Dong H. Kim
Center for Biofunctional Molecules and Department of Chemistry, Pohang University of Science and Technology, San 31 Hyojadong, Pohang 790-784, Korea

Design of Protease Inhibitors on the Basis of Substrate Stereospecificity

Abstract: *The substrate stereospecificity in enzymic reactions, which is one of characteristics of enzymes along with the substrate and regiospecificity can provide a basis for the rational design of inhibitors. This has been demonstrated using α-chymotrypsin, a prototypic serine protease as a model enzyme. On the basis of the structure–activity relationships for substrates as well as inhibitors and mechanism of the enzymic reaction, a schematic three-dimensional model of the S_1 subsite of α-chymotrypsin is constructed. It was envisioned from the three-dimensional active site model that 2-benzyl-3,4-epoxybutanoic acid methyl ester (**1**) having a (2*S*)-configuration would bind the enzyme with its oxirane ring being rested at the catalytic site, in which the oxirane ring is subject to a nucleophilic attack by the Ser-195 hydroxyl to form a ether linkage. Kinetic analysis of the enzymic reaction in the presence of the potential inhibitors showed that (2S,3R)-1 inactivates α-chymotrypsin, while (2S,3S)-1 inhibits the enzyme competitively. The lack of inactivating activity in the case of (2S,3S)-1 may be due to the unfavorable alignment of the C_3—O bond with respect to the hydroxyl of Ser-195 for the S_N2-type ring cleave reaction of the oxirane moiety. When the design protocol was applied to papain, a representative cysteine protease, (2S,3S)-1 inhibited the enzyme irreversibly, while (2S,3R)-1 inhibited reversibly. On the basis of the stereospecificity shown in the inactivation of the enzymes, it was inferred that in the case of α-chymotrypsin, the nucleophilic attack of the Ser-195 hydroxyl at the scissile carbonyl carbon of substrates occurs in a* si *fashion, while the thiolate of Cys-25 in papain attacks the substrate amide bond in a* re *fashion. The inhibitor design protocol may be applied to other proteases.* © 1999 John Wiley & Sons, Inc. Biopoly 51: 3–8, 1999

Keywords: *inhibitor design; stereochemical approach; substrate stereospecificity; α-chymotrypsin; papain*

INTRODUCTION

Enzymes have unusual ability to recognize their substrates and act selectively on them in a stereospecific manner. The substrate stereospecificity is one of characteristics of enzymic reactions along with the substrate and regiospecificity.[1] The active site of enzymes including proteases is an asymmetric three-dimensional entity with binding and catalytic sites being uniquely and characteristically arranged in space. Substrates are accommodated by the active site to form enzyme–substrate complexes in which the catalytic chemical reaction takes place. Different from asymmetric reactions that are encountered in organic synthesis, in which the asymmetry is conferred by the built-in chiral environment at the reaction center of the substrate, in the enzymic reaction the enzyme confers the asymmetry on the substrate. The substrate

Correspondence to: Dong H. Kim
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stereospecificity of enzymic reactions is thus rationalized on the basis of the structural complementarity between the molecular shape of substrates and the topology of the active site crevice that accommodates the substrates. However, our knowledge on the factors that control and determine the enzyme stereospecificity is still very limited, although the three-point model of Ogston[2] that led to the development of the prochiral concept in stereochemical reactions in the biological system was advanced as early as the mid-1940s. Nevertheless, the substrate stereospecificity of enzymic reactions can provide a basis for rational design of enzyme inhibitors. A novel design protocol of enzyme inhibitors based on substrate stereospecificity has been demonstrated using α-chymotrypsin, a representative serine protease as a model enzyme, and employed to the development of inhibitors for papain.

THREE-DIMENSIONAL MODEL OF THE ACTIVE SITE OF α-CHYMOTRYPSIN

α-Chymotrypsin is a prototypical serine proteolytic enzyme that has been studied most extensively.[3] The enzyme cleaves peptide bond of the amino acid residue (P_1 amino acid residue) having a hydrophobic side chain.[3] It also hydrolyzes the ester bond. The proteolytic reaction of α-chymotrypsin is initiated by the nucleophilic attack on the scissile amide (or ester) carbonyl carbon by the hydroxyl group of Ser-195 to result in acylated enzyme intermediate.[4,5] At the active site of α-chymotrypsin there is an extended binding region composed of several subsites, of which the S_1 subsite plays a dominant role in the substrate discrimination. There are present, in the S_1 subsite, three distinctive binding loci that interact with substrate in the formation of the enzyme–substrate complex in addition to the nucleophilic hydroxyl group of Ser-195[6,7]: A large hydrophobic pocket (primary substrate recognition subsite pocket) that is deeply invaginated into the core of the enzyme accommodates the hydrophobic side chain of P_1 residue of the substrate, a hydrogen-bonding site where the amide hydrogen of P_1 residue forms a hydrogen bond with the backbone peptide carbonyl oxygen of Ser-214, and a cavity of restricted volume for the α-hydrogen of P_1 residue.[6,7]

Previously, we have proposed a three-dimensional schematic representation of the active site of α-chymotrypsin.[8] Figure 1 depicts the active site model that is occupied by a substrate. In binding of a substrate to the enzyme, the hydrophobic side chain of P_1 residue having the L-stereochemistry is inserted into the hydrophobic pocket and the amino hydrogen of P_1 residue forms a hydrogen bond with the peptide carbonyl of Ser-214. The α-hydrogen is fitted into the small cavity. These interactions between the enzyme and the substrate at three sites would place the scissile peptide bond at the catalytic site in which the hydroxyl group of Ser-195 would attack the carbonyl carbon of the substrate. A ligand whose stereochemistry of P_1 residue belongs to the D-series binds the enzyme differently. For example, in the case of D-AcNPheOMe, none of possible conformers can bind the enzyme in a productive fashion, that is, in a fashion that the scissile ester bond is rested in the catalytic site. If the binding would occur, the acetylamide group rather than the scissile bond is placed in the catalytic site.

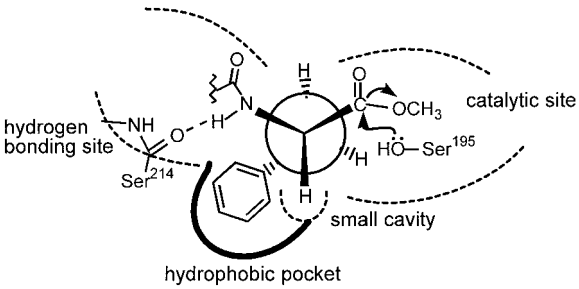


FIGURE 1 A schematic representation of the spatial arrangement of the catalytic site (Ser-195) and binding sites, constituting the S_1 subsite of α-chymotrypsin. The subsite is occupied by a substrate.

DESIGN OF α-CHYMOTRYPSIN INHIBITORS ON THE BASIS OF SUBSTRATE STEREOSPECIFICITY

Since there are two stereogenic centers in 2-benzyl-3,4-epoxybutanoic acid (BEBA) ester, four diastereoisomers are possible. Out of four stereoisomers, two isomers having the *R*-configuration at the 2-position are expected to bind to α-chymotrypsin with their ester bond being placed at the catalytic site, and then the hydroxyl of Ser-195 attacks the ester bond, leading to catalytic hydrolysis. Indeed, these isomers were found to be substrates for the enzyme.[9] On the other hand, in the case of the other two isomers having the *S*-configuration, bindings were expected to take place with the oxirane ring being rested at the catalytic site. If such a mode of binding takes place, then the nucleophilic attack of the Ser-195 hydroxyl occurs on the oxirane moiety to cleave the ring with the formation of an ether linkage between the ligand and the enzyme (Figure 2). The BEBA ester is then covalently

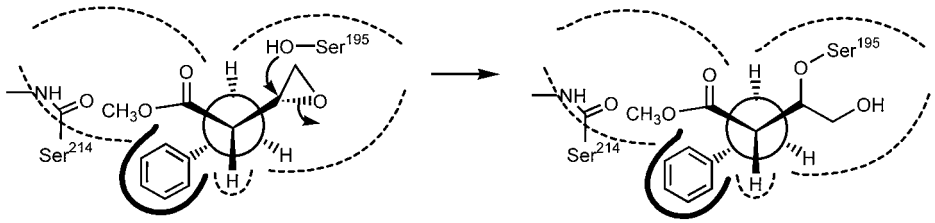
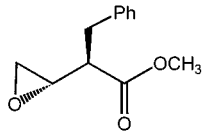
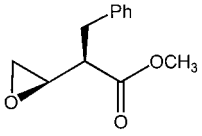


FIGURE 2 (2*S*,3*R*)-2-Benzyl-3,4-epoxybutanoic acid methyl ester anchors in the active site of α-chymotrypsin with the oxirane ring being positioned at the catalytic site. The C_3—O bond is aligned periplanar to the Ser-195 hydroxide nucleophile so that there occurs a S_N2 type ring cleavage reaction, leading to covalent modification at the hydroxyl.

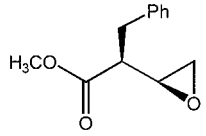
tethered to the enzyme, and as a result the enzyme loses its catalytic activity.



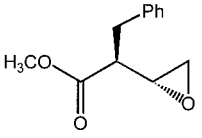
(2*R*,3*S*)-**1**



(2*R*,3*R*)-**1**



(2*S*,3*S*)-**1**



(2*S*,3*R*)-**1**

As expected, the two diastereoisomers of BEBA methyl ester (**1**) having the *S*-configuration at the 2-position were found to have an inhibitory activity against α-chymotrypsin.[9] Further examination of the inhibitory action revealed that (2*S*,3*S*)-**1** is a competitive inhibitor having K_i value of 9.95 m*M* when analyzed by the plots of Lineweaver–Burk[10] and Dixon,[11] while (2*S*,3*R*)-**1** showed a time-dependent loss of the enzymic activity with the k_{inact}/K_I value of $27M^{-1}$ s^{-1}, suggesting that there occurs a covalent modification at the hydroxyl of Ser-195.[12] The rate of the irreversible inhibition was reduced by increasing the concentration of the substrate to suggest that the active site is involved in the inactivation. The covalent modification was further supported by the dialysis experiment. Thus, no return of the enzymic activity was observed after 24 h dialysis of the incubation mixture. The inactivation reaction was found to proceed with 1 : 1 stoichiometry when analyzed by the method of Levy et al.[13] Furthermore, the chemical modification of Ser-195 hydroxyl with the formation of an ether linkage was substantiated by the electrospray ionization mass spectra of the inactivated enzyme and its degradation fragments.[14] The observation that the inactivation of α-chymotrypsin occurs only with (2*S*,3*R*)-**1** strongly suggests that the nucleophilic attack on the oxirane ring by the hydroxyl occurs at the 3-position of the BEBA ester. It is worthy of noting that while (2*S*,3*R*)-**1** inactivates the enzyme, its diastereoismer having (2*S*,3*S*)-configuration is a competitive inhibitor for the enzyme. The observed stereospecificity at the 3-position of (2*S*)-BEBA methyl ester suggests that the C_3—O bond of (2*S*,3*R*)-**1** is oriented periplanar to the hydroxyl group, meeting the requirement for the S_N2-type ring opening reaction when the inactivator binds the active site. The lack of α-chymotrypsin inactivating property shown by (2*S*,3*S*)-**1** may then be ascribed to the unfavorable stereochemical orientation of the C_3—O bond with respect to the nucleophilic hydroxyl of Ser-195 for the S_N2 type ring cleavage reaction (Figure 3). The stereospecificity shown at the 3-position of the inhibitor for the inactivation of α-chymotrypsin bears an important implication with regard to the stereochemistry of the initial step of the enzymic hydrolysis reaction, suggesting that the nucleophilic attack of the Ser-195 hydroxyl at the scissile carbonyl carbon of substrates occurs in a *si* fashion.

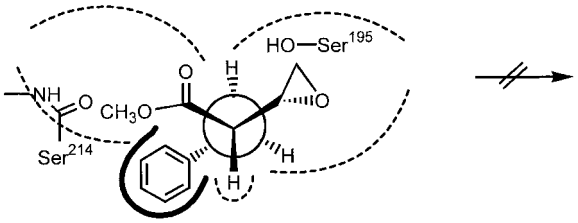
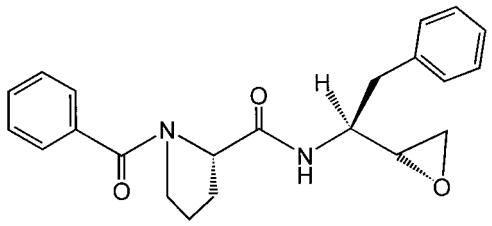


FIGURE 3 (2*S*,3*S*)-2-Benzyl-3,4-epoxybutanoic acid methyl ester anchors in the active site of α-chymotrypsin with the oxirane moiety being positioned in the catalytic site, but the C_3—O bond is not aligned favorably for the S_N2 type ring cleavage reaction. No nucleophilic attack of the Ser-195 hydroxyl on the oxirane ring ensues.

It was thought that an improvement of the inactivating potency of the prototype inhibitor might be achieved by lowering the K_i value, which may be attained by replacing the ester moiety in (2*S*,3*R*)-**1** with a peptidyl group that can interact with the auxiliary binding subsites at the active site of the enzyme. The ester moiety in (2*S*,3*R*)-**1** was thus replaced with *N*-benzoylpyrrolidylcarboxamide to obtain **2**. The replacement was made in light of the report of Segal and others.[15–17] They showed that substrates having a proline at the P_2 position bind the enzyme with high affinity by virtue of its hydrophobic interactions possibly with the side chain of Ile-99 as well as a smaller entropy decrease upon binding to the enzyme due to its restricted rotational freedom, in addition to hydrogen bondings between the substrate and active site backbone residues of the enzyme.



(2*S*,3*R*)-2

Contrary to expectation, however, inhibitor **2** failed to exhibit a time-dependent loss of the enzymic activity, indicating that the inhibitor in not an inactivator but a competitive reversible inhibitor for the enzyme.[18] The K_i value of 0.82 mm was estimated for the inhibition.[18]

The observed difference in the inhibitory property between (2*S*,3*R*)-**1** and (2*S*,3*R*)-**2** is surprising, but can be envisaged: The failure of the irreversible inhibition in the case of (2*S*,3*R*)-**2** is ascribed to the enhanced binding affinity as a result of the additional interactions between the peptidyl moiety of the inhibitor and the auxiliary binding subsites at the active site of the enzyme. This improved binding in the case of **2** prevents its oxirane ring from approaching close to the Ser-195 hydroxyl to undergo the chemical reaction for the epoxide ring cleavage.

DESIGN OF PAPAIN INHIBITORS

Cysteine proteases, which may be represented by papain, are enzymes of considerable pharmacological interest as they are implicated in a number of pathological states such as progressive cartilage and bone degradation associated with arthritis.[19] Structures of the active site of cystein proteases are known to be very similar to those of serine proteases. Both families of enzymes effect proteolysis by a common catalytic pathway involving a nucleophilic attack at the scissile peptide bond of the bound substrates to form acylated enzyme intermediates, although the nature of the nucleophile is different. Thus, for cystein proteases, the thiol group of cysteine (Cys-25 for papain) rather than the hydroxyl group as in the case of α-chymotrypsin initiates the catalytic bond cleavage. Accordingly, it was expected that the BEBA methyl ester having the *S*-configuration at the 2-position would bind papain[19] in the same way as it does to α-chymotrypsin, and then there would follow an inactivation of the enzyme.

As expected, both BEBA methyl esters showed inhibitory activity against papain when assayed at pH 7.0 using benzyloxycarbonyl-glycine *o*-nitrophenyl ester as substrate, and further examination of the mode of inhibition indicated that (2*S*,3*R*)-**1** is a competitive inhibitor for the enzyme having the K_i value of 0.71 m*M*.[21] The other inhibitor, (2*S*,3*S*)-**1** caused the loss of the enzymic activity in a time- and concentration-dependent manner, suggesting that there occurs an irreversible inhibition. Kinetic parameters for the irreversible inhibition were determined to be K_I = 0.132 mm and k_{inact} = 0.14 min^{-1}.[21] The inactivated enzyme failed to regain its enzymic activity upon dialysis, inconsistent with the irreversible nature of the inhibition, and the rate of the irreversible inhibition was reduced by increasing the concentration of the substrate, suggesting that the inhibition is active site directed. Recently, Albeck et al. have also reported that *erythro*-peptidyl epoxides exemplified by Cbz–Gly–Leu–Phe–epoxide are potent and selective irreversible inhibitors for papain.[22,23]

Unlike α-chymotrypsin in which the S_1 subsite plays a key role in substrate recognition, in the case of papain the S_2 subsite is known to function as the primary substrate recognition site discriminating for an amino acid residue having a hydrophobic side chain such as Phe and Leu. The S_2 subsite is known as a large hydrophobic pocket consisting of Tyr-69, Tyr-67, Phe-207, Pro-68, Ala-160, Val-133, and Val-157.[24] The S_1 subsite of papain is much less well defined. In the schematic representation of the active site, the two subsites of S_1 and S_2 that play an important role in the recognition of substrate are shown as pockets (Figure 4). The thiol nucleophile of Cys-25 is positioned in the catalytic site. As with α-chymotrypsin, the methyl esters of (2*S*,3*S*)- and (2*S*,3*R*)-BEBA bind the enzyme in a reverse fashion, with the oxirane

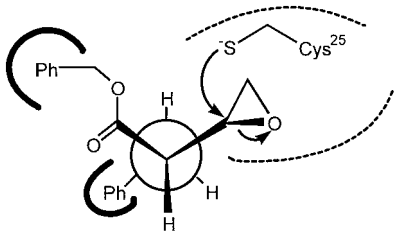


FIGURE 4 (2*S*,3*S*)-2-Benzyl-3,4-epoxybutanoic acid benzyl ester anchors in the active site of papain with the oxirane ring being positioned at the catalytic site. The C_3—O bond is aligned periplanar to the Cys-25 thiolate nucleophile so that there occurs the S_N2 type ring cleavage reaction, leading to covalent modification of the enzyme at the thiol group. The aromatic ring of the benzyl moiety in the inactivator is inserted into the S_2 subsite pocket, which improves the inactivating potency.

moiety being positioned in the catalytic site. The chemically active oxirane ring then undergoes a chemical reaction with the thiol group of Cys-25 to result in the attachment of the inhibitor to the enzyme with formation of a thioether linkage. The inactivation of papain only by (2*S*,3*S*)-**1** suggests that the ring cleavage reaction occurs again exclusively at the 3-position of the inhibitor. The 3-position of (2*S*,3*S*)-**1** corresponds to the scissile peptide carbonyl carbon of substrates. Accordingly, it can be inferred from the inactivation stereochemistry observed with the inhibitor that in the papain catalyzed proteolytic reaction the nucleophilic attack of the Cys-25 thiolate on the scissile carbonyl carbon occurs on the *re* face.

As described above, the S_2 subsite pocket of papain is known to accommodate an amino acid residue having a bulky hydrophobic side chain. Accordingly, it was thought that benzyl ester of (2*S*,3*S*)-BEBA would be an inactivator of improved potency as a result of its benzyl group to rest in the S_2 subsite, leading to the enhancement of the binding of the inhibitor to the enzyme (Figure 4). Indeed, the k_{inact}/K_I value was increased by about threefold by the replacement. The improvement of the inactivating potency is largely due to the increase of the k_{inact} value, which is in line with observations that substrates having a large hydrophobic P_2 residue results in an increase of k_{cat} value.[25]

CONCLUSION

Inhibitors of an enzyme may be designed on the basis of the substrate stereospecificity of the target enzyme. In this approach, understanding of the spatial arrangement of the catalytic site in relation to the substrate binding loci especially the primary substrate recognition pocket is essential. Construction of a three-dimensional active site model is of value. Such a model may be constructed on the basis of the three-dimensional shape of substrates or inhibitors for the enzyme and mechanism of the enzymic reaction. α-Chymotrypsin is used as a model enzyme in demonstrating the design protocol. On the basis of the three-dimensional active site model of the enzyme, BEBA methyl esters having the *S*-configuration at the 2-position were designed as inhibitors for the enzyme. Thus, for example, in the case of (2*S*,3*R*)-**1**, upon binding to the enzyme, its oxirane moiety is expected to rest at the catalytic site where the S_N2 type ring cleavage of the oxirane by the hydroxyl of Ser-195 would ensue to result in the covalent modification of the hydroxyl. Kinetic analysis of the enzymic reaction in the presence of these inhibitors revealed that (2*S*,3*R*)-**1** inactivates α-chymotrypsin, whereas (2*S*,3*S*)-**1** inhibits the enzyme reversibly. The inhibitor design approach was applied to papain, a prototypic cysteine protease to find that in this case (2*S*,3*S*)-**1** inactivates the enzyme and (2*S*,3*R*)-**1** inhibits it reversibly. This enzyme inhibitor design protocol can be applied to other proteolytic enzymes whose active site topology and catalytic mechanism are defined, and may be useful for the determination of the spatial arrangement of the active site residues and enzymic reaction mechanism.

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