Direct observation by X-ray analysis of the tetrahedral "intermediate" of aspartic proteinases

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Abstract

We report the X-ray analysis at 2.0 Å resolution for crystals of the aspartic proteinase endothiapepsin (EC 3.4.23.6) complexed with a potent difluorostatone-containing tripeptide renin inhibitor (CP-81,282). The scissile bond surrogate, an electrophilic ketone, is hydrated in the complex. The pro-(R) (statine-like) hydroxyl of the tetrahedral carbonyl hydrate is hydrogen-bonded to both active-site aspartates 32 and 215 in the position occupied by a water in the native enzyme. The second hydroxyl oxygen of the hydrate is hydrogen-bonded only to the outer oxygen of Asp 32. These experimental data provide a basis for a model of the tetrahedral intermediate in aspartic proteinase-mediated cleavage of the amide bond. This indicates a mechanism in which Asp 32 is the proton donor and Asp 215 carboxylate polarizes a bound water for nucleophilic attack. The mechanism involves a carboxylate (Asp 32) that is stabilized by extensive hydrogen bonding, rather than an oxyanion derivative of the peptide as in serine proteinase catalysis.

Keywords: aspartic proteinase; mechanism; renin inhibitor; tetrahedral intermediate; X-ray analysis

The synthesis of inhibitors that mimic the putative tetrahedral intermediate of aspartic proteinase catalysis is an important objective in drug design. The monomeric aspartic proteinase renin is an attractive target in antihypertensive therapy, and inhibition of the dimeric retroviral proteinase from human immunodeficiency virus (HIV) seems certain to be important in the treatment of acquired immune deficiency syndrome (AIDS). Both classes of aspartic proteinase are characterized by two catalytic aspartic acid residues (see Kinemage 1) at the center of an extended active-site cleft (James & Sielecki, 1983; Andreeva et al., 1984; Blundell et al., 1985; Suguna et al., 1987a; Miller et al., 1989; Wlodawer et al., 1989; Lapatto et al., 1989; Davies, 1990). X-ray analyses have defined structures of aspartic proteinase inhibitor complexes in which extended peptide inhibitors have several residues hydrogen-bonded on either side of the scissile bond surrogate (Bott et al., 1982; James et al., 1982; Suguna et al.,

1987a; Blundell et al., 1987; Foundling et al., 1987; Cooper et al., 1989; Šali et al., 1989).

Aspartic proteinases can be characterized by their inhibition by the microbially produced natural product pepstatin, which contains the dipeptide analogue statine (Umezawa et al., 1970). Early structure–activity studies on peptide inhibitors containing statine (Rich et al., 1980) and the homologous hydroxyethylene dipeptide isostere (Szelke et al., 1983), together with the X-ray structural determination of complexes of pepstatin and a pepstatin fragment (Bott et al., 1982; James et al., 1982) suggested that such structures mimic the putative tetrahedral intermediate, which forms part of most mechanistic proposals for this class of enzymes (James & Sielecki, 1985; Pearl & Blundell, 1984; Polgar, 1987; Suguna et al., 1987b). The hydroxyl oxygen of statine occupies the position of water formerly bound to the catalytic aspartates of the native enzyme. Such inhibitors, however, possess one of the two hydroxyl residues of the putative tetrahedral proteolysis intermediate.

Interest in inhibitors containing both hydroxyls (e.g., the gem-diol unit) of the tetrahedral intermediate was

stimulated by direct NMR observation (Rich et al., 1982; Holladay et al., 1985) that porcine pepsin binds ketone analogues ($-CO-CH_2-$) of these inhibitors (statones) in the tetrahedral hydrate form ($-C(OH)_2-CH_2-$). Little exchange of ^{18}O into inhibitor recovered from one such experiment indicated both hydrate formation and decomposition to be highly stereoselective and hence enzyme-catalyzed processes. The weaker binding to pepsin and renin of statones relative to the hydroxy analogues was attributed (Rich et al., 1985) to the unfavorable equilibrium for hydration of the ketone function. Indeed, peptides containing the more readily hydrated 2,2-difluorostatone moiety ($-C(OH)_2-CF_2-$) were subsequently found to be 50-1,000-fold more potent against both porcine pepsin and renin than the nonfluorinated statone analogues (Gelb et al., 1985; Thairivongs et al., 1985, 1986; Fearon et al., 1987).

The stability of these gem-diol complexes derived from a difluorostatone moiety, together with their probable resemblance to the tetrahedral proteolysis intermediate ($-C(OH)_2-NH-$), makes them attractive for crystallization and high-resolution X-ray analysis. To this end we have synthesized a potent difluorostatone-containing tripeptide, CP-81,282 (morpholino-4-carbonyl-Phe-Nle-cyclohexyldifluorostatone-N-methylamide). This substance is 10-fold more potent against renin and 90-fold more potent against endothiapepsin than the corresponding alcohol having the statine-like hydroxyl configuration (Fig. 1).

We now describe the high-resolution X-ray structure of the complex between the fungal aspartic proteinase endo-

thiapepsin and this potent difluorostatone-containing tripeptide renin inhibitor. Our study shows that the pro-(R) (statine-like) hydroxyl of the tetrahedral carbonyl hydrate is hydrogen-bonded to both active-site aspartates 32 and 215 in the position occupied by a water in the native enzyme. The second hydroxyl oxygen of the hydrate is hydrogen-bonded only to the carboxyl oxygen of Asp 32 that is most distant from Asp 215 (the outer oxygen) at the catalytic center. These experimental data provide a basis for a model of the tetrahedral intermediate in aspartic proteinase-mediated cleavage of the amide bond.

Results and discussion

The high potency of the difluorostatone analogue is reflected in the IC_{50}'s of CP-81,282 and its equivalent (R)- and (S)-alcohols. The IC_{50}'s of CP-81,282 for human plasma renin and endothiapepsin are 1 and 11 nM, respectively. The potency of the difluorostatone is most probably a consequence of its low barrier for formation of the tetrahedral hydrate ($K_{hydration} = 2.1$ in 5:1 DMSO–water and 0.6 in wet chloroform as determined by ^{19}F NMR), which binds tightly to these enzymes. Alternatively, the (R) (statine-like) alcohol has IC_{50}'s of 10 and 920 nM, whereas the (S)-alcohol has values of 420 and 6,200 nM (Fig. 1).

The complex with the difluorostatone crystallized in a form that is isomorphous to the native endothiapepsin (Blundell et al., 1990). X-ray data were collected to 2.0 Å resolution and merged resulting in a 76% complete data set with a merging R-factor of 7.4% based on the intensities. The structure was solved using difference Fourier techniques and least-squares refinement using data from 20 Å to 2.0 Å (16,999 reflections). The final R-factor of 0.18 and correlation coefficient of 0.91 were obtained for a model containing 306 water molecules. The coordinates have satisfactory geometry; root mean square (rms) residuals are 0.021 Å for bond lengths, 0.023 Å for bond 'angle' distances, 0.032 Å for nonbonded contacts, 0.005 Å for main-chain planes and 0.004 Å for side-chain planes. Unrestrained temperature factor refinement resulted in mean B_{iso} values of 17.2 $Å^2$ for the enzyme, 15.7 $Å^2$ for the inhibitor, and 43.0 $Å^2$ for the waters. The bound structure of the inhibitor is illustrated in Figure 2 and Kinemage 2.

The inhibitor is bound in an extended conformation. Residues P_3 to P_1 form an antiparallel beta-sheet with residues 217-219 of the enzyme, and a hydrogen bond is made between P_2' NH and the CO of Gly 34. Further hydrogen bonds are made with the extended hairpin known as the flap at residues 76 and 77. The interactions of the inhibitor other than those of the tetrahedral hydrate will not be described here in further detail; they are in general similar to those found in other aspartic proteinase inhibitor complexes (Bott et al., 1982; James et al., 1982; Foundling et al., 1987; Suguna et al., 1987b; Šali et al., 1989).
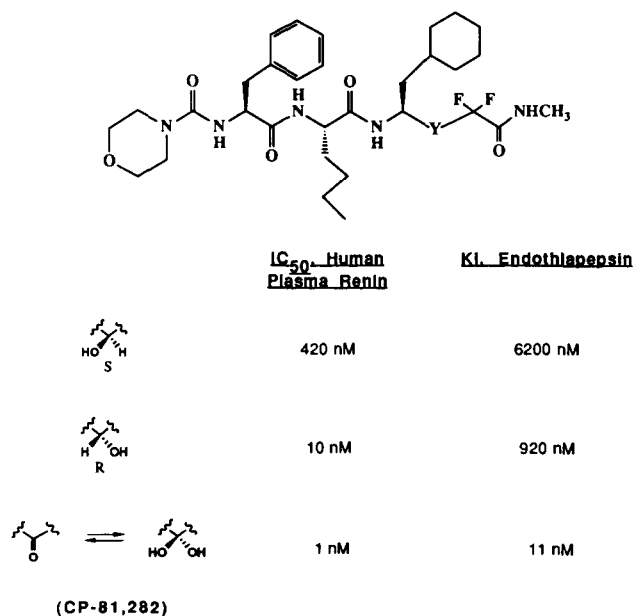

	IC_{50}, Human Plasma Renin	KI, Endothiapepsin
HO⟍⟍H S	420 nM	6200 nM
H⟍⟍OH R	10 nM	920 nM
O ⇌ HO⟍OH	1 nM	11 nM

(CP-81,282)

Fig. 1. CP-81,282 and its related alcohols. The full chemical name of CP-81,282 is 4(S)-[[[[N-(morpholino-1-carbonyl)-L-phenylalanyl]-L-nor-leucyl]amino]-5-cyclohexyl-2,2-difluoro-3-oxopentanoyl]-methylamine.
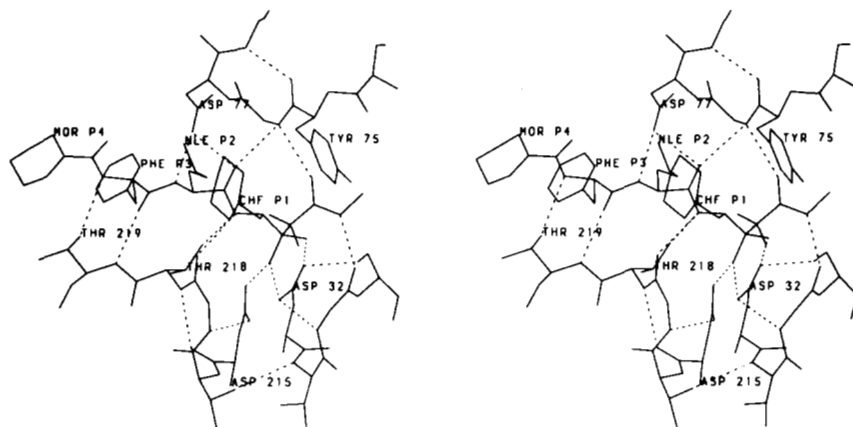
Fig. 2. Hydrogen bond interactions made by the inhibitor with the enzyme active-site cleft.

Figure 3 shows that the inhibitor is bound as the hydrate with the hydroxyls of the gem-diol function associating tightly with the catalytic carboxyl residues of aspartates 32 and 215. The pro-(R) hydroxyl oxygen occupies the same position as the corresponding hydroxyl oxygen in endothiapepsin complexes of inhibitors containing statine (e.g., Cooper et al., 1989) or the hydroxyethylene dipeptide isostere (Blundell et al., 1987; Veerapandian et al., 1990). This oxygen atom of the inhibitor is within 3.5 Å of all four carboxyl oxygens, in an equivalent position to the oxygen atom of water in the native enzyme (Pearl and Blundell, 1984; Blundell et al., 1990). The distances to the two inner carboxylate oxygens are consistent with hydrogen bonds, but the shortest distance (2.6 Å) is to the outer oxygen of Asp 215 (Fig. 3). The second hydroxyl oxygen of the hydrate is located at an equivalent distance (2.6 Å) from the outer carboxyl oxygen of Asp 32 (Fig. 3).

Because X-ray analyses of proteins at 2.0 Å resolution cannot locate hydrogens, their positions must be inferred from those of the heavier atoms. One proton originates from the catalytic aspartates at pH 4.5, and two protons derive from the hydrate hydroxyls. Their positions should
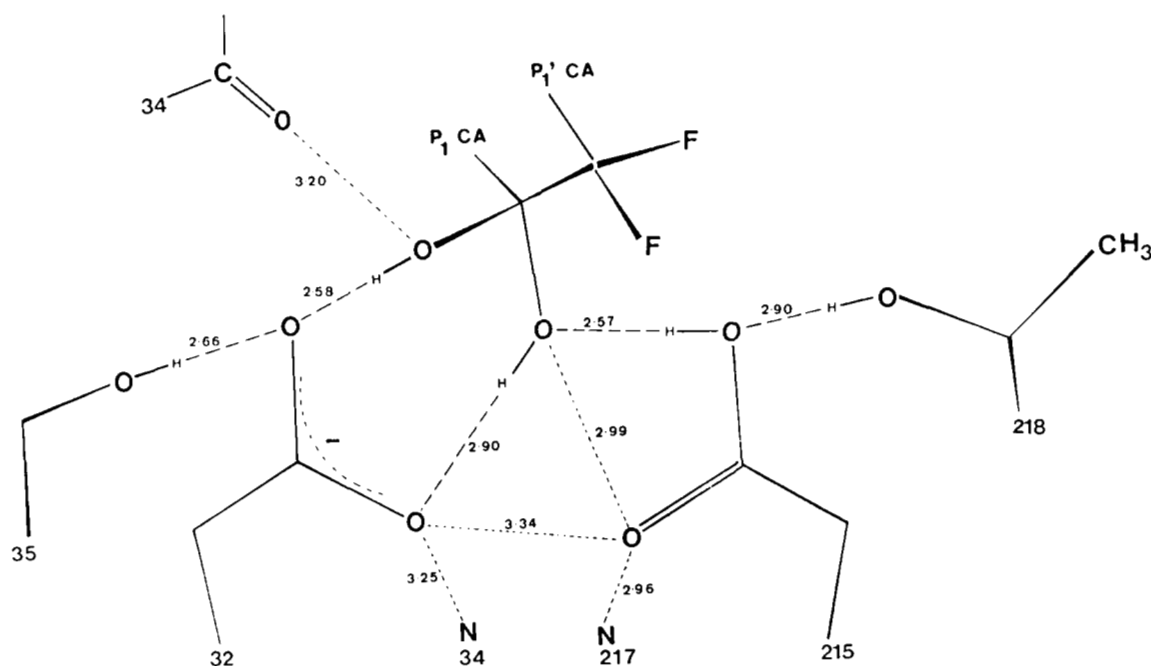

Fig. 3. The stereochemistry and interactions of the tetrahedral hydrate with the enzyme. Interatomic distances are taken from the refined structure, which has an estimated coordinate error of 0.2 Å. The input for a Gaussian 88 calculation was created from the X-ray positions for the carboxy groups of Asp 32 and 215, and, from the statine residue, the two hydroxyl oxygens, the two fluorines, and the two carbons they are attached to. Hydrogen atoms were then added using standard bond lengths and angles to form two possible complexes (the one shown, and an alternative arrangement in which Asp 32 is protonated and Asp 215 charged).

be consistent with the shorter distances that indicate hydrogen bonds stabilizing the tetrahedral hydrate. Short oxygen–oxygen interatomic distances of about 2.6 Å strongly indicate the approximate locations of two of these protons, one between the outer oxygen of Asp 215 and the statine-like (pro-R) hydroxyl oxygen of the hydrate, and the other between the outer oxygen of Asp 32 and the second (pro-S) hydrate oxygen. The observed interatomic distances suggest that the third proton is located between the statine-like hydrate oxygen and either of the inner oxygens of the aspartates. The interaction of this proton with the inner oxygen of Asp 215, however, is unlikely. At any dihedral angle of the C–O bond of the statine-like hydroxyl where an acceptable C–O–H angle (approximately 109°) is maintained, this proton is closer to either the outer oxygen of 215 or the inner of 32. This proton is therefore most probably directed instead to the inner oxygen of Asp 32 (2.9 Å). A molecular orbital calculation (see legend to Fig. 3) indicates a favorable O–H–O bond angle of 173° for this proton.

Two possible complexes with differing locations of the negative charge result from this arrangement. In the first (depicted in Fig. 3), a negatively charged Asp 32 is stabilized by four hydrogen bonds, two from the hydrate hydroxyls, one from Ser 35 γ-OH, and one from Gly 34 NH. The outer oxygen of protonated Asp 215 donates a hydrogen bond to the statine-like hydroxyl. In the second (not shown), a negatively charged Asp 215 is stabilized by three hydrogen bonds, one from the statine-like hydroxyl of the hydrate, one from Thr 218 γ-OH, and one from Gly 217 NH, whereas the inner oxygen of protonated Asp 32 donates a hydrogen to the statine-like hydroxyl, and the outer hydroxyl of the hydrate donates a further hydrogen bond to the outer oxygen of Asp 32. Thus, although these alternative complexes differ only in the proximity of each of the three protons to one or the other of the pairs of interacting oxygens, there is significant additional stabilization of the negative charge in the first. In the second, the O–H bond of the statine-like hydrate hydroxyl is also nearly eclipsed with the main-chain $CH(OH)_2$–CF_2 carbon–carbon bond (dihedral angle 33°). The preferred positions of the protons are shown in Figure 3. Molecular orbital calculations favor this arrangement by 16 kcal/mol using STO-4G basis set and by 8 kcal/mol using the 6–31G basis set and indicate that these bonds are staggered (dihedral angle 148°).

In the preferred arrangement, the strong hydrogen bond between the second hydrate oxygen and Asp 32 could contribute to its 100-fold greater binding affinity to endothiapepsin compared to the analogous difluorostatine (where this hydroxyl is replaced by a proton). The greater activity of the R-hydroxy (statine-like) alcohol compared to the S-hydroxy isomer (see legend to Fig. 1) probably originates from the binding of the hydroxyl of the former to both aspartate oxygens rather than to only one. There is also the potential for a weak interaction of the second
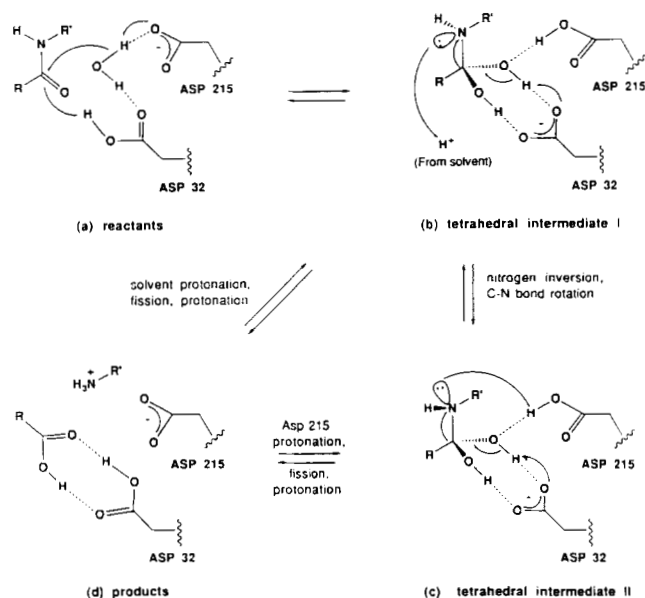

Fig. 4. The proposed mechanism for proteolytic cleavage of the amide bond by an aspartic proteinase.

hydrate oxygen with both the oxygen of 34 CO (3.2 Å) and the hydrogens on the edge of the phenol ring of Tyr 75, as suggested from a model of the intermediate based on the structure of a hydroxyethylene containing inhibitor (Blundell et al., 1987). This is the most favorable interaction of a phenol ring with an oxygen (Thomas et al., 1982) and may contribute to a small degree to the stability of the intermediate.

Figure 4 outlines a mechanism for proteolytic cleavage of the amide bond by an aspartic proteinase, which is consistent with these data and resembles that previously proposed by Suguna et al. (1987b). The scissile bond carbonyl is protonated by Asp 32 and concomitantly attacked by water polarized into a nucleophilic state by Asp 215. The attack of the nucleophilic water on the polarized carbonyl may be additionally facilitated by enzyme-induced distortion of the amide function, possibly via rigid body movement (Šali et al., 1989) in the complex. This gives a tetrahedral intermediate (b of Fig. 4) with a proton arrangement consistent with that of the bound hydrate as shown in Figure 3. Thus, the intermediate is stabilized by hydrogen bonds from the gem-diol unit to a negatively charged Asp 32 and from Asp 215 to the statine-like hydroxyl oxygen.

According to the principles of stereoelectronic control (Deslongchamps, 1975), the amide nitrogen will have been pyramidalized in this process with the lone pair disposed antiperiplanar to the newly formed carbon–oxygen bond of the tetrahedral intermediate. A well-organized water molecule can be seen in the electron density approximately 4.5 Å from the $-CF_2-$ carbon. This could mediate the transfer of a proton from bulk solvent but

only with some conformational change in the transition state complex. Alternatively, nitrogen inversion and a rotation of about 60° about the $C(OH)_2$–N bond may allow a staggered disposition of the substrate P_1 and P_1' α-carbons to be maintained while the secondary amine is rendered accessible to protonation by Asp 215. This is attractive because a model of this intermediate shows that the nitrogen of the amine is within hydrogen-bonding distance of the outer oxygen of Asp 215. If amine protonation is mediated by Asp 215, this event would be conveniently accompanied by loss of stabilization of the tetrahedral species by removal of the hydrogen bond to the statine-like hydroxyl simultaneously as the amine is protonated. Formation and stabilization of the products would be achieved by a concomitant deprotonation of the statine-like hydroxyl by Asp 32 (see c of Fig. 4). A hydrogen-bonded carboxylic acid dimer between the amino-terminal half of the product and Asp 32 (d of Fig. 4) results.

Thus, the high-resolution structure of the tetrahedral hydrate suggests a mechanism that involves the stabilization of a negatively charged aspartic acid carboxylate first at Asp 215 and then in the intermediate at Asp 32. In each case the stabilization involves complete inaccessibility of the carboxylate from bulk solvent and the formation of three or four hydrogen bonds to the two carboxylate oxygens. This is similar to the environment of several other carboxylates that are conserved in pepsins and which contribute to the negative overall charge at low pH (Cooper et al., 1990; Sielecki et al., 1990). Site-directed mutagenesis experiments of Ser 35 and Thr 218 of chymosin (Mantafounis & Pitts, 1990) and pepsin (J. Tang, unpubl.) are consistent with the role of these side-chain hydroxyl hydrogen bonds in stabilizing negatively charged carboxylates that occur in the mechanism shown in Figure 4. Their role is also consistent with the higher optimal pH for the catalysis by human renin with alanine at 218 and by the retroviral proteinases that have alanines at both equivalent positions.

The absence of any strong stabilization of a developing oxyanion at the peptide carbonyl argues against a possible analogy with serine proteinases, in which main-chain nitrogen atoms are thought to stabilize the negative charge of an oxyanion (Henderson, 1970). In fact the oxyanion is protonated and stabilized as a neutral gem-diol. In the case of the aspartic proteinases, anionic intermediates are stabilized sequentially by a complex hydrogen-bonding network at each of the active-site aspartates in two intermediates during the catalysis.

Methods and materials

The syntheses of the inhibitors have been described elsewhere (Hoover, 1989). CP-81,282 was formed in an approximately 20:1 ratio with the presumably inactive P_1

isomer by either diisopropylamine-modified Swern or DEC-modified Pfitzner–Moffatt oxidation of the (S)-alcohol precursor. The substance was stable in $CDCl_3$ or DMSO–water for weeks, but equilibrated within 24 h at 25 °C to a 1:1.3 mixture of CP-81,282:P_1 epimer (by ^{19}F NMR) in buffered solution at either pH 4.3 or 7.0. Renin inhibition was measured at pH 7.4 with angiotensin I quantitation by radioimmunoassay. Endothiapepsin activity was monitored by measuring A_{300} for cleavage of the synthetic substrate K–P–A–E–F–pNO_2F–R–L (Dunn et al., 1986) at pH 3.1. Each value was the mean of six determinations (I.M. Purcell & K.A. Simpson, unpubl.).

Crystals of endothiapepsin complexed with the inhibitor, but isomorphous with the uncomplexed enzyme, were grown by the method of Moews and Bunn (1970) using a 10-fold molar excess of inhibitor. A total of 42,676 X-ray reflections were recorded to 2.0 Å using an Enraf Nonius FAST television detector and corrected for Lorentz polarization and absorption effects. They were merged to give a unique set of 16,999 reflections with a merging R-factor of 7.4%. Fourier maps with coefficients $|Fo| - |Fc|$ and $2|Fo| - |Fc|$ were computed and displayed on an Evans and Sutherland PS300 graphics system using FRODO (Jones, 1978). Clear and contiguous electron density for the inhibitor was observed. The resulting model of the enzyme–inhibitor complex was then subjected to stereochemically restrained least-squares refinement using the program RESTRAIN (Haneef et al., 1985) and remodeling. The final R-factor was 0.18 for data in the resolution range 20 Å to 2.0 Å for reflections greater than $2\sigma(F)$. The coordinates will be deposited in the Brookhaven Protein Databank (Bernstein et al., 1977). The error in the atomic coordinates was calculated by the method of Read (1986), which gave a value of 0.22 Å.

For molecular orbital calculations, the coordinates for the two oxygen and one carbon atoms of the carboxy group of Asp 32 were extracted from the whole set of X-ray coordinates and a hydrogen atom was added to the inner carboxy carbon atom using a standard bond length of 1.09 Å and a bond angle of 112.89° to each oxygen atom. Similarly, the coordinates for the carboxy group of Asp 215 were extracted and a hydrogen atom added to the carbon atom. The coordinates of the carbon and two oxygens of the diol group and the difluoromethylene atoms of the inhibitor were also extracted and a hydrogen atom was added to each of those two carbon atoms using a standard bond length of 1.09 Å, a standard bond angle of 109.45°, and a dihedral angle that split either the two oxygens or the two fluorines. Hydrogens were then added to the two diol oxygens and one of the carboxy oxygens using a standard bond length of 0.95 Å and bond angles of 109.45° for the diol hydrogens and 120° for the carboxy hydrogen. The dihedral angles were then set to the two possible arrangements (see Results and discussion). Only the geometries of the hydrogens attached to

the oxygen atoms were allowed to optimize during the calculations, which were performed with the Gaussian 88 program on an IBM 3090. The STO-4G basis set was chosen because it has been demonstrated to be useful for describing hydrogen-bonded complexes (Del Bene & Pople, 1970).

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