Understanding the P_1' Specificity of the Matrix Metalloproteinases: Effect of S_1' Pocket Mutations in Matrilysin and Stromelysin-1†

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Received January 26, 1996; Revised Manuscript Received May 20, 1996⊗

ABSTRACT: Matrilysin (MAT) prefers leucine over residues that have aromatic side chains at the P_1' position of peptide and protein substrates, while stromelysin (HFS) has a broader specificity. The X-ray structures of these enzymes show that their respective S_1' subsites differ primarily due to the amino acids present at positions 214 and 215. To examine the role that these residues play in determining P_1' specificity, the amino acids at these positions in matrilysin have been replaced by those found in stromelysin (MAT: Y214L, MAT:A215V, and MAT:Y214L/A215V). The specificity and activity of MAT:A215V are similar to those of wild type matrilysin. Both MAT:Y214L and MAT:Y214L/A215V, however, have P_1' specificities that are more similar to stromelysin than matrilysin. Specifically, these enzymes exhibit an 8- to 9-fold reduction in k_{cat}/K_M toward a peptide substrate with Leu in subsite P_1' relative to wild type matrilysin. This is predominantly the result of an approximate 5-fold decrease in k_{cat}. The K_M values only partially increase toward the value observed for stromelysin. Studies of the pre-steady-state reaction of wild type and mutant matrilysin with substrates with Leu and Tyr residues in the P_1' position confirm that the K_M values for these reactions reflect K_D values for substrate binding. Thus, replacement of a single tyrosine residue in the S_1' pocket of matrilysin by leucine alters its P_1' specificity to resemble that of stromelysin. In contrast, alteration of the S_1' subsite of stromelysin (HFS:L214Y/V215A) to resemble matrilysin increases activity (i.e., higher k_{cat}/K_M) toward peptide substrates with both leucine and residues with aromatic side chains in the P_1' position with only a partial increase in specificity for Leu. These increases in activity are the result of decreases in the K_M values for these reactions.

Inappropriate degradation of the extracellular matrix is associated with a number of pathological processes including cancer cell metastasis, periodontitis, angiogenesis, and osteo- and rheumatoid arthritis (Woessner, 1991; Birkedal-Hansen et al., 1993). A family of zinc endopeptidases known as the matrix metalloproteinases (MMPs)[1] has been shown to contribute markedly to the catabolism of matrix proteins during these processes. Therefore, the MMPs are targets for the development of novel chemotherapeutic agents for the treatment of a variety of diseases. A detailed understanding of the structural features of these enzymes that contribute to their specificities should aid in the design of inhibitors which are specific for one MMP over another.

The recent publication of structures for several members of the MMP family allows the structural basis for their specificity to be addressed. The structures of the catalytic domains of human fibroblast stromelysin-1 (HFS, MMP-3, EC 3.4.24.17) (Gooley et al., 1994; Becker et al., 1995), human fibroblast collagenase (HFC, MMP-1, EC 3.4.24.7) (Lovejoy et al., 1994; Borkakoti et al., 1994; Spurlino et al., 1994), human neutrophil collagenase (HNC, MMP-8, EC 3.4.24.34) (Stams et al., 1994; Bode et al., 1994; Grams et al., 1995), and matrilysin (MAT, MMP-7, EC 3.4.24.3) (Browner et al., 1995) bound to substrate-analog inhibitors have been reported. These studies have revealed that the overall secondary and tertiary structures of these enzymes are conserved. The S_1' subsite (nomenclature of Schechter and Berger, 1967) is the most well-defined binding site in these MMPs, consisting of a hydrophobic pocket of variable depth. Two types of S_1' pocket architecture can be distinguished from the available structures. One type consists of a shallow pocket whose bottom is defined primarily by the side chain of residue 214 (numbering according to Browner et al. (1995) in which the first residue of the activated enzyme is residue 100). MAT (Figure 1A) and HFC possess this architecture, where the side chains of Tyr-214 and Arg-214, respectively, form the bottom of the pocket. The other type of S_1' subsite consists of a deep hydrophobic pocket that forms a channel through the enzyme. HFS (Figure 1B) and HNC fall into this class, and both have a leucine residue in position 214 that points away from the P_1' side chain of the bound inhibitor.

† This work was supported by National Institutes of Health Grant GM27939 to H.E.V.W.

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: DIEA, *N*-ethyldiisopropylamine; Dnp, 2,4-dinitrophenyl; EDT, 1,2-ethanedithiol; HFC, human fibroblast collagenase; HFS, human stromelysin-1; HNC, human neutrophil collagenase; HOBt, 1-hydroxybenzotriazole; MAT, human matrilysin; MMP, matrix metalloproteinase; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; MES, 2-(*N*-morpholino)ethanesulfonate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate; Fmoc, (9-fluorenylmethoxy)carbonyl; Boc, (*tert*-butyloxy)carbonyl; HPLC, high performance liquid chromatography; BB94, (2*S*,3*R*)-5-methyl-3-[[(α-*S*)-α-(methylcarbamoyl)-phenethyl]carbamoyl]-2-[(2-thienylthio)methyl]hexanohydroxamic acid.

S0006-2960(96)00196-1 CCC: $12.00

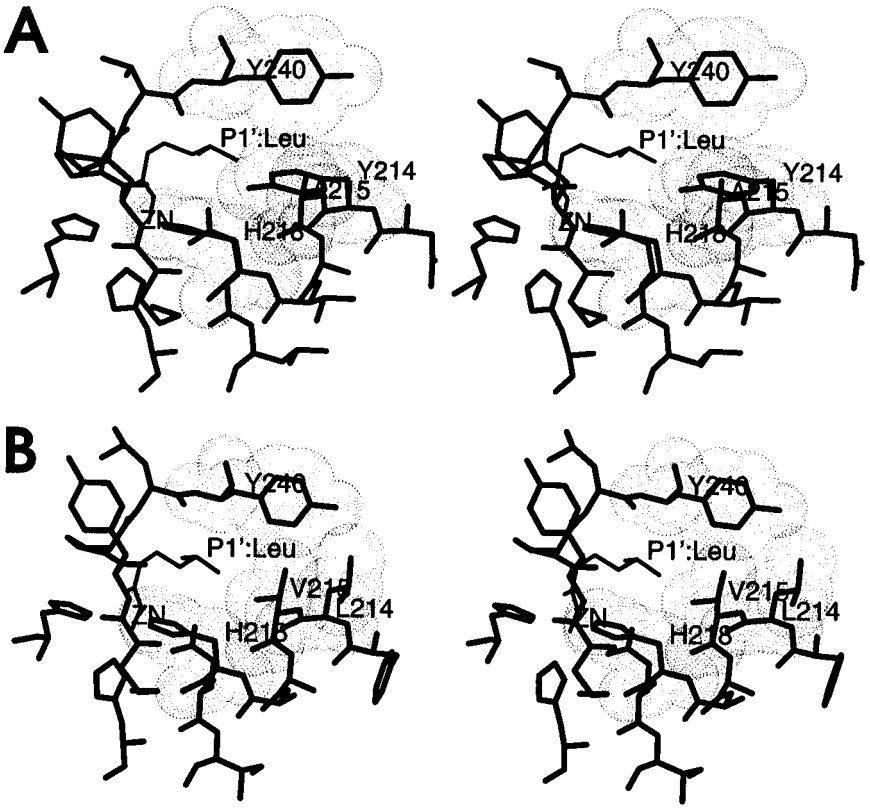

FIGURE 1: Stereo diagram of the S_1' subsites of (A) MAT and (B) HFS, including the active site zinc atom. The diagrams were constructed from the X-ray crystal structures of the MAT [MMQ from the Protein Data Bank (Bernstein et al., 1977; Abola et al., 1987)] and HFS (K. Appelt and B. Almassy, personal communication) catalytic domains complexed to hydroxamate inhibitors that contain a Leu side chain in the P_1' position (P_1':Leu). In both diagrams, residues 213−220, 222, 228, and 236−240 are shown. The dotted surface indicates the van der Waals radius of residues critical in defining the differences between the two subsites, most importantly residues 214 and 215.

The substrate specificities of the human MMPs have been examined previously against a library of octapeptides (Fields et al., 1987; Mallya et al., 1990; Netzel-Arnett et al., 1991a, 1992, 1993). MAT and HFC prefer residues with aliphatic vs aromatic side chains in the P_1' position. In particular, MAT strongly prefers substrates with a Leu residue in this P_1' position. In contrast, HNC and HFS hydrolyze substrates with a wider variety of P_1' residues with similar efficiency. The most dramatic differences in the structures of MAT and HFS lie in their different S_1' subsite architectures, making them good candidates for studies designed to address the relationship between S_1' subsite structure and P_1' substrate specificity. The S_1' pockets of MAT and HFS differ only in the residues at positions 214 and 215, which are Tyr and Ala in MAT, but Leu and Val in HFS, respectively (Figure 2). To examine the contribution of these residues in determining the preference of MAT for Leu in the P_1' position, both the single and the double mutations have been introduced into MAT and the resulting mutant enzymes examined for their specificity against a set of fluorogenic peptide substrates. Conversely, a double mutation was introduced into the S_1' subsite of HFS, and the specificity of this mutant has also been examined.

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MAT          ...LYAATHEL.....MYPTYGN...
HFC          ...HRVAAHEL.....MYPSYTF...
HS-3         ...LQVAAHEF.....MSAFYTF...
MME          ...FLTAVHEI.....MFPTYKY...

HFS          ...FLVAAHEI.....MYPLYHS...
HS-2         ...FLVAAHEL.....MYPLYNS...
HNC          ...FLVAAHEF.....MYPNYAF...
HC-3         ...FLVAAHEF.....MFPIYTY...
Gel A        ...FLVAAHEF.....MAPIYTY...
Gel B        ...FLVAAHEF.....MYPMYRF...
MT-MMP       ...FLVAVHEL.....MAPFYQW...
                    |              |
                   213            236
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FIGURE 2: Amino acid residues contributing side chains to the S_1' subsite of MAT, and by analogy, the other ten human MMPs, are highlighted in bold typeface. The MMPs in the lower group have identical residues at the five highlighted positions, while those in the upper group all differ in at least one of these positions. The abbreviations are HS-2, human stromelysin-2; HS-3, human stromelysin-3; MME, macrophage metalloelastase; HC-3, human collagenase-3; Gel A, human gelatinase A; Gel B, human gelatinase B; MT-MMP, human membrane-type MMP.

MATERIALS AND METHODS

Materials. Sepharose-Pro-Leu-Gly-NHOH was prepared as described by Moore and Spilburg (1986). The following reagents were purchased from the sources indicated: XL1-Blue cells (Stratagene); BL21(DE3) cells (Novagen); restriction enzymes (Boehringer-Mannheim); Tris, MES, HEPES, guanidine-HCl, activated Sepharose, Pro-Leu-Gly-NHOH (Sigma); Taq polymerase (Perkin Elmer Cetus); oligonucleotides (Bio-Synthesis, Lewisville, TX); amino acid derivatives (Bachem, King of Prussia, and Novabiochem, San Diego).

Mutagenesis of MAT and HFS. Site-directed mutations were introduced into MAT and HFS using the oligonucleotide-directed method as modified by Kunkel et al. (1987). The following oligonucleotides were used to introduce mutations into MAT: Y214L: 5′GGATTAACTTCCTGCTAGCTGCAACTC3′; A215V: 5′CTTCCTGTATGT-

TGCAACTCATG3′; Y214LA215V: 5′GATTAACTTC-CTGGGTACCGCAACTCATG3′; and HFS: L214Y/V215A: 5′CCAATTTATTTTACGCTGCTGCTCATGAAATTGGC-C3′.

Expression and Purification of MAT and HFS from Escherichia coli. Wild type and mutant (Y214L, A215V, Y214L/A215V) pro-MAT were expressed as N-terminal fusion proteins with yeast ubiquitin and purified from *E. coli*. The cloning, expression, activation, and purification to obtain the respective MAT catalytic domains have been described in detail previously (Welch et al., 1995). A section of DNA coding for the Ala-1 to Thr-256 portion of HFS was ligated into an expression plasmid utilizing the T7 gene 10 promoter designed to express the truncated form (i.e., with the hemopexin-like domain deleted) of the enzyme. Both the wild type and mutant (L214Y/V215A) truncated forms of pro-HFS were expressed and purified from *E. coli* and the respective catalytic domains obtained after activation with aminophenylmercuric acetate, as described previously (Marcy et al., 1991; Holman et al., in preparation). In all subsequent sections, HFS refers to the stromelysin-1 catalytic domain.

Synthesis and Characterization of Dnp-Arg-Pro-Leu-Ala-X-Trp-Arg-Ser-Y. The peptide with X = Leu and Y = COOH was synthesized as described previously (Welch et al., 1995). The homologs with X = Tyr, Phe and Trp, and Y = NH_2 were synthesized by stepwise solid-phase synthesis using a Vega Coupler 250 (Vega Biotechnologies, Tucson) in the semiautomated mode in which activated amino acid derivatives were added manually. Fmoc protection was used for α-amino groups and Pmc and *tert*-butyl protection for Arg and Ser side chains, respectively. Due to the presence of the two Arg(Pmc) residues in each peptide, *N*-Boc protection of the indole ring of Trp was used in order to minimize side reactions during final deprotection (White, 1992). *p*-[(*R*,*S*)-α-[1-(9*H*-fluoren-9-yl)methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid (Novabiochem, San Diego) was used as a linker to yield C-terminal amides upon cleavage of the peptides from the resin. All amino acids were double coupled in 3-fold molar excess using TBTU/HOBt activation. After completion of the synthesis, the Dnp group was introduced by reacting the α-amino group of the resin-bound peptides with 10 equiv of 2,4-dinitrofluorobenzene in the presence of 15 equiv of DIEA.

Peptides were deprotected and cleaved from the resin by treatment with TFA/EDT/H_2O (76:20:4) for 2 h at 40 °C. The resin was filtered and washed, and the combined filtrate and washings were concentrated *in vacuo*. The residue was diluted with 20% acetic acid, the solution was extracted with ether, and the aqueous layer was lyophilized. Crude products were redissolved in A/B (70:30) at ∼40 mg/mL, where A was 0.1% TFA in H_2O and B was 0.1% TFA in 95% CH_3-CN. The peptides were purified by reverse phase HPLC on a Rainin Dynamax C-8 column (5 μm particle size, 300 Å pore size, 21.4 × 250 mm) using gradients of 0.5% B/min in A at a flow rate of 14 mL/min. Purity of the products was checked by analytical reverse phase HPLC using gradients of 1% B/min in A on a Rainin Microsorb-MV C-8 column (5 μm particle size, 300 Å pore size, 4.6 × 250 mm). Identity of the peptides was confirmed by mass spectrometry.

Enzyme Assays and Determination of Steady-State Kinetic Parameters. The hydrolysis of Dnp-RPLAXWRS (X = Leu, Tyr, Phe, Trp) was monitored fluorometrically using excitation at 278 nm and emission at 358 nm (Stack & Gray, 1989). Assays were carried out in 50 mM HEPES, 200 mM NaCl, 1 mM $CaCl_2$, 0.01% Brij-35, pH 7.5 at 30 °C. The final $[E_0]$ in the assays was 50 nM, with the exception of assays for the hydrolysis of Dnp-RPLALWRS by MAT, where it was 10 nM. Initial rates (v) were calculated from the changes in fluorescence as described previously (Netzel-Arnett et al., 1991b). To evaluate the steady-state kinetic parameters, v was measured at values of $[S_0]$ of 5, 10, 20, 60, 120, and 200 μM for Dnp-RPLAXWRS, where X = Leu, Tyr, and Phe, and 5, 10, 20, 60, and 120 μM where X = Trp. Four assays were performed at each $[S_0]$, and the kinetic parameters V_{max} and K_M were estimated by nonlinear curve fitting to the equation $v = V_{max}[S_0]/(K_M + [S_0])$ (Cleland, 1979) using the program KinetAsyst. Values of k_{cat} were calculated by dividing the V_{max} values by the concentration of enzyme active sites, as determined by active site titration with the inhibitor BB94 (Davies et al., 1993).

Stopped-Flow Measurements and Determination of Pre-Steady-State Kinetic Parameters. Both the Applied Photophysics SX-17MV and KinTek Model SF-2001 stopped-flow instruments were used to obtain pre-steady-state fluorescence transients for the reaction of HFS and MAT with the Dnp substrates. An excitation wavelength of 280 nm was selected with a monochrometer using a bandwidth of 2−10 nm. The emission wavelength was selected with a broadband 360 nm bandpass filter (Oriel #51660, Stratford, CT) to allow observation of Trp fluorescence. All reactions were carried out at 30 °C. Values of $[E_0]$ after mixing were either 0.5 or 1 μM. The transients obtained were biphasic, consisting of a rapid initial phase in which Trp fluorescence decreased followed by a much slower phase of increasing fluorescence resulting from substrate hydrolysis. The rapid phase of the transients was fit to a single exponential, or in cases where the two phases were not well separated, the transient was fit to a sum of an exponential and a linear phase. Three to four replicate shots were averaged before curve fitting. The observed first-order rate constant (k_{obs}) for the rapid phase was measured at five values of $[S_0]$ in the 20−500 μM range that straddled the K_M values for these reactions. Plots of k_{obs} vs $[S_0]$ were linear, and values of the rate constants for substrate binding, k_{on}, and substrate release, k_{off}, were obtained from the slope and intercept, respectively (Johnson, 1992). Values of the substrate dissociation constant (K_D) were calcuated from the ratio k_{off}/k_{on}.

RESULTS

P_1' Specificities of MAT and HFS. The P_1' residues found at the sites that MAT (Netzel-Arnett et al., 1993) and HFS (Nagase et al., 1994) hydrolyze protein substrates have been cataloged previously. These data indicate that MAT exhibits a marked preference for Leu in the P_1' position, but that HFS has a much broader specificity. In particular, HFS accepts substrates with Phe, Tyr, Leu, Ser, and Met residues in subsite P_1'. This conclusion is also consistent with specificity studies using peptide substrates (Netzel-Arnett et al., 1992, 1993).

To facilitate kinetic studies of MAT and HFS, we have recently described a new fluorogenic substrate with the sequence Dnp-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Ser (Welch et al., 1995). This substrate is hydrolyzed efficiently at the Ala-Leu bond by both MAT ($k_{cat}/K_M = 1.9 \times 10^5$ M^{-1} s^{-1}) and HFS ($k_{cat}/K_M = 1.0 \times 10^4$ M^{-1} s^{-1}). The solubility of

Table 1: P_1' Specificity of Wild Type MAT and HFS

P_1' residue	act. (%) (k_{cat}/K_M (M^{-1} s^{-1}))	
	MAT	HFS
Leu	100 (1.9×10^5)	100 (1.0×10^4)
Tyr	2.1	69
Phe	1.8	98
Trp	0.53	23

this substrate in assay buffer (50 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$, 0.01% Brij-35, pH 7.5) is 38- and 8.5-fold greater than the K_M value for its hydrolysis by MAT and HFS, respectively, allowing accurate steady-state kinetic parameters for these reactions to be measured. The Dnp group of this substrate quenches the intrinsic Trp fluorescence of these enzymes on binding. Thus, this quenching can be used to measure the pre-steady-state equilibration of substrate with these enzymes and allows evaluation of k_{on}, k_{off}, and K_D (Brenner et al., in preparation).

To assist in the present investigation, new substrates with Tyr, Phe, and Trp residues in place of Leu in the P_1' position (also designated residue X) have been synthesized. Using these substrates, the P_1' specificities of both wild type and mutant MAT and HFS have been examined with respect to their preference for Leu vs residues with aromatic side chains in the P_1' position. All four substrates are hydrolyzed by these enzymes exclusively at the Ala-X bond. The relative rates of hydrolysis of these substrates by wild type MAT and HFS are shown in Table 1, where the rate for the substrate with Leu in P_1' is taken as 100%. These data confirm the conclusion reached previously from an examination of the protein cleavage sites for these two MMP that MAT exhibits a marked preference for Leu in subsite P_1', but that HFS has a broader specificity and also accommodates Tyr, Phe, and Trp residues at this position.

Differences in the S_1' Subsites of MAT and HFS. Comparison of the structures of HFS (Gooley et al., 1994; Becker et al., 1995; Appelt et al., personal communication) and MAT (Browner et al., 1995) shows that there are differences in their respective S_1' subsites that are likely to be the basis for their differences in P_1' specificity. The five amino acid side chains that define the S_1' subsite of MAT (i.e., those within 5 Å of the P_1' Leu side chain of the bound inhibitor; Browner et al., 1995), and by extrapolation, the other MMPs, are highlighted in bold typeface in Figure 2. HFS is representative of a group of seven human MMPs (also including stromelysin-2, neutrophil collagenase, collagenase-3, gelatinase A, gelatinase B, and membrane-type MMP) in which the five S_1' pocket residues are identical. MAT is one of four MMPs (also including fibroblast collagenase, stromelysin-3, and macrophage metalloelastase) in which there is a residue that is different from HFS in at least one of these five positions.

Of the five residues highlighted, only those in positions 214 and 215 are different between MAT and HFS. These residues are Tyr and Ala in MAT, and Leu and Val in HFS, respectively. In MAT, the side chain of Tyr-214 defines the bottom of the S_1' pocket and is in close contact with the Leu side chain in the P_1' subsite of the bound inhibitor (Figure 1A). In contrast, the side chain of Leu-214 of HFS points away from the P_1' side chain of the inhibitor and is part of the side of a deep channel that extends from the active site (Figure 1B). Thus, we hypothesize that the differences in P_1' specificity between MAT and HFS are due to the different residues at positions 214 and 215 in these two MMPs.

Effect of Mutations at Positions 214 and 215 on the P_1' Specificity of MAT. To investigate the hypothesis that the residues at positions 214 and 215 are responsible for the differences in specificity between MAT and HFS, the three mutants MAT:Y214L, MAT:A215V, and MAT:Y214L/A215V have been constructed, expressed in *E. coli*, and purified. The P_1' specificities of these mutants have been examined by measuring the k_{cat}/K_M values for the hydrolysis of the four fluorogenic peptides described above. The specificities of the engineered proteins are compared with those of wild type MAT and HFS in Table 2. Replacement of Ala-215 by Val has very little effect on the specificity of MAT. In contrast, both of the mutants involving replacement of Tyr-214 by Leu (MAT:Y214L and MAT:Y214L/A215V) exhibit a loss of specificity for Leu and a broadening of P_1' specificity to resemble that of HFS. This is primarily the result of decreasing k_{cat}/K_M for the Leu substrate as opposed to an increase for the others. The similarity in the P_1' specificities of MAT:Y214L and MAT:Y214L/A215V indicates that Tyr-214 is the most important residue in determining the strong preference of MAT for Leu.

Effect of Mutations at Positions 214 and 215 in MAT on the Steady-State and Pre-Steady-State Kinetic Parameters for Hydrolysis of Substrates with P_1' Leu and Tyr Residues. To examine the effect of these S_1' pocket mutations on the activity of MAT toward substrates with P_1' Leu and Tyr residues in more detail, additional kinetic measurements were performed. These included measurement of the individual steady-state kinetic parameters, k_{cat} and K_M, and evaluation of the k_{on} and k_{off} rate constants (and, hence, K_D values) from the pre-steady-state reactions (Table 3). The A215V mutation has very little effect on any of the parameters for interaction of MAT with either substrate. There is virtually no effect on the Leu substrate, and only an approximate 2-fold increase in k_{cat}/K_M toward the Tyr substrate that is due to an increase in k_{cat}. For these and all of the other reactions shown in this table, there is a reasonably good

Table 2: Effect of S_1' Subsite Mutations on the P_1' Specificity of MAT

P_1' residue	act. (%) (k_{cat}/K_M (M^{-1} s^{-1}))				
	MAT	A215V	Y214L	Y214L A215V	HFS
Leu	100 (1.9×10^5)	100 (1.7×10^5)	100 (2.3×10^4)	100 (2.1×10^4)	100 (1.0×10^4)
Tyr	2.1	5.1	83	90	69
Phe	1.8	6.5	25	33	98
Trp	0.53	0.59	11	4.8	23

Table 3: Steady-State and Pre-Steady-State Kinetic Parameters for the Hydrolysis of Dnp-Arg-Pro-Leu-Ala-X-Trp-Arg-Ser by MAT, MAT Mutants, and HFS

enzyme	P_1' residue (X)	k_{cat}/K_M ($\times 10^{-4}$) (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_M (μM)	K_D (μM)	k_{on} ($\times 10^{-5}$) (M^{-1} s^{-1})	k_{off} (s^{-1})
MAT:							
wild type	Leu	19	5.0	26	39	4.1	16
	Tyr	0.40	0.45	110	150	1.2	18
A215V	Leu	17	8.4	49	31	5.5	17
	Tyr	0.87	0.87	100	170	2.6	45
Y214L	Leu	2.3	1.0	44	63	3.0	19
	Tyr	1.9	0.80	42	73	1.5	11
Y214L/A215V	Leu	2.1	1.4	65	74	7.2	53
	Tyr	1.9	1.1	57	100	3.5	36
HFS:							
wild type	Leu	1.0	1.2	120	110	0.25	2.8
	Tyr	0.69			570	0.068	3.9

agreement between the values of K_M and K_D, indicating that there is rapid equilibrium between the free enzyme, free substrate, and enzyme−substrate complex.

In contrast, MAT:Y214L and MAT:Y214L/A215V exhibit an 8- to 9-fold decrease in k_{cat}/K_M relative to wild type MAT toward the substrate with a Leu residue in the P_1' position. This is primarily the result of a decrease in k_{cat} to almost the same value as observed for HFS. This is further support for the conclusion that the residue at position 214 of MAT plays the dominant role in determining its preference for Leu in position P_1'. The K_M values for hydrolysis of this substrate by these two MAT mutants only partially increase (less than 2-fold) toward the value observed for HFS. This small increase in K_M is paralleled by a similar increase in K_D. It is interesting that although the K_D values of HFS and MAT:Y214L/A215V are similar for the substrate with Leu in subsite P_1', the k_{on} and k_{off} values for HFS are both more than 10-fold lower than for MAT.

Both MAT:Y214L and MAT:Y214L/A215V exhibit an approximate 4-fold increase in k_{cat}/K_M toward a substrate containing a Tyr residue in the P_1' position. For MAT:Y214L, this is due to a 2.5-fold decrease in K_M combined with an approximate 2-fold increase in k_{cat}. A similar trend is observed for MAT:Y214L/A215V. The shift in k_{cat} for these enzymes is in the direction of the value observed for HFS. The decrease in K_M, however, does not mimic the situation for HFS. The K_M value for hydrolysis of this substrate by HFS is >200 μM and could not be determined due to the limited solubility of the peptide. Thus, it appears that these mutations in MAT increase the strength of substrate binding through interactions that are not present in HFS.

Effect of Mutations at Positions 214 and 215 on the P_1' Specificity of HFS. In light of the results presented above for MAT that show that its P_1' specificity can be partly converted into that for HFS through mutations at positions 214 and 215, the reverse experiment was carried out to determine whether the specificity of HFS could be changed to resemble that of MAT. Thus, the double S_1' pocket mutant HFS:L214Y/V215A was prepared and its P_1' specificity assessed using the substrates described above (Table 4). The k_{cat}/K_M value for the mutant increased significantly (9.6-fold) toward the Leu substrate in the direction observed for MAT. Unexpectedly, however, the k_{cat}/K_M values also increased for the substrates with the P_1' residues with aromatic side chains. As a result, this mutant only partially gained the increased specificity for Leu that is characteristic of MAT. Moreover,

Table 4: Steady-State Kinetic Parameters for the Hydrolysis of Dnp-Arg-Pro-Leu-Ala-X-Trp-Arg-Ser by HFS, HFS (L214Y,V215A), and MAT

enzyme	P_1' residue (X)	act. (%)	k_{cat}/K_M ($\times 10^{-4}$) (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)
HFS:					
wild type	Leu	100	1.0	1.2	120
	Tyr	69	0.69		
	Phe	98	0.98		
	Trp	23	0.23		
HFS:					
L214Y/V215A	Leu	100	9.6	1.0	10
	Tyr	18	1.7	0.35	20
	Phe	45	4.3	0.79	18
	Trp	4.6	0.44		
MAT:					
wild type	Leu	100	19	5.0	26
	Tyr	2.3	0.40	0.45	110
	Phe	1.8	0.35		
	Trp	0.53	0.10		

in contrast to MAT for which the specificity for Leu in position P_1' is the result of a higher k_{cat}, the HFS mutant has achieved higher activity toward this substrate exclusively by decreasing K_M. In fact, the HFS mutant also binds the substrates with Tyr and Phe residues in position P_1' more efficiently, as evidenced by the lowered K_M values for these reactions.

DISCUSSION

Previous studies have demonstrated that MAT has a marked preference for Leu in the P_1' position of protein and peptide substrates (Netzel-Arnett et al., 1993). To examine the structural basis for this specificity, the S_1' subsite of MAT has been compared with that of HFS, a homologous enzyme with broader P_1' specificity. The major difference in the S_1' subsites is due to the side chain of residue 214 which is Tyr in MAT, but Leu in HFS. Indeed, mutation of Tyr-214 to Leu in MAT gives an enzyme with a specificity profile very similar to that of HFS. The observed broadening of specificity is largely the result of a 9-fold decrease in k_{cat}/K_M toward the substrate with a P_1' Leu residue caused primarily by a 5-fold decrease in k_{cat}. Thus, the specificity of wild type MAT for Leu residues in subsite P_1' is attributable to a specific interaction with Tyr-214. This interaction generates binding energy that can be used to stabilize the transition state. This interaction is lost on replacement of this Tyr residue by Leu.

These results are very similar to those reported for the S_2 specificity of cathepsin S, a cysteine proteinase which prefers Leu at the P_2 position of peptide substrates (Bromme et al., 1994). Mutations introduced into the S_2 subsite of cathepsin S in an attempt to alter its specificity to resemble that of the homologous enzyme, cathepsin L, resulted in a decrease in k_{cat}/K_M toward the substrate with a Leu residue in subsite P_2. As with MAT, this decrease was the result of a reduced k_{cat}, with little effect on K_M. Thus, MAT and cathepsin S are examples of unrelated proteinases which have evolved specificity toward a Leu residue in a specific subsite by using binding energy to stabilize the transition state and increase k_{cat}. In both cases, it has proven possible to *engineer out* this interaction by making mutations in a subsite of the enzyme.

In contrast, our attempt with HFS to *engineer in* a preference for Leu has been less successful. Changing the S_1' pocket residues of HFS into those found in MAT (HFS: L214Y/V215A), in fact, increases the k_{cat}/K_M values for all four substrates, but gives only a small increase in specificity toward Leu. The increase in k_{cat}/K_M is the result of a decrease in K_M toward these substrates. A similar result has been reported in mutational studies of the hydrophobic S_1 subsite of α-lytic protease (Bone et al., 1989). The mutations introduced also resulted in an increase in k_{cat}/K_M for hydrolysis of substrates with large P_1 residues. As with the HFS mutant described here, these increases were primarily due to decreased K_M values. Thus, while it has proven possible to *engineer out* the preference for Leu in MAT by lowering k_{cat}, we have only been able to *engineer in* tighter binding in HFS, as reflected by lowered values of K_M.

In view of these results, it is appropriate to distinguish two aspects of specificity. One aspect pertains to the ability to increase k_{cat}/K_M, which can often be achieved for multiple substrates of similar structure. A second distinct aspect pertains to the ability to discriminate between a group of good substrates of closely related structure. Protein engineering studies of proteases have demonstrated that it is considerably easier to achieve the former than the latter. Mutation of residues that are in direct contact with the substrate in α-lytic protease (Bone et al., 1989), subtilisin (Wells et al., 1987), and HFS (this study) have produced increases in k_{cat}/K_M that were due predominantly to decreases in K_M. Although some of the changes in activity observed in these studies were quite large, there were few corresponding changes in the ability of the engineered enzymes to discriminate between closely related substrates.

With respect to substrate discrimination, recent protein engineering studies have revealed the importance of structural elements of the enzyme that are not in direct contact with the substrate. Studies of the specificity of chymotrypsin and trypsin (Hedstrom et al., 1992), and α-lytic protease (Mace et al., 1995), have shown that loops within the respective enzymes contribute significantly to their specificities. Mutations in surface residues are translated into subtle changes in the structure of the substrate binding pocket. Interestingly, the increases in k_{cat}/K_M observed for individual substrates as the result of surface loop mutations were most often due to increases in k_{cat}.

The importance of loop structures in MAT and their role in determining the substrate binding site structure have been investigated (Browner et al., in preparation). MAT has a surface loop that consists of residues 240−249 (referred to as the S_1' loop) which appears to be essential for maintaining the appropriate orientation of the Tyr-214 side chain in the S_1' pocket. The side chain of Phe-249 interacts with the aromatic ring of Tyr-214 by creating an edge-to-face stacking interaction. This interaction positions the Tyr-214 side chain perpendicular to the S_1' subsite such that one edge of the ring is pointing directly toward the incoming P_1' residue. This orientation of Tyr-214 is also stabilized by a water molecule which bridges the hydroxyl group of Tyr-214 to the carbonyl oxygen of Tyr-237. It is possible that the failure of the HFS L214Y/V215A mutant to acquire the preference of MAT for Leu in subsite P_1' is due to differences in these other structural features of the S_1' subsite. To alter more subtly the P_1' specificity of HFS, the possibility of changing residues within the S_1' loop, along with those within the S_1' subsite, should be considered.

This study also revealed an important difference between the way that HFS and MAT bind substrates. HFS shows significantly slower k_{on} and k_{off} rates for substrates with both Leu and Tyr residues in position P_1' compared with MAT. These differences could be due to subtle differences in the construction of the S_1' pocket. Alternatively, the active site clefts of these two MMPs differ in that there is a Phe residue at position 227 of HFS, but a Gly residue in MAT. In HFS, the side chain of Phe-227 is positioned directly over the active site cleft in a manner that could slow down both substrate binding and release. In contrast, the Gly-227 residue in MAT provides no restriction to the active site cleft. Future mutagenesis studies will test this possibility.

The structural analyses of several MMPs including MAT (Browner et al., 1995), HFS (Gooley et al., 1994; Becker et al., 1995; Appelt et al., personal communication), HNC (Stams et al., 1994; Bode et al., 1994; Grams et al., 1995), and HFC (Lovejoy et al., 1994; Borkakoti et al., 1994; Spurlino et al., 1994) reveal that the S_1' subsite is the most well-defined specificity pocket in these MMPs. In this subsite, the residue in position 214 appears to play an important role in shaping the pocket and influencing P_1' specificity. For MMPs of known sequence, but whose structures have not been solved, the identity of the residue at this position allows one to speculate as to the general shape of the S_1' pocket. This assumes, of course, that there are no large changes in the conformation of the backbone due to movements of loops. Thus, for the group of seven MMPs (Figure 2) that have Leu at position 214, the S_1' subsite is predicted to have a deep hydrophobic pocket. This includes the recently described collagenase-3 (Freije et al., 1994) and membrane-type MMP (Sato et al., 1994). In contrast, if there is a residue with a polar side chain at position 214, the pocket is predicted to be shallower. By these criteria, the S_1' subsite of HFS-3 (Basset et al., 1990) is predicted to be the shallow type. This generalization may aid in predicting the potential P_1' specificities of new MMPs.

ACKNOWLEDGMENT

The authors thank Drs. Krzysztof Appelt and Bob Almassy for kindly providing access to the coordinates of the X-ray structure of the HFS−inhibitor complex, Dr. Vera Imper for initiating the synthesis of the fluorogenic peptides used in this study, and Dr. Steve Margosiak for use of the Applied Photophysics stopped-flow instrument. We would also like to acknowledge Dr. Sarah Netzel-Arnett, Dr. James Fann,

Dr. Mauricio Sankovic, and Marianna Dioszegi for helpful discussions and criticism during this study.

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BI9601969