Differences in Binding Modes of Enantiomers of 1-Acetamido Boronic Acid Based Protease Inhibitors: Crystal Structures of γ-Chymotrypsin and Subtilisin Carlsberg Complexes[†,‡]

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ABSTRACT: In order to probe the structural basis of stereoselectivity in the serine protease family, a series of enantiomeric boronic acids $RCH_2CH(NHCOCH_3)B(OH)_2$ has been synthesized and kinetically characterized as transition-state analog inhibitors using α-chymotrypsin and subtilisin Carlsberg as model systems. When the R-substituent in this series was changed from a *p*-chlorophenyl to a 1-naphthyl group, α-chymotrypsin, but not subtilisin, reversed its usual preference for L-enantiomers and bound more tightly to the D-enantiomer [Martichonok, V., & Jones, J. B. (1996) *J. Am. Chem. Soc. 118*, 950−958]. The structural factors responsible for the differences in stereoselectivity between the two enzymes have been explored by X-ray crystallographic examination of subtilisin Carlsberg and γ-chymotrypsin complexes of the L- and D-enantiomers of *p*-chlorophenyl and 1-naphthyl boronic acid derivatives. In both enzymes, the L-isomers of the inhibitors, which are more closely related to the natural L-amino acid substrates, form tetrahedral adducts, covalently linking the central boron atom and O_γ of the catalytic serine. The D-isomers, however, differ in the way they interact with subtilisin or γ-chymotrypsin. With subtilisin, both the D-*p*-chlorophenyl and D-1-naphthyl inhibitor complexes form covalent Ser O_γ-to-boron bonds, but with γ-chymotrypsin, the same inhibitors lead to novel tetrahedral adducts covalently linking both Ser195 O_γ and His57 $N_{\epsilon 2}$ covalently via the boron atom.

Two important fields for the use or manipulation of enzymes are synthetic organic chemistry (Faber, 1995; Wong & Whitesides, 1994) and regulation of enzymatic activity by drugs (Verlinde & Hol, 1994; Kurinov & Harrison, 1994). In either case the substrate is generally a small organic compound. Organic synthesis involves the formation of unique molecules, especially in asymmetric synthesis with its broad demand for chiral synthons. In the search for promising drugs, pharmacological considerations often require selection of single enantiomers. While much structural information is available on the interaction of enzymes with their natural substrates or effectors, less is known about the binding and recognition of designed, unnatural ligands and the catalytic mechanisms involved in their reactions.

Hydrolytic enzymes have become popular tools in synthetic chemistry due to their selectivity and stereospecificity (Faber, 1995; Wong & Whitesides, 1994). They also play essential roles in a multitude of medically important processes, ranging from blood clotting and coagulation (Grutter et al., 1990) to Alzheimer's disease (Hynes et al., 1990) and brain tumor development (Yakamoto et al., 1994), making this class of enzymes an obvious focus for structure-based drug design. The factors determining the structural specificities and stereoselectivities of the enzymes need to be identified and understood in order to provide tailormade chiral catalysts to the synthetic chemist or to achieve the goal of designing effective drugs.

Serine proteases are one of the most widely studied classes of hydrolytic enzymes. A large body of information has been assembled on their crystal structures as well as on ligand binding and kinetic mechanisms (Fersht, 1985). Subtilisin Carlsberg and chymotrypsin are classical representatives of this enzyme class and are well suited to identify the

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[‡] Atomic coordinates have been deposited with the Brookhaven Protein Data Bank under entry codes 1VGC, 2VGC, 3VGC, 4VGC, 1VSB, 1AV7, 3VSB, and 1AVT.

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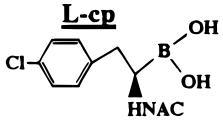
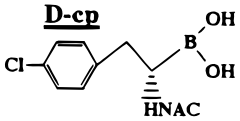
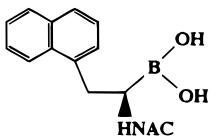
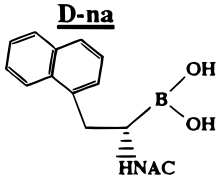
Table 1: Structure of Inhibitors and Kinetic Data[a]

inhibitor	γ-chymotrypsin K_i (μM)	subtilisin
L-cp 	1.20 ± 0.05	0.15 ± 0.01
D-cp 	5.76 ± 0.23	91.3 ± 5.4
L-na 	3.11 ± 0.09	0.88 ± 0.02
D-na 	0.127 ± 0.03	56.4 ± 3.0

[a] Data from Martichonok and Jones (1996).

determinants of enzyme specificity. Both enzymes have highly specific cleavage sites, preferring large hydrophobic amino acids in the S_1 site [S_1 is the specificity subsite; P_1 is the amino acid side chain binding in S_1 (Schechter & Berger, 1967)]. Furthermore, both are highly stereoselective, exhibiting a strong catalytic preference for L- over D-amino acid substrates, and both use the same chemical hydrolysis mechanism, involving the classical Ser-His-Asp triad.

Boronic acids are potent transition-state inhibitors of serine proteases (Bone et al., 1989; Bachovchin et al., 1988; Tsilikounas et al., 1992) and have been applied extensively in our laboratories as probes of serine protease specificity (Seufer-Wasserthal et al., 1994; Martichonok & Jones, 1996). Recently, a kinetic evaluation of the factors controlling the stereoselectivities of subtilisin Carlsberg and α-chymotrypsin was made with a series of novel L- and D-boronic acid inhibitors. These included L-(1*R*)-(L-cp)[1] and D-[(1*S*)-1-acetamido-2-(*p*-chlorophenyl)ethyl]boronic acid (D-cp) as well as L-(1*R*)- (L-na) and D-[(1*S*)-1-acetamido-2-(1-naphthyl)ethyl]boronic acid (D-na) (Table 1). Subtilisin maintained its natural L-enantiomer preference for both the cp− and na−inhibitor series. The stereoselectivity of chymotrypsin, however, switched from L for the chlorophenyl compounds to D for the naphthyl-substituted inhibitors (Martichonok & Jones, 1996). This unexpected reversal of stereochemical preference was examined by molecular modeling studies in a first attempt to identify the factors responsible. These computational studies on the enzyme−inhibitor (EI) complexes pointed to variations in the hydrogen-bonding patterns and to a reversal of the orientation of the naphthyl ring as potential determinants of the switch in stereo preference for chymotrypsin. A limitation of this approach was the lack of proper potential functions which restricted calculations to covalent serine adducts. There is, however, evidence from NMR and X-ray crystallography that other covalent complexes are possible between serine proteases and boronic acid inhibitors (Bone et al., 1987, 1989; Takahashi et al., 1989; Tsilikounas et al., 1992). Tsilikounas et al. (1992) proposed that the properties of the specificity pockets are responsible for placing those inhibitors which closely mimic natural substrates into a position favoring a covalent bond between boron and the catalytic serine residue. Inhibitors that deviate from native substrate-like structures may be forced into a binding mode that could lead to the formation of histidine−boron adducts. Studies have shown that a covalent bond from the serine O_γ to the boron atom can coexist with a long coordinate−covalent bond between boron and histidine $N_{\epsilon 2}$ (Bachovchin et al., 1988; Bone et al., 1989; Takahashi et al., 1989).

In order to answer these questions of binding mode and stereoselectivity reversal (Martichonok & Jones, 1996), we have determined the crystal structures of subtilisin Carlsberg and γ-chymotrypsin complexed with the L- and D-enantiomers of both the *p*-chlorophenyl- and the naphthyl-1-acetamido boronic acids.

EXPERIMENTAL PROCEDURES

Enzyme Crystallizations and Inhibitor Complexes. Subtilisin Carlsberg (type VIII; EC 3.4.21.62) was purchased from Sigma (St. Louis, MO) and cocrystallized with inhibitors in large-volume batch setups using conditions as described by Petsko and Tsernoglou (1976). The crystals are orthorhombic, space group $P2_12_12_1$, with unit cell axes $a = 53.0$ Å, $b = 55.4$ Å, and $c = 76.6$ Å. To conform to the axis labeling convention $a < b < c$, the starting model was reindexed. α-Chymotrypsin (EC 3.4.21.1) was purchased from Sigma, converted to γ-chymotrypsin, and crystallized as previously described (Stoddard et al., 1990). The crystals are tetragonal, $P4_22_12$, with unit cell axes of $a = b = 69.5$ Å and $c = 97.5$ Å.

The inhibitors were synthesized and purified as previously described (Martichonok & Jones, 1996). Crystals of γ-chymotrypsin were harvested and soaked in standard solutions of 2.9 M ammonium sulfate, 50 mM cacodylate, pH 6.5, and 5−10% dimethyl sulfoxide with inhibitor concentrations ranging from 1 to 17 mM.

Data Collection and Structure Refinement. Crystals were mounted in thin-walled glass capillaries (Supper, Natwick, MA). Data for all chymotrypsin complexes and for the complexes of subtilisin Carlsberg with the L- and D-naphthyl inhibitors were collected on a Siemens X1000 multiwire area detector at room temperature using Cu Kα radiation from a rotating anode (RU200H, Rigaku, Japan; 0.2 × 2 mm^2 focal spot, 37 kV, 70 mA). Radiation was Ni-filtered and focused using Franks mirror optics. Data were reduced with an updated version of the program package XDS (Kabsch, 1988a,b).

Data for the subtilisin L- and D-*p*-chlorophenyl inhibitor complexes were collected at Synchrotron radiation sources, the D-cp complexes at CHESS (F_1 beamline, Cornell University, Ithaca, NY), and the L-cp complex at the X11 beamline of EMBL/DESY (Hamburg, FRG). Data were

[1] Abbreviations: L-cp, L-[(1*R*)-1-acetamido-2-(*p*-chlorophenyl)ethyl]-boronic acid; D-cap, D-[(1*S*)-1-acetamido-2-(*p*-chlorophenyl)ethyl]-boronic acid; L-na, L-[(1*R*)-1-acetamido-2-(1-naphthyl)ethyl]boronic acid; D-na, D-[(1*S*)-1-acetamido-2-(1-naphthyl)ethyl]boronic acid.

Table 2: Data Collection and Crystallographic Refinement Statistics for All Inhibitors Bound to either Chymotrypsin or Subtilisin[a]

	chymotryspin				subtilisin			
inhibitor	L-cp	D-cp	L-na	D-na	L-cp	D-cp	L-na	D-na
resolution range refinement (Å)	10−1.9	10−1.8	10−1.7	10−2.1	8−2.1	8−2.0	8−2.65	8−2.6
unique reflections	21972	22354	25288	14525	15529	14662	5346	5426
completeness (%)	95.5	88.4	85.2	89.7	98.0	95.6	83.2	77.0
R_{sym}	6.8	8.2	6.6	7.5	7.8	8.2	12.1	9.1
R_{cryst} (%)	19.6	21.0	20.1	19.3	17.1	15.2	15.0	15.5
R_{free} (%)	25.0	25.4	24.3	26.8	24.2	22.0	27.4	29.9
esd of bonds (Å)	0.011	0.010	0.010	0.011	0.010	0.009	0.011	0.011
esd of angles (deg)	1.67	1.69	1.61	1.67	1.65	1.48	1.71	1.81
sulfate/metals[b]	2	2	2	2	2	2	2	2
waters	88	95	95	83	84	158	25	22

[a] $R_{sym} = \sum_{i,hkl} (|I(i,hkl) - \langle I(i,hkl)\rangle|/\sum_{i,hkl}\langle I(hkl)\rangle$. $R_{cryst} = \sum|F_o - F_c|/\sum F_o$. Only reflections with $I > 1.0\sigma I$ were used in the analysis. [b] Chymotrypsin has two sulfates and subtilisin two sodium ions bound.

processed using the program package DENZO (Otwinowski, 1993; Minor, 1993).

All complex crystals were isomorphous with the free enzymes. Atomic models obtained from the Brookhaven Protein Data Bank [3GCH with cinnimate inhibitor omitted for γ-chymotrypsin (Stoddard et al., 1990) and 1SCA for subtilisin (Fitzpatrick et al., 1993)] were used as a starting point for refinement. The structure of native γ-chymotrypsin, including a cleaved peptide product, was refined using the coordinates 1GMC (Yennawar et al., 1993). As this peptide overlaps with the inhibitor binding site, we took great care in our study to distinguish between the two, based on the presence of inhibitor-specific electron density. The program suite X-PLOR (Brünger, 1992) was used for all refinements. Manual fitting was performed using the graphics programs TURBO-FRODO (Jones, 1978) and O (Jones et al., 1991). The weights restraining the B−N and B−O bonds were greatly relaxed in the final cycles of refinement. Waters, metal atoms, and sulfates were included if they showed good electron density at a level of 1.0σ and were at an appropriate distance from donor/acceptor atoms. Only water molecules with $B < 60$ Å^2 were accepted in the final models. The R_{free} value was followed throughout the course of the refinement to monitor progress and avoid overfitting (Kleywegt & Brünger, 1996). For subtilisin, loop 152−162 was poorly defined in the original maps and was modeled into a simulated annealing omit map. Inhibitors were initially modeled into simulated annealing omit maps, followed by iterative rounds of refinement and manual fitting. Data collection parameters and refinement statistics are given in Table 2.

RESULTS

Electron Density Maps. In all cases, electron density for the bond between the O_γ of the catalytic serine and boron and the arrangement of the boron substituents were well-defined, along with the aromatic ring groups (Figures 1 and 2) in simulated annealing omit maps. For both D-complexes with γ-chymotrypsin, electron density unambiguously showed two covalent bonds between boron of the inhibitor and Ser195 O_γ and His57 $N_{\epsilon 2}$ (Figure 2C,D).

Subtilisin Carlsberg. The four subtilisin structures all contained a covalent bond from Ser221 O_γ to boron, forming tetrahedral complexes. One of the boron hydroxyl groups interacts with the catalytic histidine, His64 $N_{\epsilon 2}$ and a water molecule, and the second hydroxyl occupies the oxyanion hole, with bonds to Ser221 NH and Asn155 $N_{\delta 2}$. The aromatic rings of the inhibitors occupy the S_1 binding pocket of the enzyme (Figure 3).

For the L-inhibitors, L-*p*-chlorophenyl (L-cp) and L-naphthyl (L-na) bind as the natural peptide substrates, with strong hydrogen bonds from the 1-acetamido amide to the backbone carbonyl of Ser125. The D-inhibitors, D-*p*-chlorophenyl (D-cp) and D-naphthyl (D-na), are not analogous to the natural substrate. In these complexes, the interaction between inhibitor amide and protein backbone is absent.

The aromatic rings of the inhibitors interact with the S_1 pocket primarily through van der Waals contacts to main chain Cα's of Gly127 and Gly154. In L-cp and D-cp, the chlorine atom is hydrogen-bonded to Gly128 NH at distances of 3.5 and 3.8 Å, respectively. The chlorophenyl rings are slightly tilted with respect to each other, causing this variation. In L-na and D-na, the two naphthyl rings are in different orientations but overall make the same contacts with the pocket (Figure 4). No other atoms are closer than 3.5 Å to the inhibitor, demonstrating the open nature of the site. Overlaying the two L-structures and or two D-structures shows that the inhibitors of one enantiomer superimpose well, with the *p*-chlorophenyl rings centrally positioned over the naphthyl rings.

γ-Chymotrypsin Complexes. Five chymotrypsin structures were determined, one native structure including peptide bound in the active site and four inhibitor complexes. Electron density for the native structure was compatible with the peptide sequence Tyr-Ala-Gly-Pro given by Yennawar et al. (1993). This native density was used as a benchmark for subsequent comparison with possible inhibitor complex structures.

All four inhibitor complexes involve a 1.4 Å covalent bond from boron to the catalytic serine Ser195 O_γ, as for a natural peptide substrate. The aromatic ring groups all bind in the S_1 pocket (Figure 5A,B). For the L-inhibitors, the boron forms a tetrahedral arrangement with one hydroxyl group hydrogen-bonded to His57 $N_{\epsilon 2}$, displacing a water molecule. The other binds in the oxyanion hole, with bonds to backbone amides of Gly193, Asp194, and Ser195. The 1-acetamido groups mimic a peptide backbone, hydrogen bonding (3.0 and 3.5 Å, respectively) with Ser214 O. The aromatic rings make van der Waals contacts with residues Val213 C_γ and Gly216 C_α of the S_1 pocket. This arrangement is analogous to the tetrahedral transition state in the catalytic cycle of the enzyme (Matthews et al., 1975; Bone et al., 1987; Tulinsky

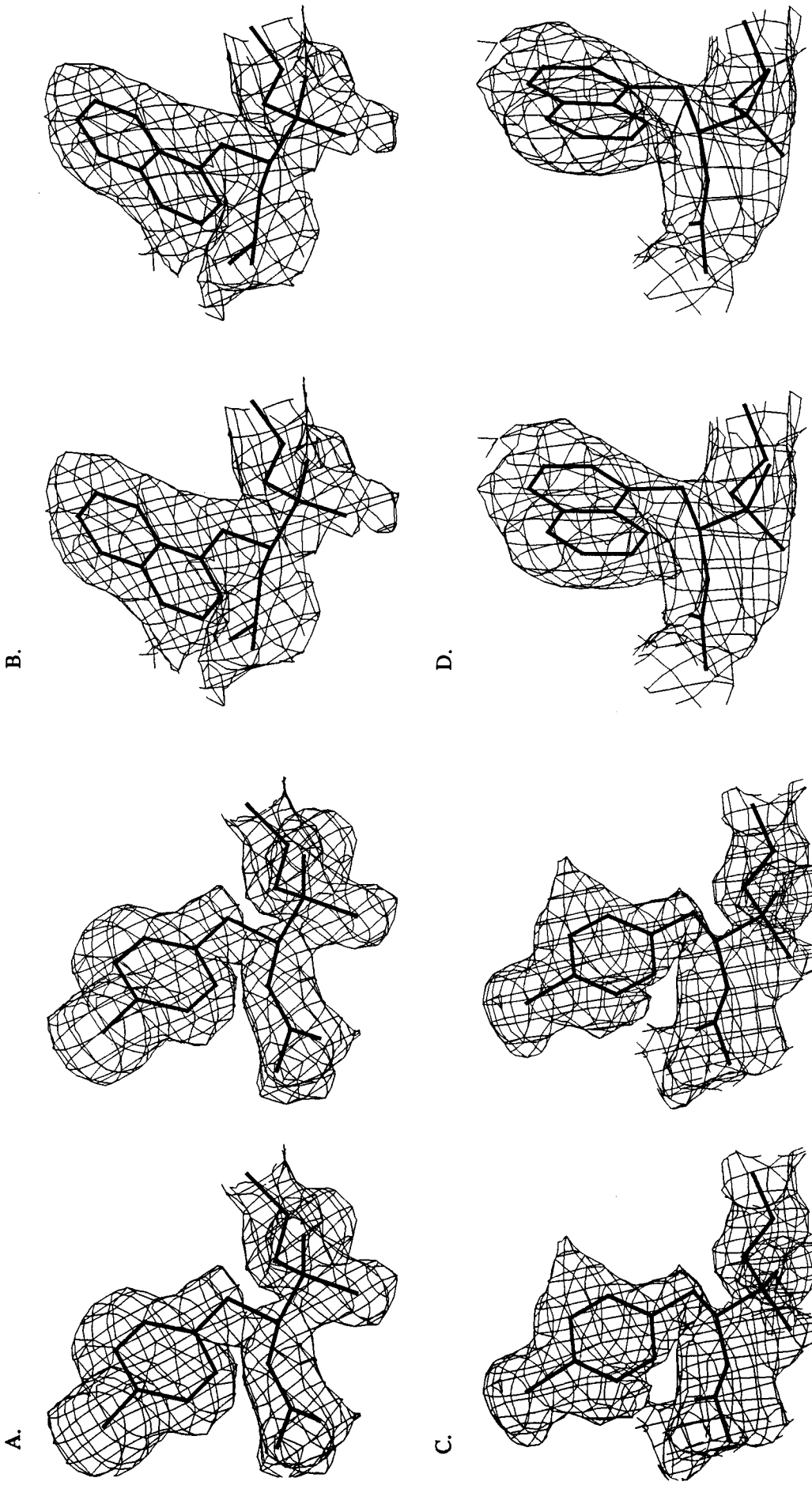


FIGURE 1: Electron density for subtilisin complexes, from $F_o - F_c$ maps contoured at a 2.0σ level where all atoms of the inhibitor were removed from structure factor calculation: (A) L-cp; (B) L-na; (C) D-cp; (D) D-na.

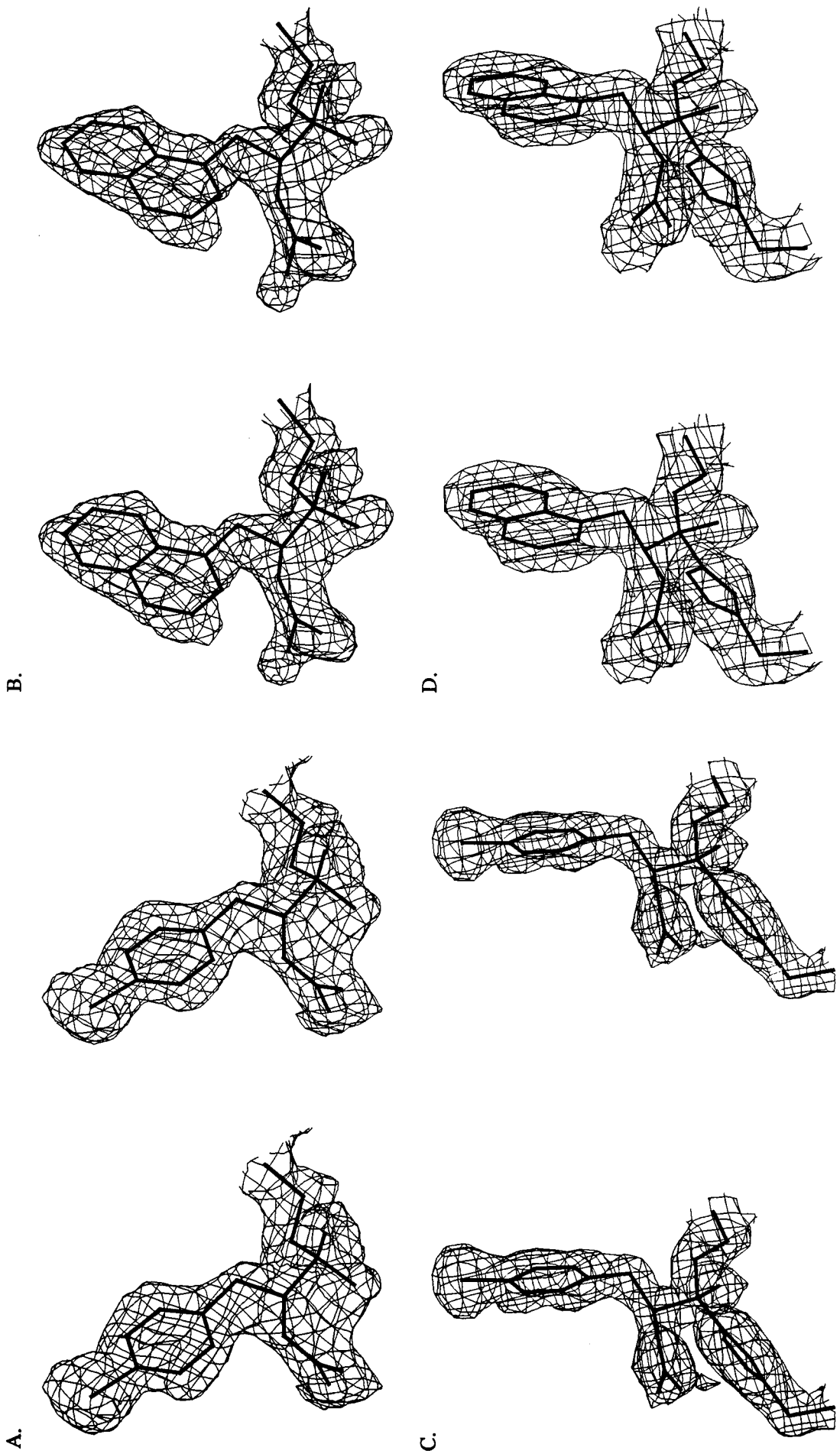


FIGURE 2: Electron density for chymotrypsin complexes, from $F_o - F_c$ maps contoured at a 2.0σ level where all atoms of the inhibitor were removed from structure factor calculation: (A) L-cp; (B) L-na; (C) D-cp; (D) D-na.

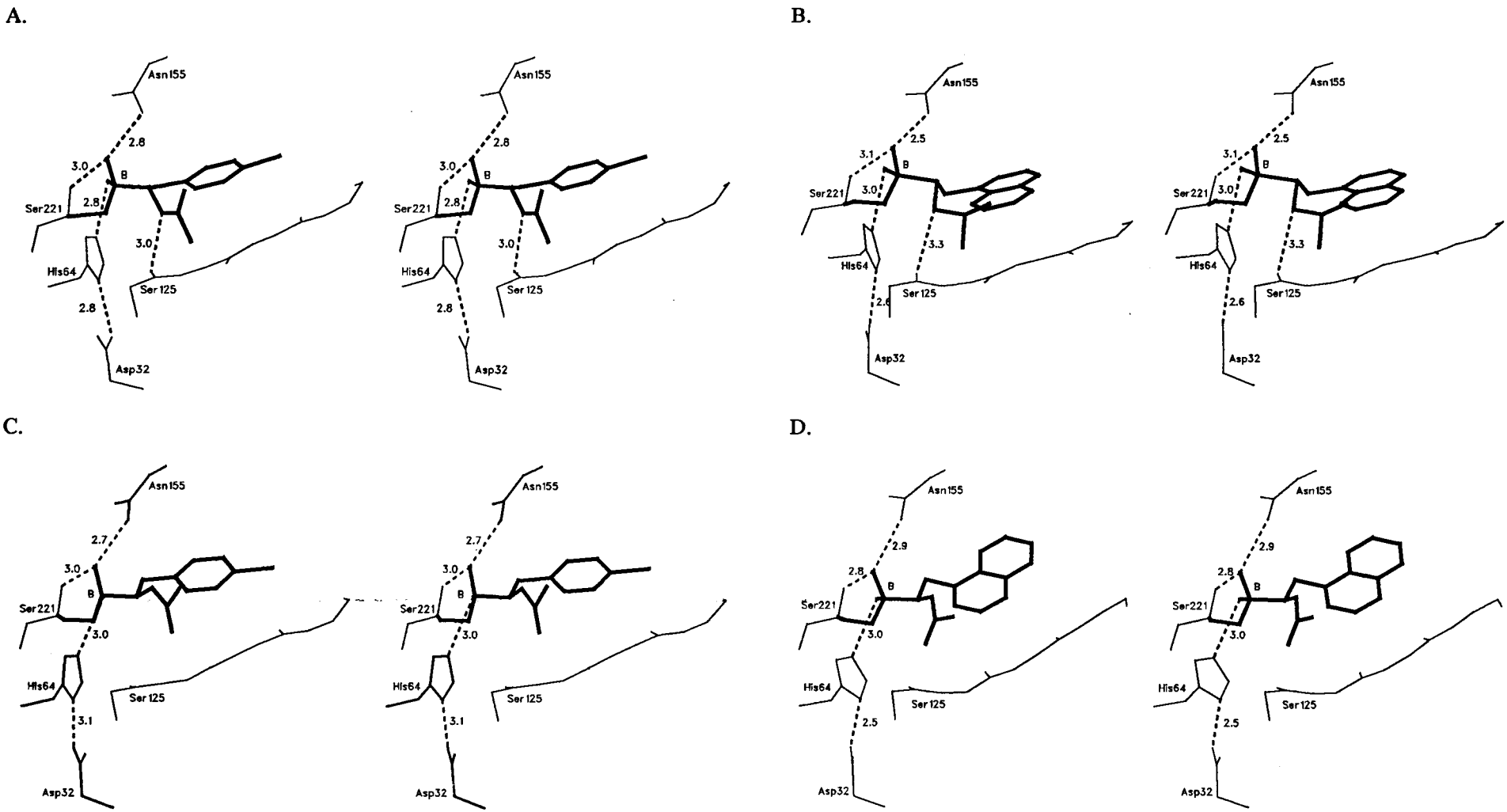


FIGURE 3: Subtilisin Carlsberg complexes showing tetrahedral coordination at boron and the covalent bond to Ser221: (A) L-cp; (B) L-na; (C) D-cp; (D) D-na.

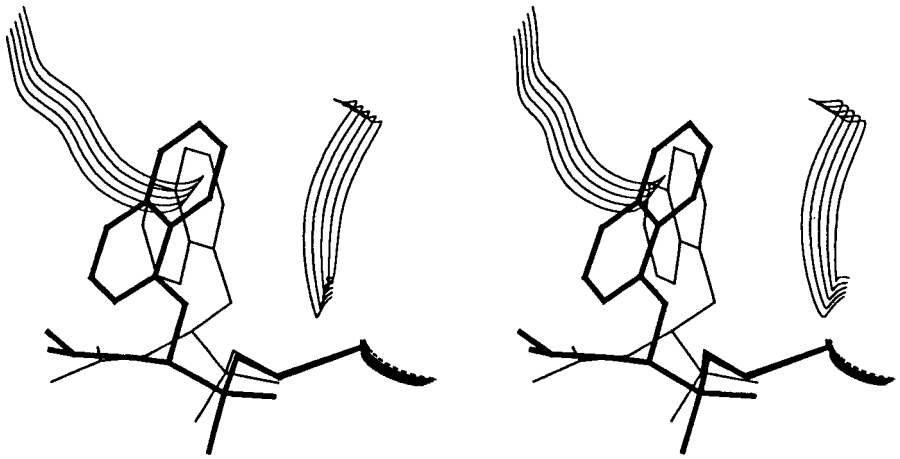


FIGURE 4: Overlay of L-na (thin lines) and D-na (thick lines) complexes with subtilisin Carlsberg.

& Blevins, 1987). In L-cp, the chlorine atom is hydrogen-bonded to Ser217 NH, Ser189 O_γ and several water molecules. Both L-cp and L-na superimpose well with a bound natural peptide substrate where tyrosine occupies the S_1 site. Overlaying L-cp and the tyrosine gives an rmsd of 0.4 Å for the rings and 0.6 Å for rings plus acetamido groups.

For the D-inhibitors, a second covalent bond from boron to His57 $N_{\epsilon 2}$ (bond distance 1.6 Å) is formed (Figure 5C,D). The geometry about boron remains tetrahedral, with the displacement of one hydroxyl group. The second covalent bond in the diadduct requires a movement of the inhibitor with a slight shift away from the S_1 pocket toward the catalytic triad (Figure 6). In the case of D-na, this results in a rotation of Met192 about the $C_\alpha-C_\beta$ bond which then makes a hydrophobic contact to the naphthyl ring. The hydrogen bonds to the chlorine of the *p*-chlorophenyl group are maintained for D-cp as in L-cp. There is no hydrogen bond from the acetamido amide to Ser214 O in the D enantiomers.

DISCUSSION

Subtilisin Carlsberg Structures. Kinetic experiments (Martichonok & Jones, 1996) showed that the L-isomers in our series of boronic acids are better inhibitors of subtilisin than the D-enantiomers (0.15 and 0.88 μM vs 91.3 and 56.4 μM), combining appropriate stereochemistry with large aromatic side chains preferred by subtilisin for binding in the S_1 pocket. While the K_i of L-cp is 6 times lower than that of L-na, both are good inhibitors with submicromolar inhibition constants. The key to the stability of these complexes seems to be the hydrogen bond from the acetamido ligand to the backbone carbonyl of Ser125. The slight preference for L-cp over L-na may be attributable to the presence of the electronegative chlorine, which can interact with the backbone amide of Gly128. Kinetic studies of a series of aryl boronic acids have shown that addition of an electronegative substituent improves binding to subtilisin (Seufer-Wasserthal et al., 1994). Both D-cp and D-na are poor inhibitors, with comparable K_i's and no interaction between inhibitor amide and protein backbone. For both the D- and L-series, the structure of the enzyme remains essentially unchanged in all inhibitor complexes; a coordinate difference plot for C_α atoms shows a maximum deviation of 0.7 Å, and the side chains of the catalytic triad superimpose well.

γ-Chymotrypsin Structures. Although α-chymotrypsin was used for the initial kinetic analyses (Martichonok & Jones, 1996), γ-chymotrypsin was chosen for the structural studies because in this crystal form the active site of the enzyme is more accessible to ligands (Dixon & Matthews, 1989). Structural differences in the active sites of the α- and γ-forms of chymotrypsin are minimal (Matthews et al., 1967; Cohen et al., 1981; Dixon & Matthews, 1989; Harel et al., 1991). The crystallographic asymmetry of the α-chymotrypsin dimer (Tulinsky et al., 1987) would have added another complication to the interpretation of the soaking experiments, which was avoided by using γ-chymotrypsin.

In both L-complexes, one of the boron hydroxyl groups displaces a water molecule found close to His57 in both α- and γ-chymotrypsin (Tulinsky & Blevins, 1987; Nakagawa et al., 1993) which has been implicated in the catalytic mechanism (Nakagawa et al., 1993; Singer et al., 1993). The presence of a boron hydroxyl group in the oxyanion hole is a standard feature found with many boronic acid inhibitor complexes of serine proteases (Bachovchin et al., 1988; Bone et al., 1989; Tsilikounas et al., 1992). The low inhibition constants of the L-inhibitors reflect the accuracy with which they mimic the natural peptide substrate's covalent intermediate.

In contrast to our findings for the L-series, complexes of γ-chymotrypsin with the D-series of inhibitors form two covalent bonds to Ser195 O_γ and His57 $N_{\epsilon 2}$. The remaining ligand in the tetrahedral configuration about boron is a single hydroxyl group. For both the D-cp and the D-na inhibitors, we observe $B-N_{\epsilon 2}$ bond lengths of 1.6 Å, far shorter than other B−His57 $N_{\epsilon 2}$ reported interactions (Bone et al., 1989; Takahashi et al., 1989; Tsilikounas et al., 1992). This represents the first time an EI complex with two covalent bonds (a dicovalent adduct) has been observed crystallographically. $B-N_{\epsilon 2}$ interactions of approximately 2.2 Å have previously been described as a dative coordinate−covalent bond for α-lytic protease and elastase (Bone et al., 1989; Takahashi et al., 1989; Tsilikounas et al., 1992). The Ser $O_\gamma-B$ bond distance in our dicovalent adduct structures is approximately 1.4 Å, consistent with known values of an O−B single bond (Allen & Kennard, 1993). Previous crystallographic studies could not unequivocally distinguish between trigonal or tetrahedral configurations of the boron ligands (Bone et al., 1989; Takahashi et al., 1989); however,

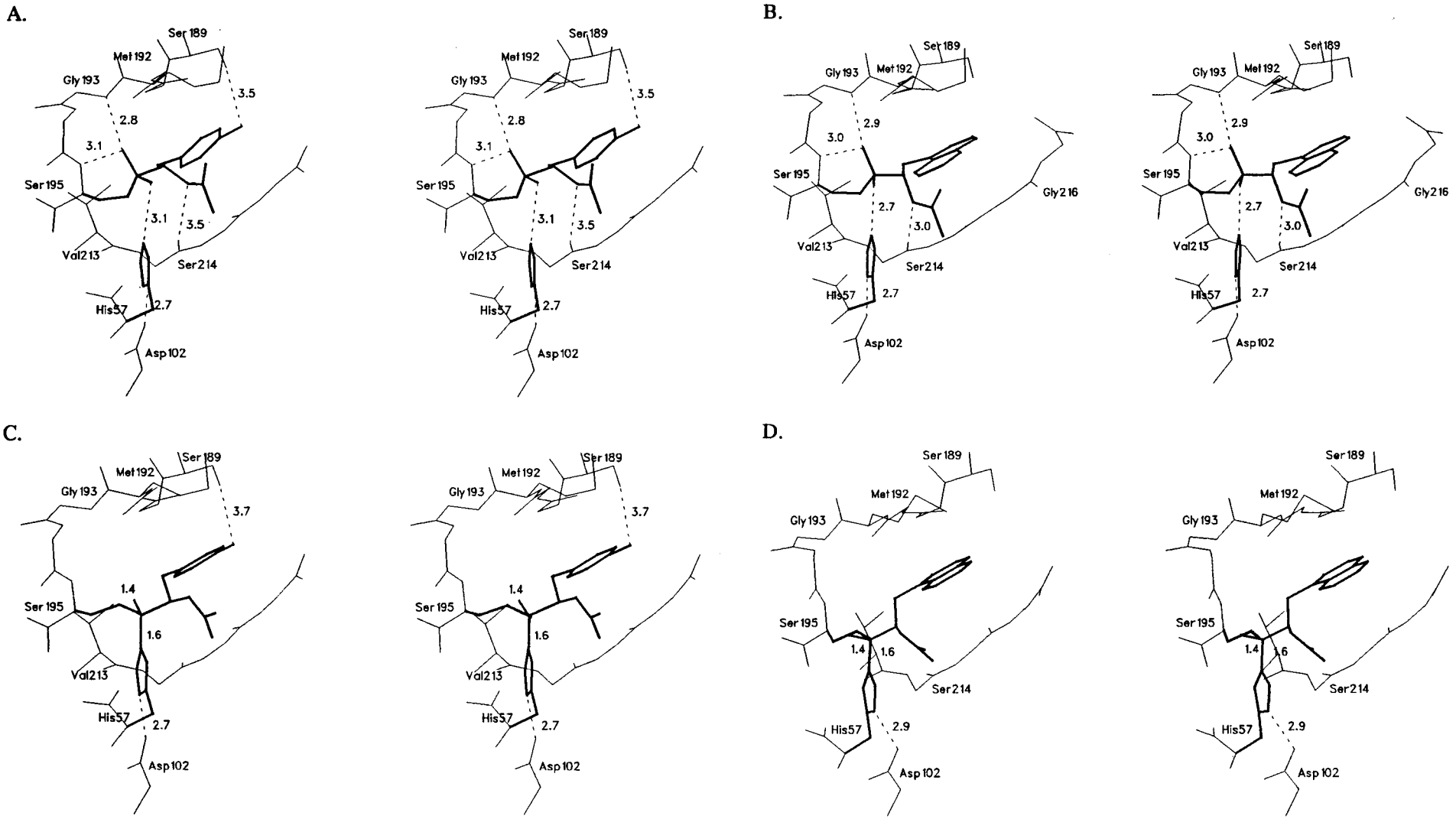


FIGURE 5: γ-Chymotrypsin complexes showing tetrahedral coordination at boron and the covalent bond to Ser195 for L complexes, (A) L-cp and (B) L-na, and dicovalent adducts with bonds to Ser195 and His57 in the D complexes, (C) D-cp and (D) D-na.

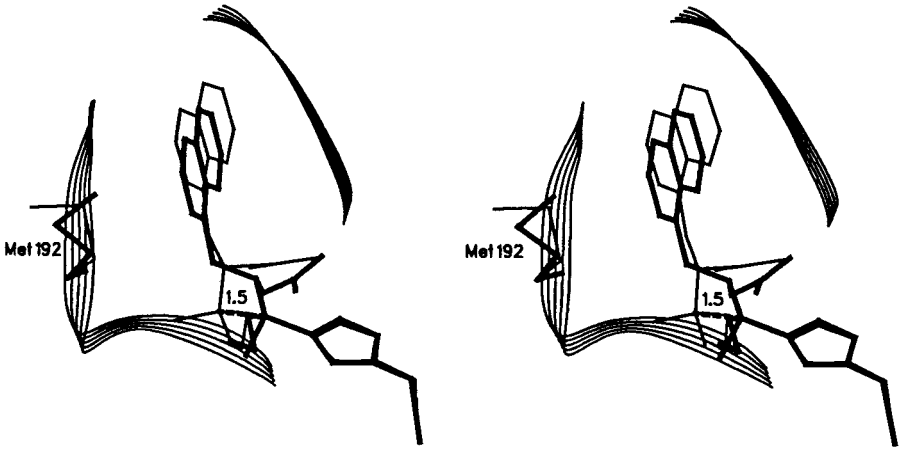


FIGURE 6: Overlay of L-na (thin lines) and D-na (thick lines) complexes with γ-chymotrypsin.

subsequent ^{11}B NMR studies have resolved the debate in favor of a tetrahedral boron configuration (Tsilikounas et al., 1993). In our crystallographic study, the configuration of the boron substituents was determined to be a slightly distorted tetrahedron.

Binding Modes of Boronic Acid Inhibitors. The exact mode of binding of boronic acid inhibitors to serine proteases has been the subject of intense and controversial discussions, with the nature of covalent bonds (serine vs histidine; mixture of both or true dicovalent adducts) at the center of these disputes. Tsilikounas et al. (1992) have suggested that compounds resembling natural substrates will make good transition-state analogs, binding to the catalytic serine within the family of trypsin-like serine proteases. Nonsubstrate-like compounds are proposed to form unspecified histidine adducts. This hypothesis was based on the premise that the binding of P_1 in the S_1 pocket determines the orientation of the boron atom. Good substrate analogs bind in the S_1 binding pocket with the boron atom positioned for nucleophilic attack by the serine. Nonsubstrate analogs can bind in the S_1 site with the boron atom closer to the histidine, making $B-N_{\epsilon 2}$ bond formation possible.

Our crystallographic results support and extend this picture. Chymotrypsin conforms with the proposal of Tsilikounas et al. (1992), with the L-inhibitors, which mimic the natural substrates, forming tetrahedral serine adducts, while the D-inhibitors form dicovalent adducts to both His57 and Ser195. This model, however, cannot be extended to the family of serine proteases as a whole. Subtilisin, which is not a trypsin-like serine protease (Rawlings & Barrett, 1994), does not conform, and both the L- and D-series form serine adducts.

Active Site Topology. Comparing the topology of the active sites provides some insight to the absence of histidine adducts with subtilisin. Subtilisin's substrate binding site is a relatively shallow groove on the protein surface (Figure 7, bottom) with fewer constraints on P_1 binding and more conformational freedom of the remainder of the inhibitor. In contrast, chymotrypsin's active site is a deep cave (Figure 7, top), and the binding of the P_1 side chain is more restricted. Consistent with this, the L- and D-naphthyl rings in the subtilisin complexes have weaker electron densities and higher *B*-factors than their chymotrypsin counterparts (average 30.74 vs 19.98 Å^2). Furthermore, only serine adducts have ever been observed in reactions of boronic acids with

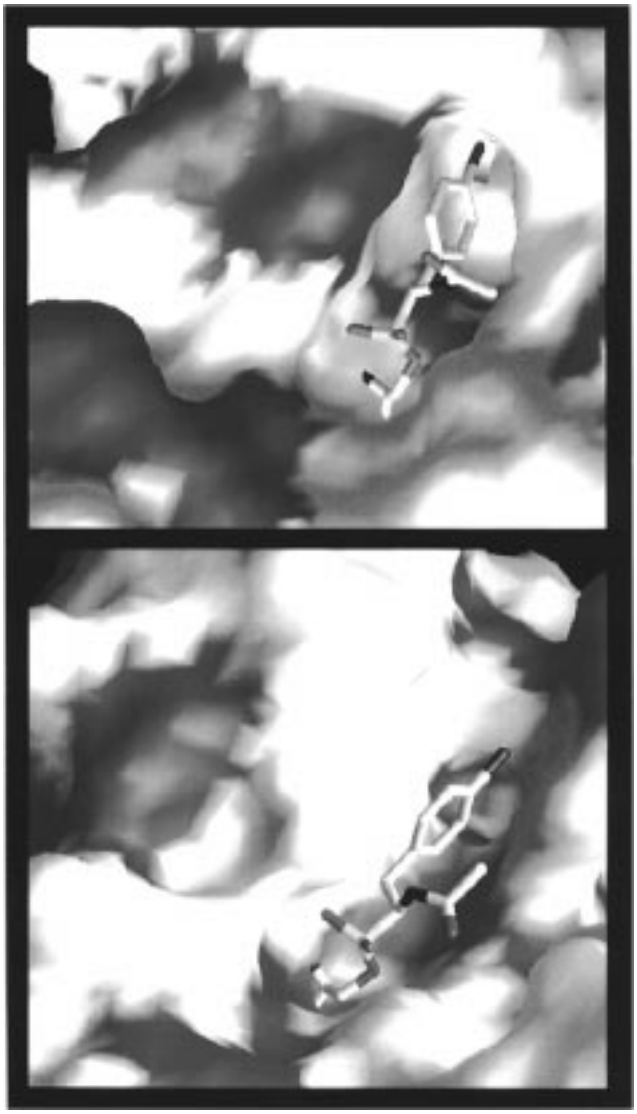


FIGURE 7: Active site surfaces of the enzymes showing difference in topology: (top) γ-chymotrypsin; (bottom) subtilisin Carlsberg. Hydrophobic regions are shown in cyan.

subtilisin (Matthews et al., 1975). A histidine adduct for a complex with a benzeneboronic acid was proposed (Phillip & Bender, 1971) but was not supported by subsequent crystallographic studies (Matthews et al., 1975). The constraints on P_1 binding in the S_1 pocket (i.e., the shape of the active site) do appear to influence the type of adduct

that is formed by nonsubstrate-like analogs. In other words, an enzyme with a more open active site (e.g., in subtilisin) is less likely to form histidine adducts with nonsubstrate-like inhibitors than an enzyme with a more constrained active site (e.g., chymotrypsin). Active site topology and surface area are clearly important considerations when designing inhibitors for this class of enzymes (Nienaber et al., 1996).

While generally described as a hydrophobic pocket, the S_1 binding site in chymotrypsin and subtilisin is more neutral than oily. Such hydrophobic contacts as exist involve C_α carbons of nearby glycine residues or the carbon of a backbone carbonyl oriented with the oxygen away from the pocket. The pocket is lined with a relatively high proportion of glycines (2/10 for chymotrypsin and 4/10 for subtilisin). For most of the remaining residues, side chains are oriented away from the pocket and have no interactions with the inhibitor. Exceptions are Met192, Val213, and Ser189 in chymotrypsin. In chymotrypsin, the bottom of the "cave" is formed by a tyrosine ring; however, this is ≈6 Å away from the bound inhibitors so it cannot be contributing any stabilizing interactions.

In addition to binding site topology, subtle movements in the active sites of serine proteases upon binding of substrates or inhibitors (Tsilikounas et al., 1992; Katz et al., 1995) may contribute to the type of adduct formed. Tsilikounas et al. (1992) proposed that a synergistic change in the protein structure occurs in the S_1 pocket upon side chain binding. Good inhibitors (or natural substrates), having an appropriate side chain in the specificity subsite, will cause a conformational change in the region of the catalytic serine and oxyanion hole which stabilizes the tetrahedral transition state. The evolutionary advantage of this induced fit mechanism would be to improve the specificity of the enzyme. This is a difficult point to address with chymotrypsin as the native enzyme contains residual bound peptide, and whatever changes occur upon substrate binding may already have taken place. However, because the peptide in uninhibited γ-chymotrypsin is bound to Ser195 in a trigonal ester arrangement, it is possible that additional active site movements take place to accommodate the tetrahedral transition state or transition-state analog. A coordinate difference plot of C_α positions comparing native chymotrypsin and its D-na complex gives an rmsd of ≈0.4 Å in the region of the active site compared to ≈0.2 Å for other regions. This is consistent with the scale of movements observed for trypsin bound to a series of boronic acid inhibitors (Katz et al., 1995). As well, there is the rotation of Met192 in the D-na complex mentioned previously which may contribute to its higher stability over D-cp. Such subtle changes were also observed in α-lytic protease and in trypsinogen where the rotation of a methionine generated a hydrophobic surface, thereby improving the binding for hydrophobic inhibitors (Bone et al., 1989; Tsilikounas et al., 1992). No trends of changes in enzyme structure were observed in subtilisin, further evidence that the open binding pocket is less demanding in its P_1 requirements.

Kinetics. While our crystallographic structures are necessarily an equilibrium state, they can be used in interpreting the kinetic data since the reactions with our inhibitors were clearly competitive (Martichonok & Jones, 1996). There was no evidence of slow binding, consistent with other studies of boronic acid inhibitors where slow−fast binding was observed only in cases where inhibitors incorporated three or more amino acids [for example, Bone et al. (1991)].

When comparing K_i's for the enantiomeric pairs of inhibitors, we see a much larger range for subtilisin than for chymotrypsin. Subtilisin shows a 616-fold increase in K_i on going from L-cp to D-cp and a 64-fold increase for the related naphthyl compounds. The kinetic data in Table 1 show that chymotrypsin undergoes a switch in stereo preference from L- to D-inhibitors when the *p*-chlorophenyl ring of the inhibitor is substituted by a naphthyl group, but the differences are much less dramatic. L-cp is only 5-fold higher than D-cp, and L-na is 24-fold lower than D-na. We propose that the relatively small differences in K_i's for chymotrypsin are the result of the formation of an additional covalent bond with His57, stabilizing the EI complex and compensating for the loss of the acetamido amide−Ser214 carbonyl hydrogen bond. The larger differences in K_i's for subtilisin reflect that no histidine adducts are formed.

The kinetic analysis of subtilisin with a series of boronic acid inhibitors with and without the acetamido group allows us to evaluate the contributions of the various functional groups and their interactions to binding specificity. The data show that a hydrogen bond between an acetamido amide and backbone carbonyl oxygen of the enzyme can lower the K_i 100-fold (Seufer-Wasserthal et al., 1994; Martichonok & Jones, 1996). The formation of the serine−boron bond alone does not contribute as much to the binding energy of these inhibitors as either the acetamido interaction or the addition of the His57 $N_{\epsilon 2}$−B bond. In their kinetic analysis of subtilisin, Seufer-Wasserthal et al. (1994) found that a boronic acid, in which a methyl had been substituted for the acetamido group, had a K_i of 13 ± 1.3 mM. Given the small size of the methyl group, most of the overall binding energy can be attributed to the formation of the serine−boron covalent bond. A comparison of this K_i to that of *p*-chlorobenzyl boronic acid for subtilisin ($K_i = 19 \pm 1\ \mu$M) demonstrates the contributions of the van der Waals interactions of the aromatic ring. Again, this is independent of the presence or absence of the acetamido group.

The situation with chymotrypsin is more complicated because two effects counteract each other. Although the D-inhibitors lack the acetamido amide hydrogen bond, they form an additional covalent bond to His57. Comparing the K_i's of L-na to D-na allows a qualitative estimate of the respective contributions of the covalent bond to histidine and the acetamido hydrogen bond. The naphthyl rings of these two inhibitors are in a similar environment (Figure 8), eliminating hydrophobic contributions as an explanation for the inversion of the K_i's. There is a weak hydrogen bond (3.6 Å) between the carbonyl of the acetamido group of D-na and His57 $N_{\epsilon 2}$, but we conclude from the 24-fold difference in K_i that the formation of the second covalent bond contributes more to the stability of the D-na complex than the acetamido hydrogen bond in the L-na complex. In the *p*-chlorophenyl complexes, the two interactions effectively counterbalance each other. The energetic advantage of the His−B bond formation is offset, possibly by a less favorable position of the *p*-chlorophenyl group of D-cp, which is displaced 0.7 Å toward the S_1 pocket opening.

Within the D-series, D-na has a lower K_i than D-cp. We attribute this difference to the additional van der Waals interaction of the naphthyl ring with Met192. This, in

conjunction with the formation of a dicovalent adduct, makes D-na the most potent inhibitor tested in this study and, in fact, one of the best inhibitors of chymotrypsin yet observed.

Comparison to Modeling Studies. As our experiments were prompted by the unexpected kinetic results of Martichonok and Jones, it is of interest to compare the predictions of their modeling studies with the crystallographic results presented here, bearing in mind that the computational approach was restricted to covalent serine adducts. In the case of the L-series of inhibitors where these restrictions were valid, our experiments are in general agreement. The hydrogen bond between the enzyme and the inhibitor's acetamido group as well as the predicted interactions of the oxygen atoms of the boronic acids is observed. However, since covalent bonding to the active site histidine residue was not considered, in cases where histidine adducts are formed, the computational approach was based on invalid premises.

In the case of subtilisin, modeling studies predicted the absence of the acetamido hydrogen bond between the inhibitor and Ser125 O in the D-complexes, with no difference in binding of boron hydroxyl groups in the oxyanion hole. For both enzymes, possible orientations (left- vs right-handed) of the naphthyl rings in the S_1 pockets were considered. The calculations suggested that the naphthyl rings for L- and D-inhibitors would be oriented in opposite directions for subtilisin (Figure 4) but not for chymotrypsin (Figure 6). Our crystal structure analyses fully concurred with these aspects of the modeling studies.

CONCLUSIONS

We have identified a novel dicovalent adduct formed by the reaction of D-boronic acid inhibitors with γ-chymotrypsin, in which the boron atom bridges the serine and histidine side chains of the catalytic triad. For all subtilisin complexes and for chymotrypsin with L-boronic acids, the expected monoadducts are formed with covalent bonds to serine only. The fact that we observe a true covalent cross-link, His57−B−Ser195, with chymotrypsin and not with subtilisin is attributed to the topology of the active site, where the S_1 specificity subsite in chymotrypsin is more enclosed, placing greater constraints on P_1 orientation. The open "groove" of subtilisin is more forgiving in its substrate requirements. This underscores the differences between classes of serine proteases, reminding us that they cannot all be treated alike. The kinetic data can be interpreted in light of these results. The narrow range of K_i and the fact that all four boronic acids are good inhibitors of chymotrypsin reflect the energetic advantage of the second covalent bond in the D-isomers, which compensates for the absent acetamido−backbone carbonyl hydrogen bond of the L-isomers. In subtilisin, since no diadducts are formed, the D-isomers are poor inhibitors of the enzyme. Finally, the switch in stereospecificity for chymotrypsin in going from the *p*-chlorophenyl to naphthyl group bound in S_1 is attributed to the introduction of one extra van der Waals interaction, resulting from the rotation of the side chain of Met192 in the D-na complex. This illustrates the importance of subtle changes in protein structure upon substrate binding which, along with active site topology and surface area, must be considered when designing truly discriminating inhibitors of serine proteases.

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