Origin of Chiral Pharmacology: Stereochemistry in Metalloprotease Inhibition

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Abstract: The stereospecificity shown by a wide variety of inhibitors that are effective for carboxypeptidase A (CPA), a representative zinc protease is analyzed on the basis of inhibitor type. In cases of ground state analog inhibitors and transition state analog inhibitors, the stereoisomers having the stereochemistry that corresponds to stereochemistry of substrate are more potent, but in the case of irreversible inhibitors including mechanism-based inactivators the preferred inhibitory stereochemistry cannot be predicted simply from the substrate stereospecificity. The Ogston's three point fit concept may be of great value in understanding the inhibitory stereochemistry of reversible competitive inhibitors. On the other hand, the stereochemistry of irreversible inhibitors may possibly be predicted on the ground of the transition state structure that plays a critical role in the inactivation pathway.

The biological activity exhibited by each enantiomer of chiral drugs would be dissimilar sometimes to show response of opposite nature. Thus, valsartan is an angiotensin II antagonist highly specific for the AT_1 receptor and is used for the treatment of hypertension. Of a pair of its stereoisomers, the (*S*)-form is more potent than the (*R*)-isomer [1]. On the other hand, in the case of picenadrol, while (+)-enantiomer is a potent agonist working at the μ− opiate receptor, (–)-picenadrol is an antagonist for the same receptor [2]. Both drug discoverers and the regulatory authorities are increasingly concerned about marketing racemic drugs and pharmaceutical industry is much interested in developing the active single enantiomer as therapeutic agent [3]. Accordingly, understanding of the unlike pharmacological effect caused by the stereochemical difference of chiral drugs is of paramount importance [4,5].

Many diseases are caused by excessive production of certain metabolites and such disease states can be remedied by normalization of the metabolic process. Enzymes are in the limelight as the attractive targets for development of therapeutic agents because the production of the pathologically important metabolite can be controlled by modulation of the catalytic activity of the enzyme that is involved in the metabolite production [6]. In fact, most of chemotherapeutic agents manifest their therapeutic effects by interfering with the catalytic activity of the enzyme that is associated with the pathological conditions. For example, the widely prescribed antihypertensive agent, captopril lowers the high blood pressure by moderating the activity of angiotensin converting enzyme that is involved in the production of potent vasoconstrictive octapeptide, angiotensin II [7].

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Proteases catalyze the hydrolysis of all sorts of proteins and play crucial roles in maintaining normal functioning of biological system. They are involved in such diverse processes as blood clotting, immunological reactions, and production of hormones [8,9]. Among vast number of proteases known, metalloproteases constitute the largest enzyme family and have received much attention owing to their association with etiology of a variety of hard to cure diseases [10].

Carboxypeptidase A (CPA) is a much studied and well characterized zinc containing metalloprotease that catalyzes the removal of the carboxy-terminal amino acid residue having a hydrophobic aromatic side chain, and serves as a prototypical enzyme for many pathologically important zinc containing proteolytic enzymes such as angiotensin converting enzyme and matrix metalloproteases [11]. As a well characterized proteolytic enzyme, CPA has been used as a model for developing inhibitor design protocols that can be applied to other zinc proteases of medicinal interest.

One of important characteristic features of enzymic reactions is substrate specificity that includes stereospecificity. That is, only those compounds whose molecular structures including stereochemistry are complementary to the active site structure of an enzyme can be recognized by the enzyme and served as substrates to undergo chemical transformations. Although the substrate stereospecificity for enzymic reaction has been well established, the stereochemisty associated with enzyme inhibition received only scant attention in spite of its enormous importance especially with regard to new drug discovery. Because of lack of synthetic methodology, most of inhibitors especially those reported in early days have been evaluated as a racemic mixture. Some of inhibitors were evaluated as an optically active form, but since their antipodes were not available, stereochemistry associated with the inhibition could not be assessed. In this article we wish

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to report the stereochemistry that we found in the inhibition of the prototypic metalloenzyme, CPA.

Three binding and one catalytic site have been identified at the active site of CPA. The zinc ion that is coordinated tightly to the backbone amino acid residues of His-69, Glu-72 and His-196 is essential for the enzymic catalytic hydrolysis reaction. A molecule of water is bound loosely to the zinc ion as the fourth ligand. The zinc ion activates the water molecule in collaboration with the carboxylate of Glu-270, generating a nucleophilic hydroxyl group that attacks at the carbonyl carbon of the scissile peptide bond to generate a tetrahedral transition state. It has been known that the guanidinium moiety of Arg-127 forms hydrogen bonds to the carbonyl group of the scissile peptide bond of substrate, whereby activating the carbonyl for a nucleophilic attack. The other principal binding sites are Arg-145 and the S_1' pocket. The latter is shaped complementary to the aromatic ring present in the side chain of the P_1' amino acid residue such as Phe, and serves as the primary substrate recognition site, and the former is involved in forming bifurcated hydrogen bonds with the terminal carboxylate of substrate (Fig. **1**). The X-ray crystal structure of the enzyme revealed that the guanidinium moiety of Arg-145 and the carboxylate of Glu-270 are located at the surface of the enzyme molecule but the substrate recognition pocket is invaginated in its core. The zinc is found deep in the active site [12]. The topology of the CPA active site is thought to be responsible for the substrate specificity. That is, only substrate having molecular configuration that is compatible with the topology of the active site can be accommodated by CPA and undergoes the catalytic hydrolysis reaction. Accordingly, the C-terminal amino acid residue (P_1' residue) that bears stereochemistry of the L-series can be removed by CPA [11].

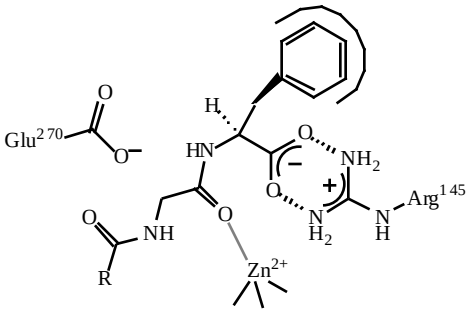

Fig. (1). Schematic representations of the active site of CPA, which is occupied by a substrate, *N*-acyl-Gly-L-Phe.

SUBSTRATE ANALOG INHIBITORS (GROUND STATE ANALOG INHIBITORS)

In order for a compound to function as an inhibitor for proteolytic enzyme, the molecule should be recognized by the target enzyme and form a complex with it but resists to be hydrolyzed. Therefore, the three dimensional shape of the inhibitor molecule should be compatible with the active site topology where the inhibitor competes with substrate for binding. Accordingly, inhibitors of zinc-containing proteases can be prepared by replacing scissile peptide bond of a substrate with an isostere that resists to the enzymic cleavage reaction, or by incorporating a zinc ligating functionality into the structural frame of a substrate-like molecule. In the case of CPA, virtually all of potent inhibitors reported to date are those that contain a zinc ligating moiety. Most of substrate analogs inhibitors having an isostere in place of scissile peptide bond bind the enzyme poorly. These types of inhibitors are commonly referred to as substrate analog inhibitors or ground state analog inhibitors and bind the enzyme reversibly.

2-Benzylsuccinic acid (**1**) is a potent competitive inhibitor of CPA designed by Byers and Wolfenden on the basis of a proposed catalytic mechanism of CPA and is one of the earliest and representative substrate analog inhibitors for zinc proteases [13]. As a racemic form, it inhibits CPA with the K_i value of 1.1 μM, but when it was resolved, the (*R*)-isomer that belongs to the L-series was found to be more potent by 6.7-fold than its enantiomer. Replacement of the α-H in (*R*)-**1** with a methyl group did not improve the inhibitory potency to any significant extent [14]. The X-ray structural analysis of CPA·**1** complex obtained by soaking CPA crystal in a solution of the racemic mixture of the inhibitor revealed that the bound inhibitor bears the (*R*)-configuration in consistent with the kinetic results [15]. The carboxylate at the β-position of **1** coordinates to the active site zinc ion in an asymmetric bidentate fashion with the two oxygen atoms at 2.3 and 2.6 Å distances adapting *syn* stereochemistry. It may be worth mentioning that surprisingly the careful analysis of the well resolved X-ray crystal structure of thermolysin inhibited with racemic **1** showed that the D-isomer rather than the L-isomer binds the enzyme preferentially [16]. Thermolysin is a zinc containing protease of bacterial origin whose active site structure and mechanism of the catalytic action resemble those of CPA. [17].

After finding that a sulfhydryl group has a high propensity to bind to zinc ion in enzyme from the work devoted to the development of therapeutically useful inhibitors of angiotensin converting enzyme [18]. Ondetti *et al.* synthesized 2-benzyl-3-mercaptopropanoic acid (**3**) as a potent CPA inhibitor having the K_i value of 0.011 μM [19]. The high inhibitory activity arises apparently from the sulfhydryl group that ligates strongly to the active site zinc ion. The inhibitory activity of **3** was shown to reside mostly on the (*S*)-form that corresponds to the L-configuration [20]. It is interesting to note that *N*-mercaptoacetylphenylalanine (**4**) obtained from D-Phe is more potent compared to its L-enantiomer by about 23-fold (Table 1) [21].

The high binding affinity of hydroxamate for metal ions such as zinc has been extensively utilized as a metal coordination functionality in designing inhibitors of metalloenzymes. Hydroxamate is known to coordinate to zinc ion in a bidentate fashion and substrate analogs containing hydroxamate moiety are in general highly potent as inhibitors effective against a wide variety of zinc proteases [22]. In fact, hydroxamate group has been exclusively utilized in the design of inhibitors for matrix metalloproteases, culminating to produce several potential therapeutic agents which are currently in clinical trial [23]. Despite such frequent uses of the hydroxamate as a zinc chelating group in the design of zinc protease inhibitors,

Table 1. Carboxypeptidase A Inhibitors and Inhibitory Constants

inhibitor no.	inhibitor	K_i (μM)	references
rac-**1**	(±)-$HO_2CCH_2CH(CH_2Ph)CO_2H$	1.1 ± 0.3	[14]
L-**1**	L-$HO_2CCH_2CH(CH_2Ph)CO_2H$	0.45	[14]
D-**1**	D-$HO_2CCH_2CH(CH_2Ph)CO_2H$	3	[14]
rac-**2**	(±)-$HO_2CCH_2CMe(CH_2Ph)CO_2H$	0.28	[13]
L-**2**	L-$HO_2CCH_2CMe(CH_2Ph)CO_2H$	0.15	[13]
D-**2**	D-$HO_2CCH_2CMe(CH_2Ph)CO_2H$	17	[13]
rac-**3**	(±)-$HSCH_2CH(CH_2Ph)CO_2H$	0.011	[19]
L-**3**	L-$HSCH_2CH(CH_2Ph)CO_2H$	0.0078	[20]
L-**4**	L-$HSCH_2CO$-Phe	5.0	[21]
D-**4**	D-$HSCH_2CO$-Phe	0.22	[21]
rac-**5**	(±)-$HONHCOCH_2CH(CH_2Ph)CO_2H$	24.2	[24]
rac-**6**	(±)-HCON(OH)-β-Phe	0.98	[25]
L-**6**	L-HCON(OH)-β-Phe	0.56	[25]
D-**6**	D-HCON(OH)-β-Phe	4.95	[25]
L-**7**	$CbzNHCH_2PO_2$-L-Phe	0.09	[31]
L-**8**	L-$(PhO)PO_2$-Phe	2.1	[21]
D-**8**	D-$(PhO)PO_2$-Phe	10	[21]
rac-**9**	(±)-$HCOCH_2CH(CH_2Ph)CO_2H$	0.48 ± 0.1	[32]
L-**9**	L-$HCOCH_2CH(CH_2Ph)CO_2^-K^+$	0.38± 0.03	[33]
D-**9**	D-$HCOCH_2CH(CH_2Ph)CO_2^-K^+$	256 ± 19	[33]

hydroxamate-type inhibitor for CPA has only very recently been reported. *rac*-$HONHCOCH_2CH(CH_2Ph)COOH$ (**5**) is moderately active as inhibitor for CPA with the K_i value of 24.2 μM. The corresponding hydroxamate prepared from malonic acid could not be tested because of its unstable nature [24]. *N*-Formyl-*N*-hydroxyl-β-Phe (**6**) in which the carbonyl and hydroxylamine are interchanged compared with the hydroxamate inhibitor of more common form was found to be a potent inhibitor for CPA having the K_i value of 0.98 μM [25]. Again, the inhibitory activity resides mostly on the (*R*)-form that belongs to the L-series with the K_i value of 0.56 μM (Table 1) [25]. When the formyl group was replaced with a bulkier acyl group such as acetyl or propanoyl group, the potency was reduced progressively [25].

It is of interest examining the stereochemistry in the inhibition of thermolysin, with hydroxamate-type inhibitors. Hydroxamates of both α- and β-amino acids were evaluated to find that they possess potent inhibitory activity against the enzyme. The inhibition of thermolysin by *N*-formyl-*N*-hydroxy-LeuOMe (**10**) is highly stereospecific with the inhibitory activity being vested mainly with the D-series with the K_i value of 44 μM [26]. The corresponding hydroxamate prepared from methylamine amide of β-Phe is much more potent for thermolysin than the hydroxamate of the corresponding α-amino acid, but interestingly the activity resides on the L-isomer (L-**11**) with the inhibitory constant of 1.66 μM (Table 2) [27]. From the above observations it may be concluded at least for thermolysin that when the zinc ligating group in the substrate analog inhibitors derived from α-amino acid functions as a chelator, the inhibitory stereochemistry reverses. That is, the D-isomer binds more tightly than the inhibitor derived from the L-amino acid.

TRANSITION STATE ANALOG INHIBITORS

An enzyme accelerates the rate of its catalytic reaction by stabilizing the transition state involved in the enzyme catalyzed chemical transformation pathway. The enzyme achieves this by altering the conformation of enzyme · substrate complex in such a way that the interactions between the enzyme and substrate become strongest at the transition state. It is therefore expected that compounds

Table 2. Thermolysin Inhibitors and Inhibitory Constants

inhibitor no.	inhibitor	K_i (μM)	references
L-**10**	L-HCON(OH)-Leu-OMe	3070	[26]
D-**10**	D-HCON(OH)-Leu-OMe	44	[26]
L-**11**	L-HCON(OH)CH_2CH(CH_2Ph)CONHMe	1.66	[27]
D-**11**	D-HCON(OH)CH_2CH(CH_2Ph)CONHMe	68.2	[27]

whose structures resemble the transition state would be much more tightly bound to the enzyme than the substrate would [28]. Hence, analogs of a transition state in the pathway would be potent inhibitors for the enzyme by virtue of their high binding affinity towards the active site of the enzyme. Since the stereochemistry of substrate remains unchanged at the transition state, the transition state analog having the same stereochemistry as that of the substrate is expected to bind the enzyme more tightly than its enantiomer.

Numerous phosphorus derivatives of amino acids and peptides have been developed as inhibitors of zinc proteases since the discovery that phosphoramidon, *N*-(α-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan isolated from *Actinomycetes* culture filtrates is a potent inhibitor of thermolysin [29]. The X-ray crystallographic study of thermolysin·phosphoramidon complex established that the phosphoramidate group which ligates the active site zinc ion resembles the transition state for the enzyme catalyzed hydrolysis reaction [30]. A phosphonamidate analog of a CPA substrate, CbzGly-L-Phe, i.e., $CbzNHCH_2PO_2$-L-Phe (**7**) is a transition state analog inhibitor of CPA with the K_i value of 0.09 μM at pH 7.5 [31]. The strong affinity of this compound is attributed to the phosphonamidate moiety that resembles the tetrahedral transition state in the proposed mechanism for CPA catalyzed peptide hydrolysis (Fig. **2**). However, the mirror image form of this inhibitor has not been evaluated. Holmquist and Vallee reported that phenylphosphoramidate of Phe, i.e., $(PhO)PO_2$–Phe (**8**) is a potent CPA inhibitor [21]. As expected, the L-isomer is more potent with the K_i value of 2.1 μM than the D-isomer, the K_i value of which is 10 μM (Table 1).

2-Benzyl-3-formylpropanoic acid (**9**) was reported to be a potent competitive inhibitor for CPA by Garlardy and Kortylewicz who attributed the high binding affinity to the facile tendency of its formyl group, in aqueous solution, to form a *gem*-diol that mimics the tetrahedral transition state formed in the catalytic process [32]. Recently, Kim and Chung have found that its inhibitory activity resides mostly with the (*R*)-isomer that belongs to the L-series with the K_i value of 0.38 μM. The K_i value of (*S*)-isomer was found to be 256 μM (Table 1) [33].

IRREVERSIBLE INHIBITORS

Various types of inactivators for CPA have been reported in the literature, but most of them were evaluated as a racemic form or as single enantiomers having the same stereochemistry as that of substrate, i.e., the L-configuration. 2-Benzyl-3,4-epoxybutanoic acid (BEBA) is a highly potent and extremely fast acting CPA inactivator which has been designed by Kim and Kim [34,35]. In binding of the inactivator to the enzyme, its carboxylate forms hydrogen bonds with the guanidinium moiety of Arg-145 and the phenyl ring is fitted in the primary substrate recognition pocket (S_1' pocket). This binding mode would place the oxirane ring of BEBA at a position proximal to the active site zinc ion so that there forms a coordinative bond between them. As a result, the oxirane ring becomes susceptible to a nucleophilic attack. The carboxylate of Glu-270 that attacks

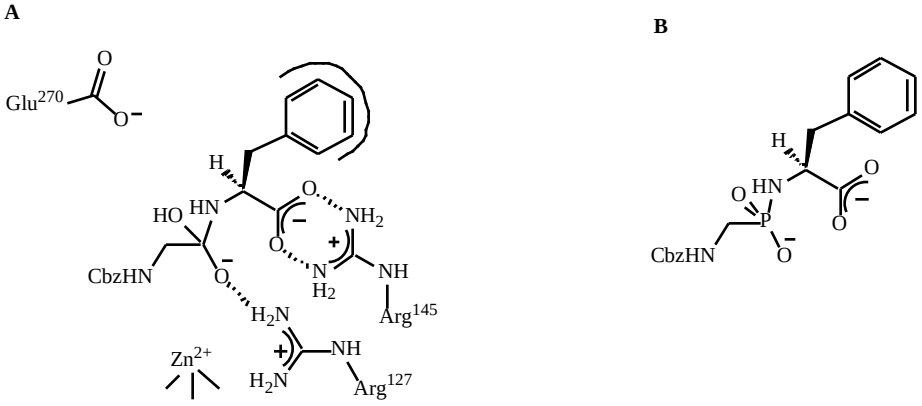

Fig. (2). (**A**) Hypothetic transition state in the CPA catalyzed hydrolysis of Cbz-Gly-L-Phe, a substrate of CPA. (**B**) The molecular structure of transition state analog inhibitor for CPA, $CbzNHCH_2PO_2$-L-Phe (7) resembles the structure of the hypothetical transition state in the CPA catalyzed hydrolysis reaction.

the scissile peptide carbonyl of substrate in the CPA catalyzed proteolytic process attacks at the 3-positon of the oxirane ring to result in a covalent attachment of BEBA to the enzyme with concurrent opening of the heterocycle (Fig. **3**). Of four stereoisomers of the inactivator, only two isomers in a mirror image relationship, namely, (2*S*,3*R*)- and (2*R*,3*S*)-BEBA were shown to have the CPA inactivating property with the k_{cat}/K_I values of 139.5 and 53.9 $M^{-1}s^{-1}$, respectively [35]. The X-ray crystal structure of the inactivated enzyme obtained by cocrystallizing the enzyme with the racemic BEBA supported the results of the kinetic studies, revealing that the carboxylate of Glu-270 in CPA is modified covalently in the form of ester by BEBA having the (2*S*,3*R*)-configuration [36]. The (2*S*)-configuration of BEBA corresponds to the configuration of D-Phe, which is not compatible with the spatial orientation of the CPA primary substrate recognition pocket that preferentially accommodates the benzyl aromatic ring of L-Phe. Mobashery and coworkers reported the design of 2-benzyl-3-iodoproanoic acid (BIPA) as a CPA affinity labeling agent [37]. Upon binding BIBA to the enzyme, the iodo group was thought to coordinate to the active site zinc ion, making it susceptible to nucleophilic substitution, possibly by the carboxylate of Glu-270 at the active site of CPA. The X-ray crystal structure of CPA inactivated by racemic BIPA established that the catalytic carboxylate of Glu-270 is modified in the form of ester with the inactivator [39]. Here again, BIPA that modified CPA was the enantiomer that belongs to the D-series.

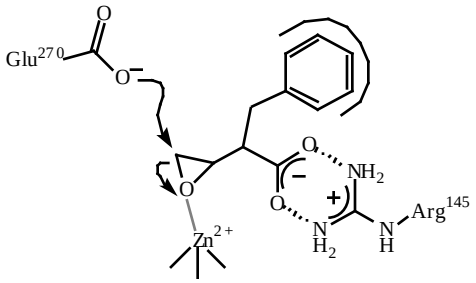

Fig. (3). The oxirane ring of CPA bound BEBA is activated by the active site zinc ion and would be subjected to a nucleophilic ring cleavage by the carboxylate of Glu-270 to result in the covalent modifiction of the carboxylate.

The X-ray crystal structure of CPA that was inactivated with BEBA having the (2*R*,3*S*)-configuration was subsequently determined and examination of the crystal structure in comparison with that inactivated with its enatiomer , i.e., (2*S*,3*R*)-BEBA showed that the two inhibitors are bound covalently to the enzyme in a symmetrical fashion [39]. When the structures of the active site region of the two inactivated CPAs were superimposed, no significant difference could be noted between the two structures except the positions of Tyr-248 and Ile-247 (Fig. **4**). The aromatic ring of Tyr-248 in the CPA inactivated by (2*S*,3*R*)-BEBA is noticeably pushed outward compared with that in the (2*R*,3*S*)-BEBA inactivated CPA [39]. It has been well established that the aromatic side chain of Tyr-248 is to function as a lid for the hydrophobic pocket at the S_1' subsite of CPA [11,40]. When a ligand anchors at the active site of CPA with its hydrophobic side chain being fitted in the pocket, the aromatic side chain of Tyr-248 moves downwards from the surface of the enzyme to the mouth of the S_1' pocket and closes it. The bound substrate is thus held at the active site until the enzymic catalytic chemistry is over. As the product is generated, the Tyr-248 side chain returns to the original position and the product is ejected into the reaction medium. However, in the inactivated CPA, the Tyr side chain remains at the mouth of the recognition pocket since the product is occupied. Expectedly, the benzyl group of (2*S*,3*R*)-BEBA would occupy the pocket in a unusual fashion: Since the carbon carrying the benzyl group has the stereochemistry corresponding to the D-series, the benzyl group in (2*S*,3*R*)-BEBA is projected into the spatial direction different from that of its preferred P_1' residue, i.e., L-Phe, and as a consequence the aromatic ring of Tyr-248 in the CPA inactivated (2*S*,3*R*)-BEBA is pushed outward in comparison with the Tyr-248 side chain in (2*R*,3*S*)-BEBA inactivated CPA. In the S_N2 type ring cleavage of oxirane, the nucleophile is expected to attack at the electrophilic center from the rear of the cleavable C–O bond. The finding that (2*S*,3*S*)- and (2*R*,3*R*)-BEBA fail to inactivate CPA may be rationalized on the ground that the C_4 reaction center of BEBA may not be rested at the position amenable for the S_N2 type ring opening reaction with the catalytic carboxylate.

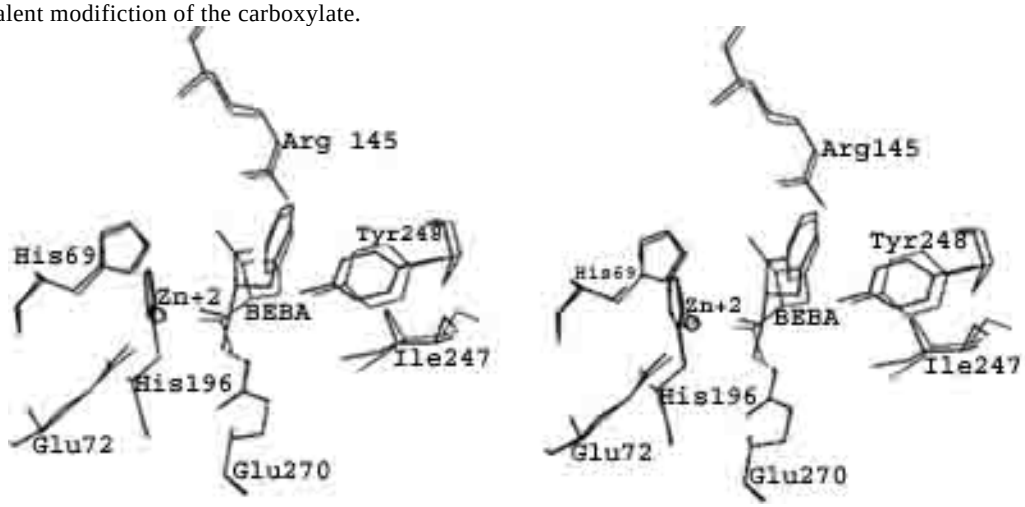

Fig. (4). Stereoview of the active site region of the inactivated CPA by the enantiomeric pair of BEBA. Thick line represents CPA inactivated by (2*R*,3*S*)-BEBA and thin line that inactivated by (2*S*,3*R*)-BEBA. Dashed line denotes the C–O covalent bond that is formed as a result of the Glu-270 carboxylate attacking on the oxirane ring of CPA bound BEBA.

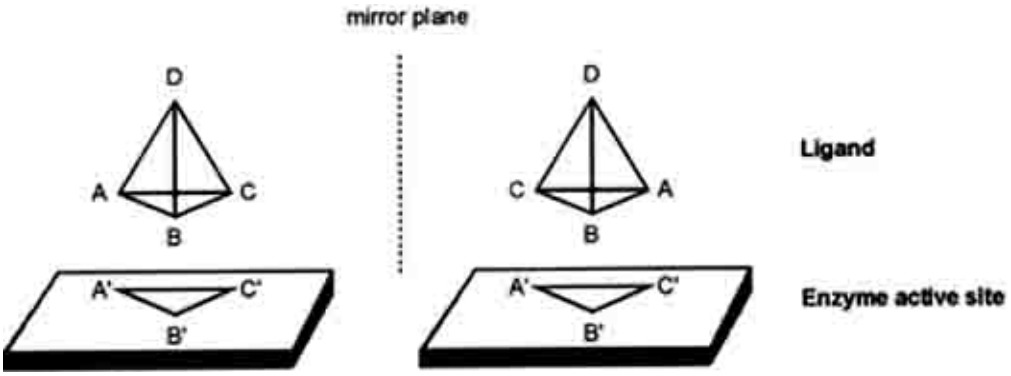

Fig. (5). Ogston's three-point fit model for enzyme stereospecificity. If one enantiomer, for example, the ligand on the left binds enzyme by interacting its three different binding groups (A, B, and C) with the respective binding sites (A′, B′, and C′) on the surface of an enzyme, its enantiomer fails to bind the enzyme because the binding groups do not match with the binding sites.

INHIBITORY STEREOCHEMISTRY AND THE THREE-POINT FIT MODEL

The substrate stereospecificity in enzymic reaction is thought to be effected because only one enantiomeric form of a dissymmetric substrate binds the enzyme at the active site, and this has been explained in terms of the three-point fit concept proposed by Ogston in 1948 [41]. Simply, the substrate stereospecificity is manifested as a result that the three different binding groups (A, B and C) at the stereogenic center of a chiral substrate can interact with the respective binding site (A′, B′ and C′) at the active site of the enzyme. Thus, the ligand on the left side in Fig. **5** can bind the active site, but its mirror image form shown on the right would not (Fig. **5**).

The Ogston's model serves well in explaining the stereospecificity of substrate analog inhibitors. This may be illustrated by binding of **3** to CPA. The L-isomer of **3** binds the active site of CPA much more strongly than its enantiomer because its three binding moieties, i.e., carboxylate, sulfhydryl, and benzene ring are arranged spatially in such a way that each of them can interact with the respective binding site present at the active site of CPA, namely, the guanidinium moiety of Arg-145, the zinc ion held by His-69, Glu-72 and His-196, and the S_1' pocket. In comparison, the three binding moieties in the mirror image form of **3**, i.e., D-**3** fails to interact simultaneously with the three binding sites at the active site in forming a complex with CPA because the spatial arrangement of the three binding groups in L-**3** does not match with the spatial arrangement of the binding sites at the CPA active site. The Ogston's concept is thus of great value for explaining the stereochemistry for substrate analog inhibitors, but may fail to explain or predict the stereochemistry of inactivators that undergo enzyme catalyzed chemical reactions in order for the enzyme to be inactivated. In the case of enzyme catalyzed substrate transformations, the step leading to product(s) should also be included in the consideration for understanding the stereospecificity. Thus, use of the specificity constant defined by the k_{cat}/K_M would be more appropriate in expressing the substrate stereospecificity [42]. The k_{cat} value reflects the unimolecular catalytic rate constant and the K_M represents the apparent equilibrium constant for the dissociation of the enzyme·substrate complex and reflects the effectiveness of binding of a substrate to the enzyme. In this connection, it may be worth noting that molecular mechanics calculations for the stereospecificity shown by α-chymotprysin in the catalytic hydrolysis of L- and D-*N*-acetyltryptophanamide revealed that the tetrahedral transition state formed by the L-substrate is energetically more stable than that formed from its enantiomer by 9.3 kcal mol^{-1}, suggesting that the stereospecificity in enzymic reaction is determined at the transition state rather than in the Michaelis complex formation [43].

CONCLUSION

As can be seen from the inhibitors of various types discussed above, the substrate analog inhibitors that bind the enzyme at the active site in competition with substrate exhibit stereospecificity that coincides with the stereochemistry of substrate, i.e., the L-configuration. However, exceptions to this general rule are found. For example, D-HCON(OH)Leu-OMe (**10**) shows much higher binding affinity (70-fold) than its enantiomer in the inhibition of thermolysin. The reversal of stereochemistry shown by the inhibitor appears to be due to the unique binding mode of the hydroxamate moiety to the active site zinc ion.

When it comes to inhibitory stereochemistry of irreversible inhibitors that modify the catalytic group by forming a covalent bond, the three-point fit concept may no longer be valid. This has been demonstrated by BEBA in which a pair of enatntionmers are nearly equally potent inactivators for CPA [35]. In fact, the most potent stereoisomer is the one in which stereochemistry at the α-position to the carboxylate bears the (*S*)-configuration that corresponds to the D-series [35,36]. Thus, prediction of inactivation stereochemistry is far more complicated and cannot be inferred simply from the stereospecificity of the substrate. As with the substrate stereospecificity, the stereochemistry of irreversible inhibitors may possibly by rationalized on the basis of the transition state structure that

plays a critical role in the covalent bond formation. It is prudent to state that medicinal chemists are urged to synthesize and evaluate all possible stereoisomers in the drug discovery research.

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