attachment of PX to the papain cavity. The modification of Cys25 abolished the proteolytic activity but did not result in large perturbations of the protein structure. The new conjugate reductively aminates α-keto acids to α-amino acids with a high catalytic efficiency. A possible model is discussed for the role of the attached PX in the papain active site.

MATERIALS AND METHODS

Materials. Papain (EC 3.4.22.2) was purchased from Sigma (USA) and methanol was obtained from Fluka (Switzerland). All other chemicals were of the highest purity commercially available. All measurements were made at room temperature unless mentioned otherwise.

Abbreviations: ALBP, adipocyte lipid binding protein; IFABP, intestinal fatty acid binding protein; PX, pyridoxamine; PXBr, 5-monobromopyridoxamine dihydrobromide.

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Preparation of 5-monobromopyridoxamine dihydrobromide (PXBr) was performed according to [20]. Pyridoxamine dihydrochloride (1 g) was dissolved in 20 ml of 48% hydrobromic acid and refluxed for 2 h. The solvent was removed, and the residue was dried under reduced pressure and dissolved in methanol. Ether was added to precipitate PXBr (melting temperature of the final product was 260-270°C).

Preparation and purification of papain–PX. Papain (100 mg, 4.3 μmol) was dissolved in 4 ml of 0.01 M Na-phosphate buffer (pH 7.5) and mixed with a 15-fold excess of PXBr (6.3 mg, 13 μmol). The mixture was stirred for 24 h in an ice bath. Papain–PX was separated from excess pyridoxamine salt by dialysis for 48 h against 0.01 M Na-phosphate buffer (pH 7.0) at 4°C. Then it was applied to a Superdex G-75 column (35 × 2.5 cm) and eluted with the same buffer to remove unreacted PX. Finally, papain–PX was dissolved in 0.1 M Na-phosphate buffer (pH 7.0). Activated Thiol-Sepharose was used to separate papain–PX from papain as the free SH group of the latter allows it to bind to the matrix.

Ion-exchange chromatography. Ion-exchange chromatography was performed with a Hitrap™ SPFF (1 ml) column using an FPLC system (Pharmacia, Sweden). Samples of pure papain or papain–PX (0.5 ml) were loaded with 50 mM Na-acetate buffer (pH 4.5) at a flow rate of 0.5 ml/min. The column was washed with 5 ml of the buffer at a flow rate to 1 ml/min and eluted with a linear gradient of 0-1.5 M NaCl (15 ml total volume).

Reductive amination reactions. The reaction of reductive amination is illustrated in Scheme 1. Briefly, all reactions were carried out at 37°C, and aliquots (10 μl) of reaction mixture were withdrawn at different times and terminated by flash freezing in N_2, and then stored at –80°C prior to HPLC analysis. The substrate pyruvate (0.3 M) or α-ketoglutarate (0.15 M) was incubated with papain–PX (160 μM) in 0.1 M Na-phosphate buffer (pH 7.5). Controls run with native papain showed no reductive amination with the same substrates.

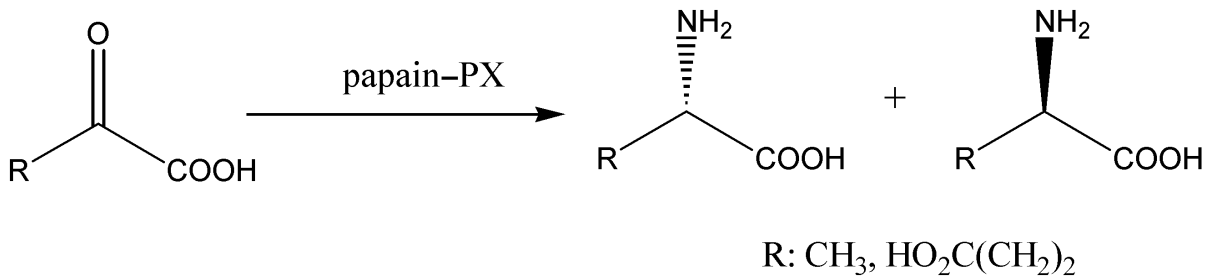
Derivatization. Immediately before analysis by HPLC, reaction samples were thawed and derivatized with 10 μl of a reagent consisting of N-acetyl-L-cysteine (10 mg) and *o*-phthaldialdehyde (10 mg) dissolved in a mixture of 1 ml ethanol and 4 ml of 0.2 M borate buffer (pH 9.8); these reagents convert enantiomeric amines into diastereomeric isoindoles. The derivatization reaction was allowed to proceed for 15 min.

HPLC analysis. All reaction mixtures were analyzed by reversed-phase HPLC using a Supelco C_{18} column (5 μm, 4.6 × 150 mm) according to the procedure of Distefano et al. [13]. Chromatography was performed by injecting a 2 μl sample, and the column was eluted at a flow rate of 1 ml/min using a gradient of solvent A (80 mM sodium citrate and 20 mM sodium phosphate, pH 6.8) and solvent B (MeOH). For alanine, A/B linear gradients (95/5 to 70/30) for 10 min, then adjusted to 70/30 to 100/0 (v/v) for 5 min were used. The retention times for D- and L-alanine derivatives were 32.4 and 34.5 min, respectively. For glutamate, a linear A/B gradient from 95/5 to 50/50 (v/v) during 20 min was used; retention times for the L- and D-glutamate derivatives were 13.7 and 19.3 min, respectively. Samples of the isoindole derivatives were detected by UV spectroscopy at 356 nm.

RESULTS AND DISCUSSION

To prepare the desired papain conjugate, PXBr was first synthesized from pyridoxamine and purified by precipitation, then reacted with papain to generate the conjugate papain–PX. Ion-exchange chromatography of papain and papain–PX under native conditions gave identical retention times, which suggested that the modification of Cys25 does not result in a large perturbation of the protein structure.

Purification. Small molecules (such as modifiers) and unreacted papain were removed by three steps of purification, which were carried out in a Na-phosphate buffer. Most of the modifiers had been removed by dialysis and gel filtration, as shown in the table. Finally, activated Thiol-Sepharose was used to remove unreacted papain, and no hydrolytic activity was observed for the papain–PX.



Enantioselective reductive amination of α-keto acids

Scheme 1

Purification of semisynthetic enzyme

Enzyme	Purification stage*	A_{253}**	Activity, %***	Yield (by protein), %****
Papain (control)		0.514	100	–
Papain–PX (during purification)	1	0.095	18	85
	2	0.021	4	79
	3	< 0.01	< 0.01	74

* Three steps for purification of semisynthetic enzymes: step 1, dialysis for 24-48 h against phosphate buffer (0.01 M, pH 7.0) at 4°C; step 2, run in Superdex-G75 column to remove small molecules; step 3, applied to activated Thiol-Sepharose to remove unreacted papain.
** Residual catalytic activity of papain was measured using BAEE (benzoyl-arginine ethyl ester) as the substrate (λ = 253 nm).
*** Activity of the papain control was defined as 100%, the remaining activity of unreacted papain was measured.
**** Yield of papain–PX after freeze-drying.

Optimal reaction conditions. A typical time course of reductive amination of pyruvate and α-ketoglutarate is shown in Fig. 1. The enantiomeric excess (*ee*) value of the reactions was high in the first 24 h and then declined slightly. The conversion increased significantly in the first 24 h, and a small increase was observed during the next 24 h.

The effect of temperature on reaction rate was investigated over the range of 35-45°C. A slight increase in activity was noted between 35 to 37°C with a decrease at 40°C. The enantioselectivity of the reactions was little affected over the temperature range used. As summarized in Fig. 2, the optimal temperature was 37°C, which is similar to that for native papain.

As shown in Fig. 3, the catalytic activity of papain–PX is greater at neutral pH. Reactions were carried out at 37°C in a 0.1 M Na-phosphate buffer. The enantioselectivity was little affected over the pH value range used. To obtain the highest conversion and enantioselectivities, pH 7.5 is optimal for the semisynthetic enzyme.

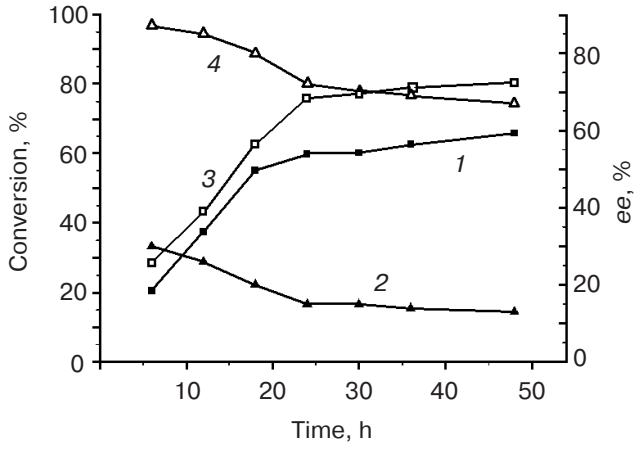


Fig. 1. Time course of reductive amination of pyruvate and α-ketoglutarate. A reaction mixture of 160 μM papain–PX and 0.15 M pyruvate or 0.3 M α-ketoglutarate was stirred in phosphate buffer (0.1 M, pH 7.5) at 37°C. Curves: *1*) conversion of pyruvate; *2*) *ee* of pyruvate; *3*) conversion of α-ketoglutarate; *4*) *ee* of α-ketoglutarate.

Effect of the geometry of the protein catalytic site cavity on the stereoselectivity of amination reaction. To examine the effect of the protein cavity on reactions, the Interchem software (Interprobe Chemical Services Ltd, UK) was used to model the interactions with the substrate and the pyridoxamine cofactor. The *ee* value of the products was determined by forming diastereomeric fluorescent derivatives and quantitating these derivatives after separation by reversed-phase HPLC.

Reactions performed with PX produced no *ee* of D-alanine and L-glutamate. In contrast, papain–PX produced a 14% *ee* of D-alanine after reacting for 24 h (59% conversion). A greater selectivity (72% *ee*) was obtained in the reaction producing a dicarboxylic amino acid, i.e. conversion of α-ketoglutarate into L-glutamate (24 h, 75% conversion).

The enantioselectivities obtained in these catalytic reactions are of particular interest. Inspection of the values obtained from the reductive amination reaction indicated that the overall transamination rates of these two substrates exhibited by papain–PX were similar, while the enantioselectivity is significantly lower with D-alanine. A possible explanation is that the structure of the papain cavity controls the selectivity of the reaction between pyridoxamine and α-keto acids. The low *ee* observed with alanine is likely due to the small size of its side chain, resulting in its limited ability to serve as a directing group, while the selectivity for D-enantiomer probably reflects a structural feature of the ketimine intermediate. Furthermore, as shown in Scheme 2, the α-carbon in the carbon–nitrogen double bond could accept a proton from both directions depending on the size of a larger group attached on the α-carbon. That explained the stereochemical selectivity resulting in D for alanine and L

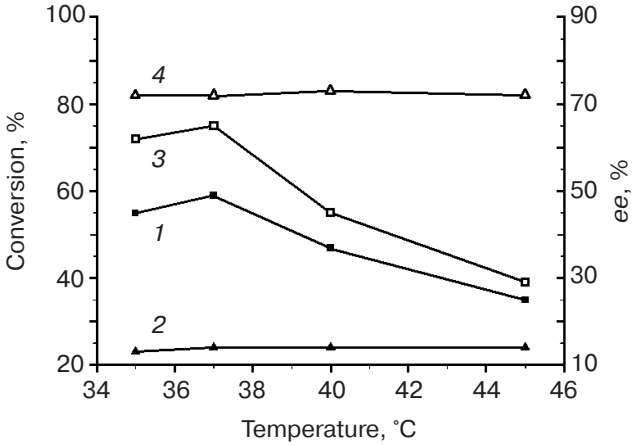


Fig. 2. Effect of temperature on the reductive amination of pyruvate and α-ketoglutarate. A reaction mixture of 160 μM papain–PX and 0.15 M pyruvate or 0.3 M α-ketoglutarate was stirred in phosphate buffer (0.1 M, pH 7.5) at different temperatures for 24 h. Curves: *1*) conversion of pyruvate; *2*) *ee* of pyruvate; *3*) conversion of α-ketoglutarate; *4*) *ee* of α-ketoglutarate.

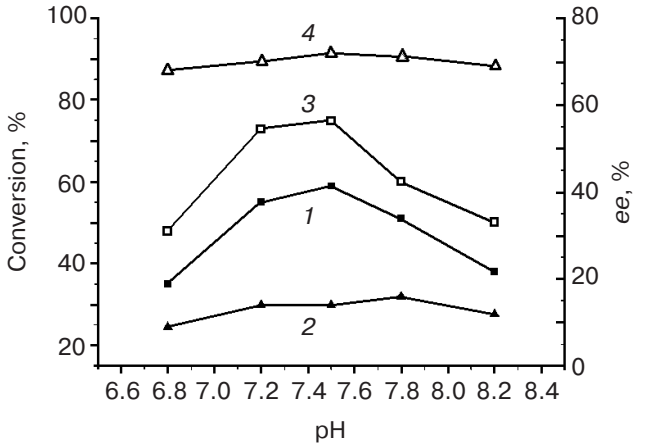
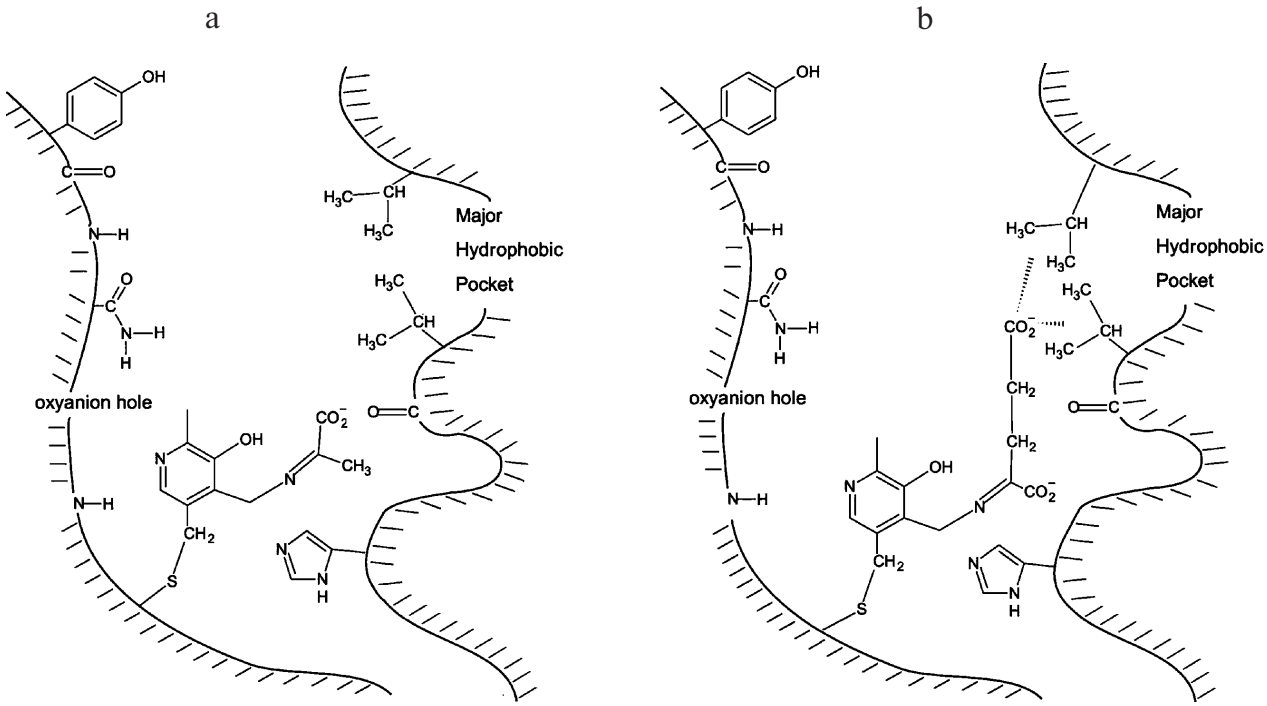


Fig. 3. Effect of pH on the reductive amination of pyruvate and α-ketoglutarate. A reaction mixture of 160 μM papain–PX and 0.15 M pyruvate or 0.3 M α-ketoglutarate was stirred at 37°C at different pH values for 24 h. Curves: *1*) conversion of pyruvate; *2*) *ee* of pyruvate; *3*) conversion of α-ketoglutarate; *4*) *ee* of α-ketoglutarate.



Models illustrating interaction of PX and pyruvate (a) or α-ketoglutarate (b) in the active site of papain–PX

Scheme 2

for glutamate. The results with alanine would also simply reflect the unusual situation in which the amino acid side chain is smaller than the carboxyl group. The carboxyl group of α-ketoglutarate is conformationally tightly locked due to interaction with the hydrophobic pocket in the active site of papain because of the longer side chain, which led to higher conversion and selectivity.

Papain–PX appears to be superior in conversion but inferior in enantioselectivity compared with ALBP–PX [14]. A possible explanation for this behavior is that ALBP and papain have analogous structures of the active site cavity that can accommodate the modifier PX and the substrates. The decrease in enantionselectivity in the papain–PX catalyzed reaction suggests that the modified

papain either alters the conformation of the quinoid intermediate (resulting in a less facial selectivity) or lowers the barrier for racemization.

The objective of this study was to develop a novel semisynthetic enzyme through chemical modification of papain by pyridoxamine cofactor. The catalytic efficiency of papain–PX is still lower than of the natural archetypes. However, the results show that pyridoxamine cofactor can be introduced into ALBP, IFABP, and papain to generate different semisynthetic enzymes, which is a step forward in semisynthetic enzyme design. In combination with further insight into the active site of enzymes, this approach of catalyst design could be promising as the catalytic efficiency of semisynthetic enzymes would be increased through using appropriate protein templates.

This work was supported by Donghua University, the Program for Changjiang Scholars and Innovative Research Team at Donghua University (No. IRT0526), NSFC (No. 50773009), UK–CHINA Joint Laboratory for Therapeutic Textiles, Biomedical Textile Materials "111 Project" from Ministry of Education of P. R. China (No. B07024) and Esquel Group.

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