Structural Analysis of the Active Site of Porcine Pancreatic Elastase Based on the X-ray Crystal Structures of Complexes with Trifluoroacetyl-Dipeptide-Anilide Inhibitors[†,‡]

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ABSTRACT: The X-ray crystal structures of two new (trifluoroacetyl)dipeptide *p*-(trifluoromethyl)anilide (TFA-dipeptide-TFM) inhibitors complexed to porcine pancreatic elastase are presented. TFA-Val-Ala-TFM and TFA-Phe-Ala-TFM both bind to elastase with the TFA group in the S1 subsite, Val or Phe in the S2 subsite, Ala in the S3 subsite, and the TFM group in the S4 subsite. Five other TFA-dipeptide-anilide/elastase crystal structures are available (two TFA-X-Ala-*p*-(trifluoromethyl)anilide, X = Lys, Leu, and three TFA-Lys-X-*p*-isopropylanilide, X = Pro, Leu, Phe). The four inhibitors with the trifluoromethyl substituent on the anilide ring bind in a single mode to elastase, whereas superposition of the three inhibitors with the isopropyl substituent on the anilide ring show three different modes of binding to the protein [Mattos, C., *et al.* (1994) *Nature Struct. Biol. 1,* 55−58]. The seven structures are taken together in a detailed analysis of the active site of porcine pancreatic elastase. The inhibition constants for the inhibitors are used in combination with the crystal structures to understand the specificity of the different elastase subsites.

Elastase is a serine protease of the trypsin family (Kraut, 1977). Different elastases have been the subject of numerous studies, due to their presumed role in several diseases such as emphysema (Powers, 1976), arthritis (Buchardi, 1984), pancreatitis (Geokas *et al.*, 1968), and cystic fibrosis (Davis, 1983). Five features are essential to catalysis in serine proteases: (1) a binding site for the portion of the substrate that forms the acyl enzyme intermediate (S subsites) and in particular (2) the specificity site, which is most commonly associated with the S1 subsite, (3) a binding site for the leaving group side of the substrate (S′ subsites), (4) the catalytic triad formed by Ser, His, and Asp, and (5) the oxyanion hole, which stabilizes the tetrahedral intermediates and transition states during catalysis. Peptide substrates are thought to bind with side chains occupying specific binding pockets in the active site (S and S′ subsites) and with main chain atoms of the acyl group side of the peptide, forming an antiparallel β-sheet with a strand (residues 222−224 (214−216))[1] on the enzyme. Elastase has a broader specificity than most other enzymes in the family, cleaving peptide bonds after any small aliphatic amino acid by the mechanism proposed for all serine proteases [for a summary, see Phillips and Fletterick (1992)]. Furthermore, kinetic studies on porcine pancreatic elastase using peptide substrates of varying lengths and compositions indicate that at least four residues preceding the cleavage site (P residues) and two residues after it (P′ residues) are necessary for proteolysis to occur optimally (Atlas, 1975).

The X-ray crystal structure of porcine pancreatic elastase (PPE)[2] was solved by Shotton and Watson (1970), and it has been used as a model for human elastases. The X-ray crystal structures of several inhibitor/PPE complexes have also been reported in the literature (Hughes *et al.*, 1982; Takahashi *et al.*, 1988; Meyer *et al.*, 1985, 1986; Mattos *et al.*, 1994). There is not a common mode of binding among the noncovalent inhibitors, and some are found to bind in two alternate conformations (Mattos & Ringe, 1993).

The (trifluoroacetyl)peptides (TFA-peptides) form an important class of potent reversible inhibitors of elastase, with K_i values on the order of 10^{-6}−10^{-8} M (Renaud *et al.*, 1983). On the basis of the X-ray crystal structure of the TFA-Lys-Ala-TFM/elastase complex (Hughes *et al.*, 1982) and ^{19}F NMR studies, which indicated a similar environment for the TFA group on elastase in all of the TFA-peptide inhibitors tested (Dimicoli *et al.*, 1980), extensive kinetic studies were interpreted to map out the active site of elastase (Renaud *et al.*, 1983). In the crystal structure (Hughes *et al.*, 1982), the TFA group could be seen in the S1 subsite, the Lys in the S2 subsite, the Ala in the S3 subsite, and the anilide group in the S4 subsite.[3] If indeed all of the TFA-peptides bound in the same mode, with the TFA group anchored in the S1 subsite, one could determine the optimal length of the peptide portion of the inhibitors, as well as probe the specificity of the S2, S3, and S4 subsites by measuring the inhibition constants of TFA-peptide inhibitors

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[‡] The crystal structure coordinates for TFA-Val-Ala-TFM and TFA-Phe-Ala-TFM have been deposited in the Brookhaven Protein Data Bank under file names 1ELE and 1ELD.

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[1] The numbering of elastase is given in terms of the consecutive numbering for porcine pancreatic elastase. The numbers in parentheses refer to the equivalent numbering in chymotrypsinogen.

[2] Abbreviations: TFA, trifluoroacetyl; ISO, *p*-isopropylanilide; TFM, *p*-(trifluoromethyl)anilide; PPE, porcine pancreatic elastase; DMSO, dimethyl sulfoxide.

[3] The authors referred to these sites as S′ subsites. The confusion in the literature regarding the nomenclature of the subsites will be addressed in the Results section.

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Table 1: Unit Cell Parameters and Refinement Statistics for Native and Inhibited Elastase Structures[a]

elastase	*a* (Å)	*b* (Å)	*c* (Å)	Res (Å)	*R*-factor	no. reflns	bond rms (Å)	angle rms (deg)
native (Meyer)[b]	52.00	58.00	75.20	1.65	0.17	17993	0.018	2.5
Ace-Ala-Pro-Val-CF_3[c]	51.4	58.2	75.4	2.57	0.14	6575	0.012	2.4
TFA-Lys-Leu-ISO	50.69	58.05	75.25	2.1	0.15	8749	0.014	2.9
TFA-Lys-Phe-ISO	51.20	58.00	75.40	1.8	0.19	23196	0.017	3.1
TFA-Lys-Pro-ISO	52.37	57.71	75.43	1.8	0.18	18391	0.014	2.7
TFA-Lys-Ala-TFM[d]	52.53	57.47	75.26	2.5	0.21	7187		
TFA-Leu-Ala-TFM[e]	52.53	57.47	75.26	1.8	0.19	13224	0.02	2
TFA-Val-Ala-TFM	52.4	57.6	75.3	1.8	0.17	11002	0.015	3.1
TFA-Phe-Ala-TFM	52.03	57.76	75.32	1.8	0.19	21511	0.015	2.8

[a] The space group in which all of the above crystallize is $P2_12_12_1$, and therefore $\alpha = 90°$, $\beta = 90°$, and $\gamma = 90°$ in all cases. [b] Meyer *et al.*, 1988. [c] Meyer *et al.*, 1986. [d] Hughes *et al.*, 1982. [e] Sierra *et al.*, 1990.

of various compositions. The most potent of the TFA-peptide inhibitors of elastase were shown to be the TFA-dipeptide-anilides with a substituent at the *para* position of the anilide ring (Renaud *et al.*, 1983). Of these inhibitors, TFA-Lys-Ala-TFM and TFA-Leu-Ala-TFM were shown to have the highest affinity for the enzyme (Renaud *et al.*, 1983).

The X-ray crystal structure of TFA-Leu-Ala-TFM complexed to elastase (Sierra *et al.*, 1990) served to support the assumptions underlying the kinetic work (Renaud *et al.*, 1983). This potent inhibitor binds in a mode identical to that of TFA-Lys-Ala-TFM. Most recently, however, the X-ray crystal structures of three additional TFA-dipeptide-anilide inhibitors have been solved, which contradict the basic assumption that the TFA-dipeptide-anilides bind in a single mode to elastase (Mattos *et al.*, 1994). While TFA-Lys-Pro-ISO binds as expected, TFA-Lys-Phe-ISO and TFA-Lys-Leu-ISO both bind very differently, in spite of the fact that the three inhibitors have similar inhibition constants. The TFA groups in both the TFA-Lys-Phe-ISO and TFA-Lys-Leu-ISO complexes bind in the oxyanion hole, rather than in the S1 subsite, and each of the two inhibitors has a different binding site for the anilide group. Although the Lys side chain in each inhibitor is found in the S2 subsite, the Phe and Leu residues, respectively, bind in the S1 subsite, rather than in the S3 subsite. Given the variety of binding modes for the different TFA-dipeptide-anilides, it becomes clear that it is not possible to use these inhibitors to map out the subsites on elastase by measuring and comparing their inhibition constants alone.

The X-ray crystal structures of two additional TFA-dipeptide-anilide inhibitors complexed to elastase are presented in this article. The coordinates for both structures have been deposited in the Brookhaven Protein Data Bank. The first is TFA-Val-Ala-TFM and the second is TFA-Phe-Ala-TFM. The binding of both of these inhibitors to elastase is discussed in detail. The seven crystal structures of TFA-dipeptide-anilide inhibitors complexed to elastase (four TFA-X-Ala-TFM, X = Lys, Leu, Val, Phe, and three TFA-Lys-X-ISO, X = Pro, Leu, Phe) are taken together in a detailed analysis of the active site of porcine pancreatic elastase. The inhibition constants published by Renaud *et al.* (1983) are used in combination with the crystal structures to understand the specificity of the different elastase subsites.

MATERIALS AND METHODS

Porcine pancreatic elastase was purchased from Worthington Biochemicals (96.5% pure) and was used without further purification. The TFA-Val-Ala-TFM and TFA-Phe-Ala-TFM inhibitors were donated by Prof. J.-L. Dimicoli at the Institut Curie in France. The inhibitors were not readily soluble in aqueous solution; therefore, dimethyl sulfoxide (DMSO) was used as a carrier solvent. A stock solution of 10 mM inhibitor in DMSO was prepared for each inhibitor. Crystallization conditions and a description of the structure determination are given for each inhibitor in this section. The inhibitor/elastase complexes all crystallized in space group $P2_12_12_1$ and were isomorphous with those of native elastase (Tables 1 and 2).

Table 2: Data Collection and Refinement Statistics for the TFA-Val-Ala-TFM/Elastase and TFA-Phe-Ala-TFM/Elastase Structures

	TFA-Val-Ala-TFM	TFA-Phe-Ala-TFM
Data Collection		
molecules per asymmetric unit	1	1
resolution (Å)	1.8	1.8
reflections		
total	NA	58417
unique	11002 ($I > 2\sigma(I)$)	21511 ($I > 0$)
R-merge (% on *I*)	NA	8.9
Refinement		
resolution range (Å)	10.0−1.8	10.0−1.8
data completeness (%)	44	86
final *R*-factor (%)	17	19
rms deviation		
bond lengths (Å)	0.015	0.015
bond angles (deg)	3.1	2.8
average *B*-factor (Å^2)		
protein	15	16
inhibitor	45	60
waters	30	29
no. of waters	137	133
no. of calcium ions	1	1
no. of sulfate ions	1	

TFA-Val-Ala-TFM. Native elastase crystals were grown by the vapor diffusion method in conditions previously described (Sawyer *et al.*, 1978). A crystal of dimensions approximately 1.0 × 0.75 × 0.5 mm was transferred to a high-salt mother liquor to which had been added enough of the inhibitor stock solution to obtain a final inhibitor concentration of 0.1 mM (~1000K_i). The crystal was soaked with the TFA-Val-Ala-TFM inhibitor for 1week. A complete data set to 1.8 Å resolution was collected from one crystal at −4 °C on a Siemens P3 diffractometer mounted on a Nicolet sealed tube generator operating at 40 kV and 50 mA. A limited ω step scan was used, so that only the peaks were measured and the background was estimated by an empirical curve. Data on a polar reflection were obtained for values

of φ between 0 and 360° and used for absorption correction. Radiation-induced decay was monitored by use of five running standard reflections at medium resolution. The unit cell parameters and the refinement statistics are given in Tables 1 and 2. After data reduction there were a total of 11 002 reflections with $I > 2\sigma(I)$.

The initial protein model used for refinement was an unpublished structure of native elastase (Navia, personal communication) that had been refined to an R-factor of 17%. Two cycles of PROLSQ restrained least-squares refinement (Hendrickson & Konnert, 1980) and four cycles of XPLOR refinement (Brünger *et al.*, 1989) took the model from an initial R-factor of 24.0% to a final R-factor of 17.1%. Water molecules taken from the fully refined structure of the TFA-Lys-Phe-ISO/elastase complex (Mattos *et al.*, 1994) were included in the model and checked against a difference Fourier electron density map with coefficients $F_o - F_c$ contoured at the 2.5σ level. Electron density for the inhibitor was visible, but a model for it was not included until the last stages of refinement.

TFA-Phe-Ala-TFM. Cocrystals of the TFA-Phe-Ala-TFM/elastase complex were grown in 36−44 μL drops containing 1.4 mM sodium sulfate and 100 mM sodium acetate buffer at pH 5. The initial inhibitor concentration was calculated to be about $1000K_i$. However, some of the TFA-Phe-Ala-TFM inhibitor precipitated as it was transferred with the carrier solvent DMSO into the aqueous crystallization solution. As a consequence, the actual concentration of the inhibitor in solution is not known, but it is safe to say that the solution was saturated with the inhibitor. The elastase concentration varied between 6.8 and 8.3 mg/mL and was measured photometrically by UV absorbtion at 280 nm using $\epsilon = 22$. Crystals grew slowly over a period of 1−2 months. The data were collected to 1.8 Å resolution at 0 °C on a Siemens X100-A multiwire area detector mounted on an Eliot GX-6 rotating anode operating at 30 kV and 30 mA. Two crystals were used. The first crystal had dimensions of 0.6 × 0.3 × 0.2 mm and was used to collect data in a single orientation. Data for a second orientation of the crystal with respect to the mounting axis were collected on a crystal measuring 0.5 × 0.2 × 0.2 mm. The two data sets were combined to give an 86% complete data set. A total of 58 417 reflections was collected, of which 21 511 are unique. The program XDS (Kabsch, 1988) was used to process and analyze the data and XSCALE was used to merge the data sets. The average correlation between the intensities of the common reflections was 98%. The merging R-factor was 8.9% on intensity.

The protein model used for refinement was taken from the TFA-Lys-Pro-ISO/elastase structure (Mattos *et al.*, 1994). The initial R-factor for this model was 28.7%. The refinement was done entirely with the program XPLOR (Brünger *et al.*, 1989). The first two rounds consisted of rigid body refinement, first including data from 10 to 3 Å resolution for better convergence and then extending to 1.8 Å resolution. A total of six rounds of XPLOR positional (least-squares) and B-factor refinement resulted in a model with an R-factor of 19.4%.

For both structures, the general strategy for refinement was first to include only the protein atoms in the model. Once the R-factor reached a minimum for this initial model, the water molecules (excluding the active site waters) were included and further refinement was carried out to obtain an optimized model that was as complete as possible without the inhibitor. This strategy is based on the observation that accounting for the bound water molecules in the model immensely improves the quality of the electron density for the inhibitor. A model for the inhibitor was created using the program QUANTA (Molecular Simulations, Inc.) and fitted into the density. Refinement was then used to optimize the complete model.

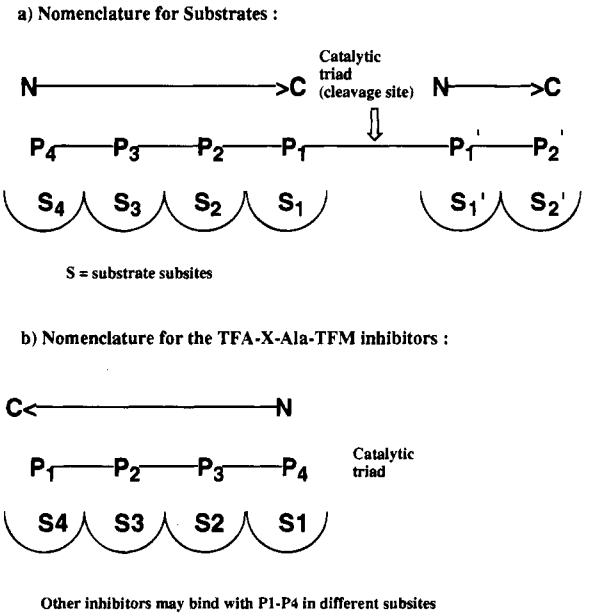

FIGURE 1: Elastase subsites nomenclature. (a) Substrate (binding occurs with the formation of an antiparallel β-sheet between protein and substrate). (b) TFA-X-Ala-TFM (X = Lys, Leu, Val, Phe). These inhibitors bind in reverse orientation, forming a parallel β-sheet with elastase. This mode of binding becomes immediatly obvious when the ligand residues are consistently named from the C-terminus toward the N-terminus of the peptide.

RESULTS

Before the structures can be described in detail, it is necessary to define a consistent nomenclature for the binding sites and a standard orientation from which to view the active site. The amino acid residues of the two new elastase structures are numbered sequentially, beginning with Val 16 (16) and ending with Asn 255 (245). In this scheme, the residues of the catalytic triad are Ser 203 (195), His 60 (57), and Asp 108 (102). The active site of elastase is referred to with respect to an orientation where Ser 203 (195) and His 60 (57) are aligned horizontally, with Ser 203 (195) on the right and His 60 (57) on the left. Asp 108 (102) is to the left and toward the back relative to Ser 203 (195) and His 60 (57). The oxyanion hole is then situated farther to the right, and the disulfide bond between Cys 45 (42) and Cys 61 (58) is above the catalytic triad. The subsites on the enzyme that bind the acyl group side of a substrate are situated below the catalytic triad and are called S1, S2, S3, S4, etc., while those that bind the leaving group side of the substrate are above the triad and are referred to as S1′, S2′, etc. (Figure 1a). These sites are located on the basis of X-ray diffraction analysis of the inhibition of elastase by a peptidyl trifluoromethyl ketone (Takahashi *et al.*, 1988) and are coincident with analogous sites on other serine proteases. Residues (or groups) occupying sites on the acyl group side are termed P1, P2, P3, P4, etc., and those on the leaving group side are P1′, P2′, etc., irrespective of whether the

molecule is an inhibitor or a substrate. The TFA-dipeptide-anilide inhibitors, however, do not behave in a manner analogous to substrate, and therefore the residues or fuctional groups will be defined in terms of where they bind relativeto the catalytic triad.

The elastase inhibitor sites are not necessarily coincident with the true substrate subsites. In a substrate, P1, P2, P3, and P4 refer to residues from the C-terminus of the peptide on the acyl group side of the scissile peptide bond back toward the N-terminus of the peptide (Figure 1a). This will also be the case for the inhibitors. For example, for the TFA-Lys-Ala-TFM inhibitor, the anilide group (TFM), which is at the C-terminus, is called P1, the Ala is P2, the Lys residue is P3, and the TFA group is P4. The corresponding sites on the enzyme in which this inhibitor binds are then S4, S3, S2, and S1, respectively (Figure 1b). The TFA-dipeptide-anilide inhibitors (at least those for which the crystal structures exist) all bind to elastase in reverse orientation relative to that expected for substrates.

The work described in this article is closely tied to the work previously done on the TFA-dipeptide-anilide inhibitors, and therefore it is important to redefine the nomenclature used for the various subsites in terms of the orientation of elastase and the nomenclature defined in the preceding paragraph. In particular, the fact that the TFA-dipeptides are found to bind in reverse orientation relative to the expected substrate orientation initially led to the erroneous conclusion that these inhibitors were binding in the S′ subsites. This misinterpretation of the subsites on elastase is found both in the description of the structure of the TFA-Lys-Ala-TFM/elastase complex (Hughes *et al.*, 1982) and in the subsequent kinetic analysis aimed at mapping out subsites on elastase (Renaud *et al.*, 1983). In both of these studies, the authors claimed that the TFA group bound in the S1 subsite, with the dipeptide and anilide groups occupying the S1′−S3′ sites. In the subsequent TFA-Leu-Ala-TFM/elastase structure (Sierra *et al.*, 1990), the correct assignment is made to the sites on elastase, with the inhibitor described as occupying subsites on the acyl group side of the catalytic triad.

The nomenclature confusion led to the comparison by Renaud *et al.* (1983) between the Lys in the TFA-Lys-X-anilide inhibitors and the Lys side chain that Atlas (1975) found at the P1′ position on substrate peptides. Although there was no crystal structure involved in Atlas' work, the Lys in her peptide substrate had, by virtue of being on a substrate, to occupy the S1′ subsite on elastase. Since Lys in the TFA-dipeptide-anilide inhibitors actually occupies the S2 subsite, the conclusions drawn from the comparison between the protein interactions involving the Lys side chain in the two studies are not valid. Renaud *et al.* (1983) further went on to compare the results that Atlas (1975) obtained for the Michaelis constants (K_m) of Ala-Ala-Ala-Ala-Lys-Phe and Ala-Ala-Ala-Ala-Lys-Ala (where Lys-Phe and Lys-Ala must occupy S′ subsites on the leaving group side of the catalytic triad) with their conclusions resulting from the inhibition constants (K_i) of TFA-Lys-Phe-ISO and TFA-Lys-Ala-TFM (where Lys-Phe and Lys-Ala now occupy sites on the acyl group side of the triad). While Atlas (1975) found that Ala-Ala-Ala-Ala-Lys-Phe was a better substrate than Ala-Ala-Ala-Ala-Lys-Ala, Renaud *et al.* (1983) found TFA-Lys-Phe-ISO to be an inhibitor 1 order of magnitude weaker than TFA-Lys-Ala-TFM (Table 3). There is, in fact, no contradiction in these results, since the sites to which Lys-Phe and Lys-Ala are binding in each study are completely different and cannot be compared to one another.

Table 3: Inhibition Constants for the Seven TFA-Dipeptide-Anilide Inhibitors[a]

inhibitor	K_i (M)
TFA-Lys-Ala-TFM	2.5×10^{-8}
TFA-Leu-Ala-TFM	2.9×10^{-8}
TFA-Lys-Leu-ISO	7.0×10^{-8}
TFA-Val-Ala-TFM	1.4×10^{-7}
TFA-Phe-Ala-TFM	2.0×10^{-7}
TFA-Lys-Pro-ISO	2.2×10^{-7}
TFA-Lys-Phe-ISO	4.4×10^{-7}

[a] The inhibitors are listed in increasing order of K_i. Data taken from Renaud *et al.* (1983).

Aside from the comparisons made with the Atlas work, the incorrect assignment of the TFA-dipeptide-anilide inhibitors to S1, S1′, S2′, and S3′ subsites does not affect in any way the valuable information resulting both from the crystal structure of the first TFA-dipeptide-anilide inhibitor complexed to PPE (Hughes *et al.*, 1982) and from the kinetic studies published thereafter (Renaud *et al.*, 1983).

TFA-Val-Ala-TFM. The structure of TFA-Val-Ala-TFM complexed to PPE was solved at 1.8 Å resolution. The final electron density map shows clear and unambiguous density for the inhibitor, as well as an alternative conformation for the side chain of Arg 226 (217A) (Figure 2). This side chain was refined with 50% occupancy in the original conformation and 50% occupancy in this new orientation. The structure includes 137 water molecules. A calcium ion and a sulfate ion are found at the positions reported previously (Sawyer *et al.*, 1978).

The inhibitor is situated in the active site below the catalytic triad on the acyl group side. It binds in the same conformation and at the same sites on elastase as the two previously published TFA-X-Ala-TFM inhibitors (Hughes *et al.*, 1982; Sierra *et al.*, 1990). The entire inhibitor, except for the Ala side chain and the Val main chain nitrogen atom, can be seen clearly in the final $2F_o - F_c$ electron density map contoured at the 1σ level (Figure 2). At lower contour levels, one sees complete density connectivity. Figure 3 shows a schematic view of the inhibitor bound to the active site, indicating the specific H-bonds and van der Waals interactions.

The TFA group (P4) binds in the S1 subsite (Table 4). The density for the CF_3 group is very well defined, but it does not present lobes for the fluorine atoms as was observed for the TFA-Lys-Phe-ISO and TFA-Lys-Leu-ISO inhibitors (Mattos *et al.*, 1994). Instead, the density is spherical around the trifluoromethyl group, suggesting that it is rotating freely. One of the fluorine atoms of TFA is 2.1 Å from Oγ of Ser 203 (195), which in turn accepts an H-bond from His 60 (57) (3.0 Å) and one from a water molecule (2.9 Å).

The Val (P3) residue of the inhibitor binds in the S2 subsite. Its main chain N-atom is 3.3 Å away from and directed toward the carbonyl oxygen of Ser 222 (214), forming the first H-bond in the parallel β-ladder between the inhibitor and the protein. The carbonyl oxygen of this Val residue is, in turn, 2.9 Å from the main chain N-atom of residue 224 (216), forming the second H-bond in the β-ladder. Except for a break at the N-atom, the Val residue is in good density and the two lobes for the Val side chain

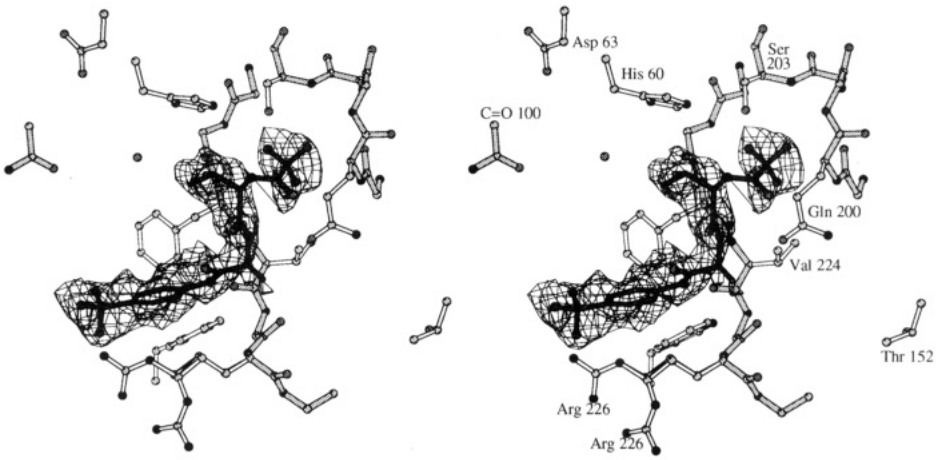

FIGURE 2: Stereoview of TFA-Val-Ala-TFM bonded to the active site of porcine pancreatic elastase. The protein backbone is shown in gray, the key protein side chains are in white, and the inhibitor is in black. The N-, O-, and F-atoms of the inhibitor are distinguished from the C-atoms by having larger radii. The electron density for the inhibitor atoms is shown. Figures 2, 4, and 6 were made with the program MOLSCRIPT (Kraulis, 1991).

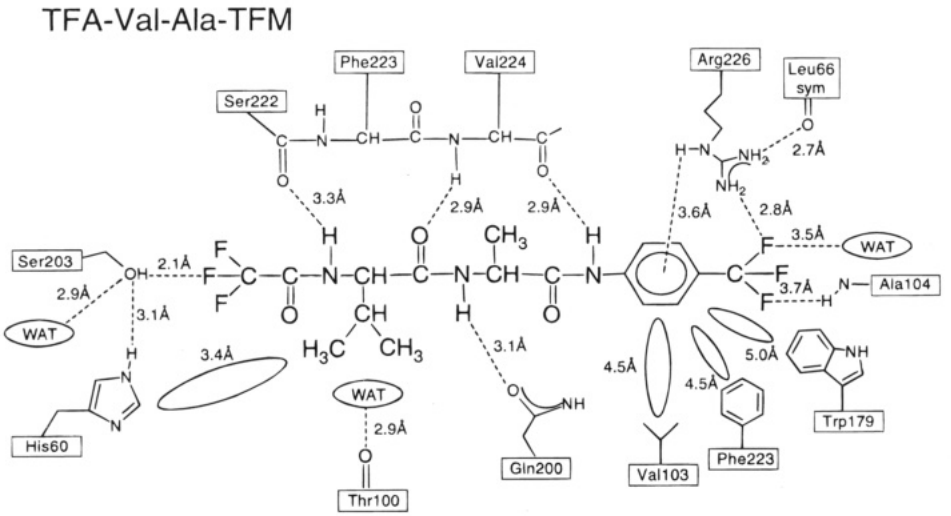

FIGURE 3: Schematic view of the H-bonds (dashed lines) and van der Waals interactions (closed lines) between TFA-Val-Ala-TFM and elastase.

Table 4: Functional Groups of the TFA-Dipeptide-Anilide Inhibitors That Are Found in Each of the Elastase Subsites

inhibitor	oxyanion hole	S1	S2	S3	S4	SA	SB
TFA-Val-Ala-TFM		TFA (P4)	Val (P3)	Ala (P2)	TFM (P1)		
TFA-Phe-Ala-TFM		TFA (P4)	Phe (P3)	Ala (P2)	TFM (P1)		
TFA-Leu-Ala-TFM		TFA (P4)	Leu (P3)	Ala (P2)	TFM (P1)		
TFA-Lys-Ala-TFM		TFA (P4)	Lys (P3)	Ala (P2)	TFM (P1)		
TFA-Lys-Pro-ISO		TFA (P4)	Lys (P3)	Pro (P2)	ISO (P1)		
TFA-Lys-Leu-ISO	TFA (P4)	Leu (P2)	Lys (P3)			ISO (P1)	
TFA-Lys-Phe-ISO	TFA (P4)	Phe (P2)	Lys (P3)				ISO (P1)

can be easily distinguished. The Val side chain fits under His 60 (57), with the Cβ-atom at a distance of 3.4 Å from the histidine ring. This hydrophobic interaction is similar to the one observed between His 60 (57) and the Lys side chains in the TFA-Lys-X-anilide inhibitors. A water molecule is in the viscinity of the position occupied by the Nζ-atom of the Lys side chain of the TFA-Lys-Ala-TFM (Hughes *et al.*, 1982) inhibitor and is involved in the same H-bond interaction with the carbonyl oxygen of residue Thr 100 (96). The H-bond length between O 100 and Nζ of Lys in the TFA-Lys-Ala-TFM inhibitor is 3.6 Å, while in the TFA-Val-Ala-TFM structure the oxygen atom of the relevant water molecule is 2.9 Å from the carbonyl O of Thr 100 (96).

The Ala (P2) residue binds in the S3 subsite. Its main chain N-atom is 3.1 Å away from Oϵ1 of Gln 200 (192) and 3.2 Å from a water molecule. An H-bonding network, similar to the one observed for TFA-Lys-Leu-ISO inhibitor, is present, involving Gln 200 (192) and two water molecules. However, in the TFA-Lys-Leu-ISO structure it was the Nϵ2, not the Oϵ1, of Gln 200 (192) that interacted with the inhibitor. The carbonyl O of the Ala residue points toward the outside of the protein and does not make any interactions that can be seen in the electron density map.

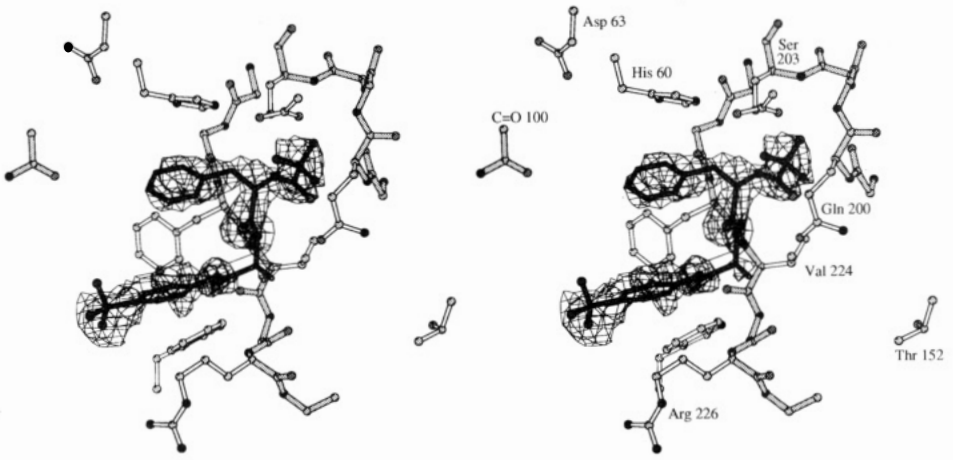

FIGURE 4: Stereoview of TFA-Phe-Ala-TFM complexed to the active site of porcine pancreatic elastase. The orientation of the protein and the protein atoms included in this figure are exactly as described for Figure 2. The electron density for the inhibitor atoms is shown.

The *p*-(trifluoromethyl)anilide (TFM) group (P1) is in the S4 subsite, where it makes contact with a symmetry-related protein molecule. This site is an excellent binding site for the TFM group. The nitrogen atom of the anilide is 2.9 Å from the carbonyl oxygen of Val 224 (216). This is the third and final H-bond in the β-ladder between inhibitor and protein (Figure 3). The anilide ring is within 4.5 Å of Phe 223 (215) and Val 103 (99) and within 5 Å of Trp 179 (172). These residues provide a series of hydrophobic interactions that are mostly above the anilide ring, in the orientation of the active site defined earlier. Underneath the TFM group, the side chain of Arg 226 (217A) is found in an alternate conformation that is not observed in the uncomplexed structure of elastase. The Nϵ of this Arg residue is 3.6 Å from the plane of the anilide ring, forming a good amino–aromatic interaction (Burley & Petsko, 1988). One of the terminal nitrogens of this arginine forms a bifurcated H-bond with one of the fluorine atoms of the para substituent of the anilide ring (2.8 Å) and one with the carbonyl O of Leu 66 (63) of a symmetry-related molecule (2.8 Å). The other terminal nitrogen of Arg 226 (217A) forms an H-bond involving the carbonyl oxygen of Leu 66 (63) (2.7 Å). The fluorine atom, which interacts with Arg 226 (217A), is also 3.5 Å away from a water molecule. One of the other fluorine atoms is 3.7 Å from the main chain N-atom of Ala 104 (99A).

TFA-Phe-Ala-TFM. The cocrystal structure of TFA-Phe-Ala-TFM complexed to elastase was solved to 1.8 Å resolution and refined to an *R*-factor of 19.4% with 133 water molecules and a calcium ion. The sulfate ion could not be seen in the electron density map for this structure, as was the case for the cocrystallized TFA-Lys-Phe-ISO/elastase structure (Mattos *et al.*, 1994). The final electron density map for this inhibitor (Figure 4) is poorer than that for the TFA-Val-Ala-TFM/elastase structure (Figure 2). This partially reflects a lower occupancy of the TFA-Phe-Ala-TFM inhibitor resulting from its relative insolubility in aqueous solution. However, the TFA-Phe portion of the inhibitor is in good density compared to the Ala-TFM portion. One therefore must not exclude the possibility that the latter half of the inhibitor has multiple binding modes. The inhibitor was built and refined in several different conformations, and the one presented in Figure 4 is the only one for which density was found in the active site. It is therefore the major, if not the only, mode of binding for this inhibitor. This result was also obtained using anneal omit maps to remove model biases from the density (Hodel *et al.*, 1992). The final electron density map in Figure 4 shows connectivity for most of the TFA-Phe-Ala-TFM model. The exception is the lack of density for the Cα- and Cβ-atoms in the Ala residue at contour levels where the rest of the inhibitor is clear. A schematic view of the interactions between this inhibitor and the protein is shown in Figure 5.

The TFA-Phe-Ala-TFM inhibitor binds to elastase on the acyl group side of the catalytic triad, as do all the TFA-dipeptide-anilides of known structure. It binds at the same subsites on elastase as the other three TFA-X-Ala-TFM inhibitors (where X = Lys, Leu, and Val). The TFA (P4) group binds in the S1 subsite (Table 4). One of the fluorine atoms is 2.9 Å from the Oγ-atom of Ser 203 (195), indicating a possible H-bonding interaction. The electron density for the entire TFA residue is very well defined, with spherical density for the CF_3 group, which is indicative of relatively free rotation.

The Phe (P3) side chain binds under the His 60 (57) ring in the S2 subsite on the enzyme (Table 4). The main chain N-atom of the Phe residue is 3.9 Å from the carbonyl oxygen of Ser 222 (214) and 3.2 Å from the Oγ-atom of Ser 203 (195). The carbonyl oxygen of Phe H-bonds to the N-atom of Ser 224 (216) on the protein (2.7 Å). The Phe ring is stacked at an angle of approximately 60° relative to the plane of the imidazole ring of His 60 (57). This is initially surprising, given that there is room for a parallel stacking of these aromatic rings. A closer structural analysis of this area reveals the presence of a ring cluster where the TFM group, the inhibitor Phe residue, and Phe 223 (215) (protein) interact with approximately 3-fold symmetry (Figure 4). In addition, Phe 223 (215) also makes a good aromatic–aromatic interaction with Trp 179 (Burley & Petsko, 1988), with the edge of Phe 223 (215) 3.8 Å from the plane of Trp 179. The TFA-Phe-Ala-TFM inhibitor binds to elastase in a manner analogous to all other TFA-dipeptide-TFM inhibitors for which crystal structures of the complex with elastase have been solved. However, the Phe aromatic ring is involved in the ring cluster interaction, which is not possible when P3 is Lys, Leu, or Val.

The Ala (P2) residue of the inhibitor binds in the S3 subsite (Table 4). Its main chain N-atom H-bonds to the side chain Oϵ1-atom of Gln 200 (192) (3.2 Å). Similar interactions with waters are found for Gln 200 (192), as observed with

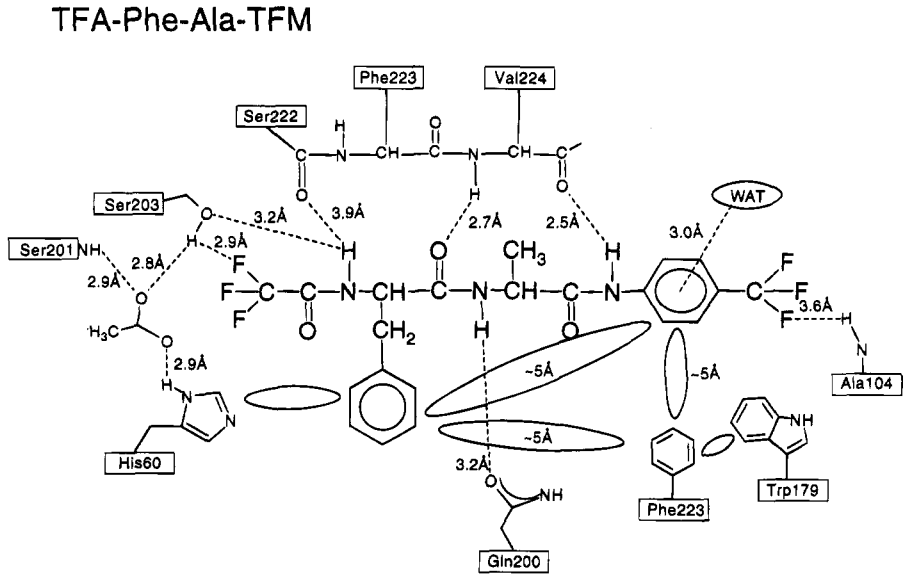

FIGURE 5: Schematic view of the H-bonds (dashed lines) and van der Waals interactions (closed lines) between TFA-Phe-Ala-TFM and elastase.

the TFA-Lys-Leu-ISO (Mattos *et al.*, 1994) and TFA-Val-Ala-TFM structures.

The (trifluoromethyl)anilide group (P1) binds in the S4 subsite. The anilide ring interacts with Phe 223 (215) and the inhibitor Phe residue to form the ring cluster described earlier. The main chain N-atom of TFM is 2.7 Å from the carbonyl O of Val 224 (216). This is the third H-bond in the parallel β-sheet formed between the inhibitor and the protein. The other two were mentioned previously: the first is between N of the inhibitor Phe residue and O of Ser 222 (214) on the protein, and the second consists of the interaction between the carbonyl O of the inhibitor Phe residue and N of Ser 224 (216). Val 103 (99) and Trp 179 (172) are also within 5 Å of the anilide ring. In this structure, unlike in the TFA-Val-Ala-TFM and TFA-Lys-Pro-ISO structures where the anilide ring also binds in the S4 subsite, Arg 226 (217A) is observed only in the conformation found in the uncomplexed enzyme. Instead of the alternate conformation for this side chain, there is a water molecule 3.0 Å from the anilide ring of TFM. The absence of density for the second conformation for Arg 226 (217A) can be interpreted in two ways. One is that this Arg does interact with the TFM group, but due to the low occupancy of the inhibitor, the water present in the uncomplexed molecules in the crystal dominates the density at this location. The second interpretation is that in this case the Arg 226 (217A) side chain does not interact with the inhibitor TFM group, because this group is instead involved in the ring cluster described earlier.

The oxyanion hole is occupied by an acetate molecule (a constituent of the crystallization mother liquor) wedged in between Gly 201 (193) and His 60 (57). One of the acetate oxygen atoms is 2.9 Å from the N-atom of Gly 201 (193) and 2.8 Å from the Oγ -atom of Ser 203 (195). The other is 2.9 Å from the Nϵ2-atom of the catalytic histidine. The electron density for the small molecule found in the oxyanion hole is very well defined, and a model for acetate was chosen over that for dimethyl sulfoxide (also in the crystallization solution) due to the presence of the ideal H-bonding interactions, which can be satisfied by the carboxyl oxygens of acetate and not by the methyl groups found at the equivalent positions in DMSO.

DISCUSSION

There are currently a total of seven crystal structures of different TFA-dipeptide-anilide inhibitors complexed to elastase. The inhibitors can be divided into two groups depending on the amino acid components of the dipeptide and on the type of substituent on the anilide ring. The first group consists of the three TFA-Lys-X-*p*-isopropylanilides (TFA-Lys-Pro-ISO, TFA-Lys-Leu-ISO, and TFA-Lys-Phe-ISO) (Mattos *et al.*, 1994). The second group consists of the TFA-X-Ala-*p*-(trifluoromethyl)anilides, two of which are presented for the first time in this manuscript (TFA-Val-Ala-TFM and TFA-Phe-Ala-TFM) and two of which have been published by others [TFA-Lys-Ala-TFM, Hughes *et al.* (1982), and TFA-Leu-Ala-TFM, Sierra *et al.* (1990)]. The structure of elastase in the complexes is virtually identical to that in the uncomplexed enzyme, with the exception of the alternate conformation of Arg 226 (217A) sometimes observed when the anilide group of the inhibitor binds in the proximity of this side chain. In the present analysis, the seven TFA-dipeptide-anilide/elastase structures are examined together in an attempt to extract general information regarding the binding of these, as well as other, ligands to the active site of elastase.

In the serine proteases, the strongest interactions available for binding a true substrate should be found in the acyl group side of the catalytic triad. After acylation occurs, the leaving group side of the peptide must leave promptly for a water molecule to attack the acyl enzyme, with subsequent completion of the reaction (Kraut, 1977). It therefore is not surprising that in most of the ligand/elastase complexes the ligand is found to bind in the S subsites on the protein.

The crystal structures of many elastase/inhibitor complexes indicate that, on the acyl group side, a series of important interactions can contribute to the binding of ligands. These interactions are exploited in different ways by different ligands, and in particular by the seven TFA-dipeptide-anilide inhibitors for which there is structural information. The sites

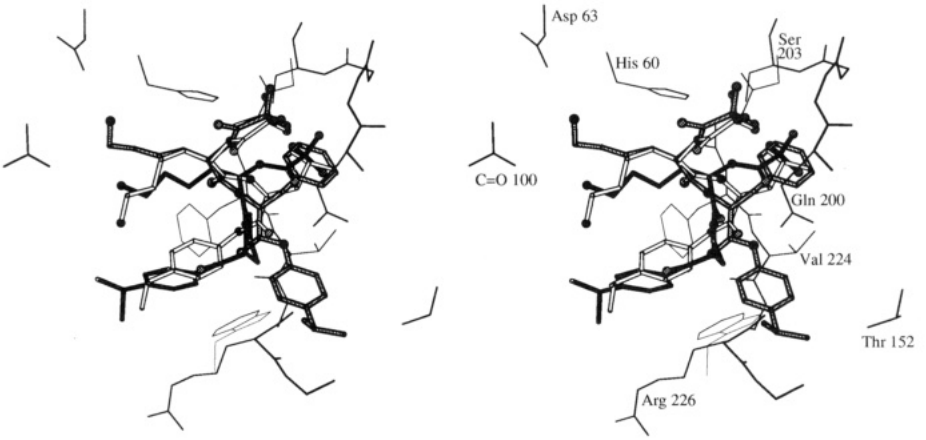

FIGURE 6: Stereoview of TFA-Lys-Leu-ISO, TFA-Lys-Phe-ISO, and TFA-Lys-Pro-ISO superimposed on the active site of PPE. The protein atoms correspond to those for the TFA-Lys-Pro-ISO/elastase complex and are shown as a line drawing for clarity. TFA-Lys-Pro-ISO is in black, TFA-Lys-Phe-ISO is in gray, and TFA-Lys-Leu-ISO is in white.

occupied by the various functional groups on the ligand are not necessarily coincident with peptide substrate subsites. For example, all of the seven TFA-dipeptide-anilides bind in a reversed main chain orientation relative to the one conducive to catalysis. The backbones of TFA-Lys-Ala-TFM, TFA-Leu-Ala-TFM, TFA-Val-Ala-TFM, TFA-Phe-Ala-TFM, and TFA-Lys-Pro-ISO make a parallel β-sheet with the appropriate strand in the protein. The backbones of TFA-Lys-Phe-ISO and TFA-Lys-Leu-ISO do not form a β-sheet with elastase, but do make other types of interactions (Mattos *et al.*, 1994).

Table 3 lists the competitive inhibition constants (K_i) for the seven TFA-dipeptide-anilide inhibitors being considered. For the four TFA-X-Ala-TFM inhibitors (X = Lys, Leu, Val, or Phe), the assumptions made by Renaud *et al.* (1983) in the kinetic mapping of the active site of elastase are valid because these inhibitors bind identically to the enzyme and differ only in the chemical composition of the X residue. The differences in K_i values therefore truly reflect the fact that Lys or Leu is preferred over Val or Phe at the S2 subsite.

The kinetic study aimed at determining the specificity of the S3 subsite by use of the TFA-Lys-X-ISO series of inhibitors (with X = Pro, Leu, and Phe) was not at all successful. The mapping of subsites on elastase by use of kinetic data is based on the assumptions that the TFA group always binds in the S1 subsite and that all of the inhibitors bind in the same mode to elastase (see introduction). While TFA-Lys-Pro-ISO binds to elastase as expected, the structures of TFA-Lys-Leu-ISO and TFA-Lys-Phe-ISO complexed to elastase reveal that these two fundamental assumptions are not general (Figure 6). The TFA group in these two inhibitors binds in the oxyanion hole rather than in the S1 pocket, and in each the anilide group is found in a different location on the enzyme. The two TFA-Lys-X-ISO inhibitors where X is either Leu or Phe bind with P3 (Lys) in the S2 subsite, but have the P2 (Leu or Phe) residue in the S1 subsite rather than in S3, as initially assumed. Clearly, nothing can be concluded about the S3 binding site on elastase, as was intended when the TFA-Lys-X-anilide inhibitors were designed.

The kinetic information is valuable in identifying which compounds are the most potent inhibitors of elastase, but because this enzyme has multiple binding sites that can be selected by ligands in different ways, the kinetic results must be combined with structural information to obtain a clear understanding of the functional group preferences at a given binding site. The differences in K_i for these inhibitors may have contributions from differences in binding throughout the entire structure. The elastase/TFA-dipeptide-anilide inhibitor example illustrates, in general, the danger of relying purely on kinetic experiments to map out the specificity of binding sites on proteins (Mattos *et al.*, 1994).

It is now useful to change perspective and focus on each of the individual subsites on elastase and the particular interactions available to accommodate the different functional groups present in the inhibitor molecules. For this purpose it is better not to subdivide the inhibitors into groups, but to analyze each functional group, from any of the seven inhibitors, that binds in the particular sites (Table 4). The oxyanion hole binds the TFA (P4) group in TFA-Lys-Leu-ISO and TFA-Lys-Phe-ISO inhibitors. In both cases, the fluorine atoms interact with the main chain N-atoms of Gly 201 (193) and Ser 203 (195), which are the two hydrogen-bond donors that stabilize the tetrahedal intermediate during normal catalysis. The Oγ-atom of Ser 203 (195) and one or more water molecules provide additional interactions to the TFA fluorine atoms. In both inhibitors, the electron density for the TFA group clearly presents three lobes, indicating that favorable interactions restrict the free rotation of the CF_3 group at this site. In the TFA-Lys-Pro-ISO/elastase structure (Mattos *et al.*, 1994) and in the TFA-Phe-Ala-TFM/elastase structure, an acetate molecule is found in the oxyanion hole. Interestingly, in both cases the protein/inhibitor complex was cocrystallized. In the other examples where the TFA group binds in the S1 subsite, the inhibitor was soaked into the protein crystal, and a water molecule, if anything, is observed in the oxyanion hole. One may argue that the presence of an acetate molecule in the oxyanion hole may influence the binding mode of the inhibitor. This may be true to some extent, but the inhibitor can compete with acetate, as is illustrated in the cocrystal structure of the TFA-Lys-Phe-ISO/elastase complex, where the TFA group and not an acetate is seen in the oxyanion hole.

The S1 site, immediately below the oxyanion hole, was observed to bind the TFA (P4) group in the TFA-Lys-Ala-TFM, TFA-Leu-Ala-TFM, TFA-Val-Ala-TFM, TFA-Phe-Ala-TFM, and TFA-Lys-Pro-ISO inhibitors (Table 4). A good interaction is seen between one of the fluorine atoms and the Oγ of Ser 203 in all five structures. Although the electron density for the two previously published structures

is not available, in the case of the TFA-Val-Ala-TFM, TFA-Phe-Ala-TFM, and TFA-Lys-Pro-ISO inhibitors the spherical shape of the density indicates a freely rotating TFA group. In the TFA-Lys-Leu-ISO inhibitor, the Leu side chain (P2) is found in the S1 subsite, and in TFA-Lys-Phe-ISO the Phe side chain (P2) is in the S1 subsite. Leucine forms a good hydrophobic interaction with Val 224 (216). The Phe side chain not only is involved in the hydrophobic interaction with Val 224 (216) but also appears to interact with the Ser 203 (195) Oγ-atom through an oxygen−aromatic H-bond of the kind described by Burley and Petsko (1988).

The S2 subsite is found to bind Lys, Leu, Val, or Phe, all of which are at the P3 position (Table 4). The S2 subsite accommodates the Lys side chain in the four TFA-Lys-X-anilide inhibitors. In TFA-Leu-Ala-TFM and TFA-Val-Ala-TFM, where Leu or Val binds at this position, a water molecule is found in the vicinity of where the Nϵ of the Lys side chain would bind in the TFA-Lys-Ala-TFM inhibitor. The aliphatic chain of Lys stacks under the His 60 (57) imidazole ring, and the Nϵ-atom interacts with a polar group in the protein or with water, depending on the inhibitor. In fact, this polar group was found to be different for each of the Lys-containing TFA inhibitors. The variation in the interactions of Lys in the S2 pocket is most likely a result of the different binding modes of the other inhibitor residues on the active site of elastase. The S2 subsite is situated under the catalytic His 60 (57) and can be thought of as a Lys binding site for inhibitors of elastase or for a functional group that has an aliphatic chain with a charged or polar group at the end. If indeed the S2 subsite is a Lys binding site on elastase, one is tempted to assume that a Lys at the P2 position on a substrate would be highly favored. This may or may not be true. Perhaps the binding of Lys at this position would lead to a geometry at the active site that is not conducive to catalysis, making the ligand a competitive inhibitor, rather than a substrate. On the other hand, the sequence of elastin contains 35 Lys side chains, 22 of which are followed by Ala, Val, or Leu (sequence data bank), residues that are commonly at the P1 position in elastase substrates. Although many of the Lys side chains are involved in cross-linking elastin, one cannot rule out the possibility that some of them actually occupy the P2 position during hydrolysis by elastase. It is possible that the Lys side chain serves as a recognition element and that elastase, contrary to other serine proteases, is an enzyme for which specificity is dominated by the P2 residue alone or in addition to the specificity at the P1 position. When the Phe side chain of TFA-Phe-Ala-TFM binds at the S2 subsite, one does not observe the water molecule that in TFA-Leu-Ala-TFM and TFA-Val-Ala-TFM occupies the position of the Nϵ-atom of lysine. The aromatic ring of the Phe residue participates in the unique ring cluster interaction described in the Results section. This type of interaction is not seen in any of the other inhibitors bound to elastase.

The S3 site is to the left of and somewhat below the S1 site. The Ala in the four TFA-X-Ala-TFM inhibitors and the Pro in the TFA-Lys-Pro-ISO occupy this site. The Ala and Pro side chains make no interactions that can be seen in the final electron density map. The only interactions observed are with backbone atoms.

There are three different subsites to which the seven anilide groups (TFM or ISO) in the TFA inhibitors bind. Only one (S4) coincides with an established substrate binding site. So as not to cause confusion, the other two will be called SA and SB. A letter (A or B) rather than a number following S serves to indicate subsites that are not used by substrates.

The S4 subsite accommodates all four TFM groups from the TFA-X-Ala-TFM inhibitors and the ISO residue in TFA-Lys-Pro-ISO (Table 4). This subsite is located below the S2 subsite. There is a series of interactions taking place in this S4 site, the most important of which involves the side chain of Arg 226 (217A). When the TFM group binds in this site, a second conformation for the Arg 226 (217A) side chain is observed in TFA-Lys-Pro-ISO and TFA-Val-Ala-TFM and possibly in TFA-Phe-Ala-TFM. In the TFA-Lys-Ala-TFM/elastase structure (Hughes *et al.*, 1982) an alternate conformation for Arg 226 (217A) is not reported, and in the TFA-Leu-Ala-TFM/elastase structure (Sierra *et al.*, 1990) a dimethylformamide (DMF) molecule is found precisely where the alternate conformation of the Arg 226 (217A) side chain would be expected. Other interactions in this site are of a hydrophobic nature and involve the side chains of Phe 223 (215), Val 103 (99), and Trp 179 (172), which are in the vicinity of the anilide ring.

The SA site is occupied by the ISO group in the TFA-Lys-Leu-ISO inhibitor (Table 4). This site partially overlaps with the S4 subsite, but the anilide ring binds at a different angle relative to its orientation in the S4 site. In the SA site, it is the isopropyl substituent of the ring that interacts with the aliphatic portion of Arg 226 (217A).

The SB site is below Gln 200 (192) and it is occupied by the anilide ring in the TFA-Lys-Phe-ISO inhibitor (Table 4). There is a strong H-bond between the main chain N-atom of the anilide group and Oϵ1 of Gln 200 (192). The Cζ2-atom of the isopropyl substituent of the anilide ring is within van der Waals contact of Cγ2 of Thr 152 (147). There is a water molecule 3.5 Å away from the plane of the anilide ring, which may be donating its hydrogen atom in an interaction with the aromatic anilide ring.

While the TFM group is always found in the same subsite on elastase, the three ISO groups are found at three different positions on the enzyme (Figure 6). The difference between the two is that in TFM the CF_3 group forms specific electrostatic interactions with the protein that are favorable in themselves, but also help to position the anilide ring for the amino−aromatic interaction with an alternate conformation of Arg 226 (217A). These interactions include the H-bond formed between the Nζ of Arg 226 (217A) and one of the fluorine atoms. The S4 site is essentially a TFM binding site that can accommodate the ISO group in TFA-Lys-Pro-ISO. It is likely that for those TFA-dipeptide-anilide inhibitors in which the anilide ring binds in the S4 subsite and that differ only with respect to having a trifluoromethyl or a isopropyl substituent on the anilide ring, the difference in K_i values would be largely a consequence of the specific interaction that the CF_3 group makes with one of the terminal nitrogens of the Arg 226 (217A) side chain. The other important interactions of the CF_3 substituent are with a symmetry-related molecule in the crystal and should be absent from the solution used to measure the inhibition constants.

Table 4 summarizes the discussion of the preceding paragraphs. The oxyanion hole can bind the P4 group (or an acetate molecule), the S2 subsite binds the P3 group, and the S3 subsite binds P2 residues on the TFA-dipeptide-anilide inhibitors, while the S1 subsite is found to accommodate P3

or P4 residues. The position of a given residue or functional group (P) in the sequence of a TFA inhibitor does not determine the S site to which it will bind. Instead, the choice of binding site is dictated by the chemical composition of the residue at the P position and that of the other functional groups on the inhibitor. It becomes clear that there are multiple binding modes for these inhibitors interacting with elastase (Figure 6). The problem of multiple binding modes of ligand to elastase and other proteins is an important topic, with consequences for drug design that have been previously discussed (Mattos *et al.*, 1994).

The functional group preferences in some of the sites have become clear through crystallographic work involving the TFA-dipeptide-anilide inhibitors. Some of the functional groups present in these ligands consistently appear in a specific site, while others appear in different sites depending on the particular inhibitor to which they belong. The lysine side chain of the four lysine-containing inhibitors are found in the S2 subsite, all four Ala residues are in the S3 subsite, and the four TFM groups occupy the S4 subsite. The other groups are found to be accommodated in more than one subsite. For example, of the two Leu side chains, one is in the S1 subsite and the other, in the S2 subsite. In the absence of the Lys side chain, the Leu in TFA-Leu-Ala-TFM binds in the S2 subsite, and the inhibitor is in the correct conformation to form a parallel β-sheet with the protein. In TFA-Lys-Leu-ISO, with the Lys side chain binding in the S2 subsite, the Leu side chain is accommodated in the S1 subsite, shifting the TFA group to the oxyanion hole. As a consequence of these rearrangements, the S3 site is unoccupied and there is no formation of the parallel β-sheet with the protein. A completely analogous situation occurs with TFA-Phe-Ala-TFM and TFA-Lys-Phe-ISO, the only difference being that in TFA-Lys-Phe-ISO the ISO group binds in the SB subsite, whereas in TFA-Lys-Leu-ISO it binds in the SA site. Of the seven TFA groups, two bind in the oxyanion hole and five bind in the S1 subsite. The TFA group is found in the S1 pocket unless this site is occupied by Phe or Leu in the two lysine-containing inhibitors TFA-Lys-Leu-ISO or TFA-Lys-Phe-ISO. Finally, the three ISO groups bind in the three different subsites on elastase, S4, SA, and SB, found to acommodate the anilide group (Figure 6). The position of this group on elastase changes depending on the composition of the remainder of the inhibitor. Pro and Val each appear only once among the seven inhibitors. By analogy with Leu and Phe, it is likely that, in a TFA-Lys-Val-TFM inhibitor, the Val would be found in the S1 subsite and the Lys in the S2 subsite.

In light of the site specificities outlined earlier, one can attempt to rationalize the results of the kinetic experiments (Table 3) in structural terms. TFA-Lys-Ala-TFM is one of the most potent of the seven TFA-dipeptide-anilide inhibitors. This is consistent with the fact that three of the four residues (Lys, Ala, and TFM) are groups that are found consistently in the S2, S3, and S4 subsites, respectively. The TFA-Leu-Ala-TFM inhibitor is also very potent, with its binding constant being indistinguishable from that of TFA-Lys-Ala-TFM. This is not surprising since these two inhibitors bind in the same conformation and make completely analogous interactions with the protein. The Lys residue is substituted by Leu in the S2 subsite. This latter side chain can still provide good hydrophobic contact with His 60 (57), and a water molecule mimics the interaction that the Lys Nϵ-atom makes with the protein. By analogy, one might expect the TFA-Val-Ala-TFM inhibitor to follow the first two in terms of potency, since like TFA-Leu-Ala-TFM it makes interactions analogous to those in TFA-Lys-Ala-TFM. Apparently, however, the smaller Val side chain does not make as good a van der Waals interaction with His 60 (57). In fact, Table 3 shows that the TFA-Lys-Leu-ISO inhibitor ranks third and TFA-Val-Ala-TFM ranks fourth in terms of potency, with the former being twice as potent as the latter. By comparing the structure and K_i values of these two inhibitors, it can be concluded that the presence of the Lys in the S2 subsite, with Leu and TFA making good interactions in their respective sites and the ISO group binding in the SA subsite interacting with Arg 226 (217A), compensates for the loss of the parallel β-sheet with elastase and of the amino−aromatic interaction involving the TFM group, both of which are present in the TFA-Val-Ala-TFM inhibitor. The Val side chain is too small to fully occupy the S2 subsite of elastase.

Weakly polar interactions, electrostatic in nature, are found for almost all of the nine aromatic groups found in the seven TFA inhibitors being studied (seven anilide rings and two Phe side chains). The (trifluoromethyl)anilide (TFM) groups and the isopropylanilide (ISO) group in the TFA-Lys-Pro-ISO inhibitor make amino−aromatic interactions with Arg 226 (217A), and the Phe in TFA-Lys-Phe-ISO makes an oxygen−aromatic interaction with Ser 203 (195). The ISO groups in TFA-Lys-Leu-ISO and TFA-Lys-Phe-ISO are the only ones not involved in these favorable interactions.

On the basis of the preceding analysis regarding the modes of binding of the TFA-dipeptide-anilide inhibitors to the active site of elastase, a "hydra-headed" inhibitor was designed to take advantage of the multiple binding sites found in elastase. The inhibitor consists of a peptide mimic predicted to have residues placed in both the S4 and SB sites simultaneously. This ligand was shown to bind as predicted (E. Peisach and D. Ringe, private communication). Other inhibitors currently are being designed according to the the principles outlined in the present article, making use of additional sites.

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