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Active site specificity of plasmepsin II

JENNIFER WESTLING,[1] PATTY CIPULLO,[1,3] SU-HWI HUNG,[1] HOWARD SAFT,[1,3]
JOHN B. DAME,[2] AND BEN M. DUNN[1]

[1]Department of Biochemistry & Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida 32610-0245
[2]Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610-0245

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Abstract

Members of the aspartic proteinase family of enzymes have very similar three-dimensional structures and catalytic mechanisms. Each, however, has unique substrate specificity. These distinctions arise from variations in amino acid residues that line the active site subsites and interact with the side chains of the amino acids of the peptides that bind to the active site. To understand the unique binding preferences of plasmepsin II, an enzyme of the aspartic proteinase class from the malaria parasite, *Plasmodium falciparum*, chromogenic octapeptides having systematic substitutions at various positions in the sequence were analyzed. This enabled the design of new, improved substrates for this enzyme (Lys-Pro-Ile-Leu-Phe*Nph-Ala/Glu-Leu-Lys, where * indicates the cleavage point). Additionally, the crystal structure of plasmepsin II was analyzed to explain the binding characteristics. Specific amino acids (Met13, Ser77, and Ile287) that were suspected of contributing to active site binding and specificity were chosen for site-directed mutagenesis experiments. The Met13Glu and Ile287Glu single mutants and the Met13Glu/Ile287Glu double mutant gain the ability to cleave substrates containing Lys residues.

Keywords: active site; aspartic protease; hemoglobin degradation; malaria; mutagenesis; plasmepsin II; oligopeptide substrates

Malaria remains a serious public health problem in the developing world, threatening half of the world's population. Each year, malaria is responsible for up to 2 million deaths and 100 million new clinical infections (World Health Organization, 1997). Unfortunately, these numbers are rising due to the resistance of parasites and insect vectors to current drugs and insecticides, respectively. Thus, there is an urgent need for the development of new antimalarial therapeutics.

Attractive targets for antimalarial agents can be found in the biochemical differences between the *Plasmodium* parasite and the human host. During its infection in humans, the parasite spends most of its life cycle in the erythrocyte. Here, the parasite degrades hemoglobin for its main source of amino acids required for new protein synthesis. The parasite relies on the enzymatic action of the aspartic proteinases, known as plasmepsins, to break down the hemoglobin into fragments (Francis et al., 1994). Inhibitors of these enzymes prevent the degradation of hemoglobin and kill the parasites in culture. Therefore, the plasmepsins have become attractive drug targets.

In previous work (Dame et al., 1994; Francis et al., 1994), genes were cloned from *Plasmodium falciparum* that encode two aspartic proteinases, termed plasmepsin I and II (Goldberg, 1998). Plasmepsin II (hereafter abbreviated PfPMII) can be produced in high yield and purity by expression of the recombinant gene in *Escherichia coli.*

To investigate the catalytic specificity of PfPMII, the recombinant enzyme was tested using synthetic chromogenic substrates. The crystal structure of PfPMII (Silva et al., 1996) was used to identify residues for mutation to verify their roles in determining substrate/enzyme interactions. This resulted in the elucidation of substrate specificity requirements unique to the plasmepsins that may contribute to the design of inhibitors with antimalarial activities.

Results

Chromogenic substrate analyses

Previous kinetic analyses using PfPMII have focused on the digestion of hemoglobin, which may be affected by the tertiary structure of this large molecule. To probe the subsite specificity of PfPMII more completely and to place PfPMII in relation to other aspartic proteinases, chromogenic octapeptides that have variations in the P_4, P_3, P_2, P_2' and P_3' positions (Schecter & Berger, 1967) based on differences in charge, size, hydrophobicity, and hydrogen bonding (Dunn et al., 1995) were analyzed. The resulting kinetic parameters of k_{cat}, K_m, and k_{cat}/K_m are shown in Table 1.

Reprint requests to: Ben M. Dunn, P.O. Box 100245, Gainesville, Florida 32610-0245; e-mail: bdunn@college.med.ufl.edu.
[3]Undergraduate research participant.

Table 1. *Kinetic parameters obtained using chromogenic octapeptides with variations in the P_4, P_3, P_2, P'_2, and P'_3 positions*

	P_5	P_4	P_3	P_2	P_1*P'_1	P'_2	P'_3	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
1	Lys	Pro	Ile	Glu	Phe*Nph[a]	Arg	Leu	2.0 ± 0.2	23 ± 3	87 ± 17
		Phe						1.1 ± 0.2	21 ± 4	50 ± 10
		Ala						2.0 ± 0.3	58 ± 11	33 ± 8
		Ser						1.3 ± 0.2	110 ± 18	12 ± 2
		Asp						1.1 ± 0.2	73 ± 12	15 ± 3
		Lys						0.17 ± 0.03	117 ± 18	1.4 ± 0.3
			Phe					2.4 ± 0.3	24 ± 3	100 ± 16
			Ala					0.5 ± 0.1	37 ± 8	14 ± 4
			Ser					0.32 ± 0.04	92 ± 9	3.4 ± 0.5
			Asp					0.40 ± 0.09	221 ± 57	1.8 ± 0.6
			Lys					0.10 ± 0.02	250 ± 60	0.4 ± 0.1
				Leu				1.7 ± 0.2	8 ± 1	213 ± 30
				Nle				3.1 ± 0.3	22 ± 2	145 ± 13
				Ala				0.73 ± 0.08	6 ± 1	117 ± 22
				Ser				0.91 ± 0.11	17 ± 1	52 ± 5
				Asp				0.25 ± 0.03	36 ± 6	8.3 ± 1.5
				Lys				0.01 ± 0.002	24 ± 3	0.4 ± 0.1
						Ala		5.7 ± 0.4	13 ± 2	438 ± 70
						Val		2.6 ± 0.2	16 ± 1	163 ± 10
						Ser		1.7 ± 0.3	52 ± 13	33 ± 10
						Glu		6.3 ± 0.8	81 ± 11	78 ± 14
						Lys		1.6 ± 0.2	56 ± 9	29 ± 6
							Ile	0.9 ± 0.1	13 ± 1	69 ± 8
							Val	0.7 ± 0.1	17 ± 2	41 ± 6
							Ser	0.44 ± 0.03	67 ± 7	6 ± 1
							Asp	1.2 ± 0.1	101 ± 18	12 ± 2
							Arg	0.53 ± 0.07	171 ± 27	3 ± 1

[a]Nph = *p*-nitrophenylalanine.

The S_4 subsite showed a preference for the proline substitution (specificity constant, $k_{cat}/K_m = 87$ $mM^{-1}s^{-1}$). This was not surprising, because other aspartic proteinases also show optimal activity with a proline residue in P_4 (pepsin: Powers et al., 1977; Rao, 1994; cathepsin D: Scarborough et al., 1993). The unique structural features of proline may help to align the peptide in the active site cleft thus facilitating cleavage. After proline, the hydrophobic residues were preferred with the Phe-containing substrate (specificity constant 50 $mM^{-1}s^{-1}$) and the Ala-containing peptide (specificity constant 33 $mM^{-1}s^{-1}$) giving roughly equivalent results. The worst substrate in this series was the Lys-containing peptide. This substrate gave the lowest k_{cat} and the highest apparent K_m, resulting in a specificity constant of only 1.4 $mM^{-1}s^{-1}$.

In turning to the P_3 substituted peptides, the hydrophobic residues gave the highest specificity constants with a preference for larger residues (Phe ≅ Ile > Ala). The k_{cat} was reduced approximately fourfold by the substitution of Ala and was also low for the rest of the substrates tested (Table 1). In addition, the Phe- and Ile-containing peptides gave the lowest values for K_m. The charged amino acid residues gave especially high K_m values (Asp221 μM and Lys250 μM) with the positively charged Lys substitution resulting in the lowest specificity constant (0.4 $mM^{-1}s^{-1}$) found among this substrate series (Table 1). Overall, amino acid variation in the P_3 position exhibited 250-fold differences in k_{cat}/K_m suggesting that this subsite has a major role in determining the specificity of PfPMII.

The P_2-substituted peptides showed similar trends with the P_3 varied substrates; however, the specificity constants exhibited an even greater magnitude of variation (530-fold). Again, the large hydrophobic residues gave the highest k_{cat}/K_m values with a substitution of Ala resulting in a marked decrease in k_{cat}. The S_2 subsite tended to be more tolerant than the S_3 pocket with respect to the Asp- and Lys-containing peptides, resulting in K_m values of 36 and 24 μM, respectively. The specificity constant for the Lys peptide was still 0.4 $mM^{-1}s^{-1}$ due to an extremely low k_{cat} (0.01 s^{-1}).

The substrates having substitutions in the P'_2 and P'_3 positions were analyzed to explore potential interactions in the prime side of the active site of PfPMII. The specificity constants showed smaller variations compared to those obtained with the P_2- and P_3-substituted peptides (15-fold for P'_2 and 29-fold for P'_3). Interestingly, the P'_2 peptides exhibited larger k_{cat} values compared to the rest of the substrates in this series. The Ala and Glu substitutions gave especially large k_{cat} values of 5.7 and 6.3 s^{-1}, respectively. This is in contrast to the results above from the substrates with variation in P_2 and P_3, where a substitution of Ala gave a marked decrease in k_{cat}. In fact, the P'_2 Ala-containing substrate results in the highest specificity constant (438 $mM^{-1}s^{-1}$) within this entire series of substrates.

Table 2. *Kinetic parameters for the original parent substrate (**1**) vs. the redesigned substrates (**2** and **3**) with PfPMII and human liver cathepsin D*[a]

	Plasmepsin II			Human cathepsin D		
Substrates	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
1 K-P-I-E-F*X-R-L	2.0 ± 0.2	23 ± 3	87 ± 17	50 ± 4[b]	28 ± 2[b]	1,800 ± 200[b]
2 K-P-I-LF*X-A-L-K	14 ± 1	18 ± 1	792 ± 78	99 ± 21	77 ± 12	1,290 ± 335
3 K-P-I-L-F*X-E-L-K	12 ± 1	19 ± 2	619 ± 68	68 ± 14	62 ± 8	1,090 ± 267

[a]Single-letter code is used for the amino acids in this table; X = *p*-nitrophenylalanine; Z = norleucine.
[b]Beyer and Dunn (1998).

Newly designed substrates

The results from Table 1 provide insight into the binding specificity of PfPMII. This knowledge can be used to design a better substrate for this enzyme. If the best substitution in each position is considered, a new substrate would be Lys (P_5)-Pro (P_4)-Phe (P_3)-Leu (P_2)-Phe (P_1)*Nph (P'_1)-Ala (P'_2)-Leu (P'_3). This substrate, however, may present problems because of the opportunity to bind in different modes leading to the cleavage between the Phe-Leu peptide bond (P_3-P_2) in addition to cleavage between the Phe*Nph (P_1*P'_1). Therefore, the second best amino acid in the P_3 position was chosen. In addition, a Lys was added at P'_4 to increase solubility, as the replacement of Arg at P'_2 by Ala increased the hydrophobicity of the peptide. The peptide, Lys (P_5)-Pro (P_4)-Ile (P_3)-Leu (P_2)-Phe (P_1)*Nph (P'_1)-Ala (P'_2)-Leu (P'_3)-Lys**2**, was synthesized and tested under the same conditions as the previous chromogenic substrates. In addition, a similar new substrate was made having P'_2 Glu**3**.

The kinetic parameters for these two new substrates are shown in Table 2, along with the parent peptide from the previous substrate series **1**. By replacing the P_2 Glu with a Leu and the P'_2 Arg with an Ala**2** or Glu**3**, there is an increase in k_{cat} by seven- and sixfold, respectively. This results in a dramatic increase in the k_{cat}/K_m values. Peptide 2 provides a valuable, sensitive, and low cost substrate for routine assay of recombinant PfPMII. To evaluate the specificity of the newly designed substrates toward PfPMII, the closely related human aspartic proteinase cathepsin D was tested also. Peptides **2** and **3**, when compared to the results with peptide **1**, showed small increases in k_{cat}. Larger effects, however, were seen in the apparent K_m, which also increased. This led to a decrease in the specificity constants for cathepsin D when compared to that for substrate **1**.

Hemoglobin-based substrate analyses

Hemoglobin digestion experiments with the isolated naturally occurring enzyme indicated that the primary cleavage site was between Phe33 and Leu34 (Goldberg et al., 1991; Gluzman et al., 1994). To test the ability of the recombinant PfPMII to cleave this site, a substrate was designed based on the surrounding region of the hemoglobin alpha chain (Ala-Leu-Glu-Arg-Met-Phe-Leu-Ser-Phe-Pro). This was converted to a chromogenic substrate by replacing the second Leu with a *p*-nitrophenylalanine and the potentially oxidizable Met with a Nle. The resulting sequence was Ala-Leu-Glu-Arg-Nle-Phe*Nph-Ser-Phe-Pro (peptide **4**), with the scissile bond between the Phe and Nph. The resulting kinetic parameters for this substrate are given in Table 3. Amino acid analysis of the products confirmed cleavage occurred between the Phe and Nph residues. This substrate was also used in the evaluation of the mutant enzymes described below.

Site-directed mutagenesis

In addition to elucidating the substrate specificity of PfPMII, an understanding of the specificity differences between PfPMII and other aspartic proteinases is useful in designing specific inhibitors that will interact with PfPMII and not human aspartic proteinases. Furthermore, for structure-based drug design, a more detailed knowledge of enzymatic specificity requirements is valuable. Therefore, structural features that play a role in determining the unique characteristics of the substrate specificity of PfPMII needed to be identified.

To elucidate differences, the structural models of PfPMII (Silva et al., 1996) were compared to those of two mammalian aspartic proteinases, porcine pepsin (Abad-Zapatero et al., 1990), and human cathepsin D (Baldwin et al., 1993), and the fungal aspartic proteinase, rhizopuspepsin (Suguna et al., 1987). Each of these enzymes has been studied in detail, thus providing significant structural and functional information useful for extensive comparative analyses (Pohl & Dunn, 1988; Lowther et al., 1992, 1995; Scarborough et al., 1993; Rao, 1994; Scarborough & Dunn, 1994; Beyer & Dunn, 1996, 1998). As results in Table 1 show the great-

Table 3. *Kinetic parameters for cleavage of the hemoglobin-based substrate by several variants of PfPMII*[a]

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Wild-type	5 ± 0.4	27 ± 6	203 ± 45
Met13Glu	5.8 ± 0.4	14.6 ± 1.7	398 ± 53
Ile287Glu	2.1 ± 0.1	13.9 ± 0.5	149 ± 8
Met13Glu/Ile287Glu	7.4 ± 0.9	33.4 ± 4.8	220 ± 42
Ser77Asp	2.7 ± 0.2	19.2 ± 2	141 ± 21

[a]The substrate **4** has sequence: Ala-Leu-Glu-Arg-Nle-Phe*Nph-Ser-Phe-Pro, where * indicates the point of cleavage. Nle = norleucine; Nph = *p*-nitrophenylalanine.

est sensitivity to substitutions that interact with the S_2 and S_3 regions, we focused our analysis in those areas of the active site. Consequently, three residues within the active site were identified that varied significantly between PfPMII and the other enzymes. Enzymes with substitutions in those positions were analyzed in two ways. First, they were studied with substrates that have variation in the part of the substrate most likely to contact the altered enzyme residue. Second, the hemoglobin-based substrate **4** was utilized to determine if the mutation had a deleterious effect on the function of the protein.

Kinetic characterization of the Met13Glu mutant

The first of the PfPMII residues altered was Met13 (residue 15 in Silva et al., 1996; we are using the numbering of porcine pepsin for the residues of PfPMII discussed here) (Fig. 1). This residue is located in the S_3 subsite pocket and may be an important factor in determining the strong hydrophobic nature at this region of the active site. The corresponding residue is a Glu in rhizopuspepsin and porcine pepsin and a Gln in human cathepsin D (Table 4). Previous kinetic analyses showed that rhizopuspepsin (Lowther et al., 1995) and porcine pepsin (Rao, 1994) could cleave substrates with an Arg in P_3 efficiently, while cathepsin D could not (Scarborough et al., 1993). In addition, porcine pepsin showed a titratable response. This pH dependence was related to the presence of Glu13 in the S_3 subsite (Rao, 1994). Therefore, there is reason to believe that the Met in PfPMII may be significant in determining differences in the S_3 specificity of PfPMII and pepsin.

A PfPMII Met13Glu mutant was compared to the wild-type enzyme using chromogenic substrates having variations in the P_3 position (Table 5). These substrates were chosen to evaluate the effect of the substitutions on interaction with the S_3 region of the enzyme. In addition, the hemoglobin-based substrate was used to assay this mutant (Table 3). The parameters reported in Table 3 indicate that the activity of the mutant is comparable to that of the wild-type enzyme. In examining the resulting S_3 substrate specificity (Table 5), the overall trends remain the same for the Met13Glu mutant as for the wild-type. However, the specificity constants have changed. This was due to changes in the apparent binding constants (K_m) because the catalytic efficiencies (k_{cat}) of the mutant enzyme were virtually unchanged from the wild-type. These similar k_{cat} values were expected because the amino acid substitution is removed from the catalytic apparatus and should not have caused structural disruptions. The replacement of the hydrophobic Met in the S_3 pocket with a Glu resulted in a decrease in the k_{cat}/K_m for the hydrophobic substrates. The k_{cat}/K_m for the Ser-containing peptide remained the same (3.4 vs. 3.8 $mM^{-1}\,s^{-1}$). The Asp-containing substrate showed a twofold increase in k_{cat}/K_m (1.8 to 3.7 $mM^{-1}\,s^{-1}$) as the result of a large decrease in K_m. The largest difference was seen with the Lys-containing peptide. The mutant showed a 5.5-fold increase in the specificity constant (0.4 to 2.2 $mM^{-1}\,s^{-1}$) due to better binding of this substrate.

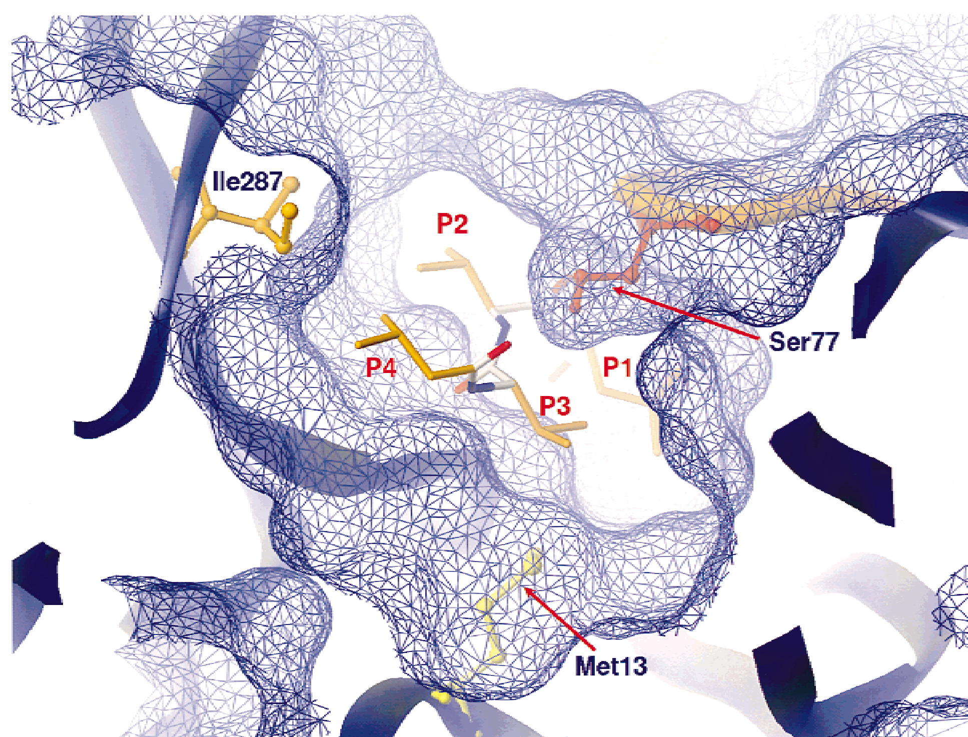

Fig. 1. Close-up view of the active site region of Plasmepsin II (PfPMII) with pepstatin bound, generated from the structure of Silva et al. (1996) (Protein Data Bank code: 1SME). The P_2-P_3-P_4 substituents on pepstatin are labeled to illustrate that each makes contact with a different part of the binding cleft. The blue Connelly solvent accessible surface net was calculated (Sybyl, version 6.4.2) with the inhibitor removed. The side chains at positions 13, 77, and 287 of PfPMII (pepsin numbering scheme) are displayed in ball-and-stick representation and labeled. The backbone of the protein is represented by a shaded ribbon. This picture is “clipped” in the Z-dimension, so that some of the ribbons are truncated. The P_3 side chain points “down” into the deep S_3 pocket in the right side on the figure, while the P_2 and P_4 side chains are more exposed to the aqueous exterior of the protein at the top of this view.

Table 4. *Amino acids of aspartic proteinases that are important for substrate binding in S_3 and S_2 (pepsin numbering scheme)*

Residue number	12	13	14	75	76	77	78		79	80	286	287	288
Porcine pepsin	M	E	Y	Y	G	T	G	•	S	M	F	E	G
Human cathepsin D	A	Q	Y	Y	G	S	G	•	S	M	F	M	G
Plasmepsin II	I	M	F	Y	V	S	G	•	T	V	I	I	G
Rhizopuspepsin	V	E	Y	Y	G	D	G	S	S	A	I	H	A

Kinetic characterization of the Ile287Glu mutant

Another residue that varied between the enzymes is at position 287 (residue 290; Silva et al., 1996) (Fig. 1). This amino acid is an Ile in PfPMII but a Met in cathepsin D, a Glu in porcine pepsin and a His in rhizopuspepsin (Table 4). Pepsin and rhizopuspepsin can both cleave substrates having a positive charge in the P_2 position. Cathepsin D and PfPMII (Table 1), on the other hand, cleave peptides having positive charges in the P_2 position inefficiently or not at all. Site-directed mutagenesis experiments on cathepsin D proved that if the Met287 was changed to a Glu then this enzyme could more efficiently cleave a substrate with a Lys in the P_2 position (Scarborough & Dunn, 1994). This suggested that the Ile287 in PfPMII may also be important in determining the preference for hydrophobic residues in the P_2 position. In addition, from the crystal structure, Ile287 appears to also interact with the P_4 side chain of bound pepstatin (Silva et al., 1996). Therefore, this residue may be important in determining both S_2 and S_4 substrate specificity.

Kinetic analyses were performed with the PfPMII Ile287Glu mutant enzyme and compared to those using the wild-type enzyme to assess the role of Ile287 in determining substrate specificity. Substrates were used that had variations in the amino acids in the P_2 and P_4 positions to probe for changes in both S_2 and S_4 enzymatic specificity (Table 6). In addition, the hemoglobin-based substrate **4** was evaluated (Table 3) with the parameters for the mutant similar to those obtained with the wild-type enzyme. In both series of substrates in Table 6, the trends in the preferred residues remained unchanged, Phe, Ala > Ser > Asp > Lys. Furthermore, in most instances, the k_{cat} values also remained unchanged.

In both series, the specificity constants for the hydrophobic-containing substrates decreased with the replacement of the hydrophobic Ile by a Glu. The P_2 Ser substrate showed a drop in k_{cat}/K_m from 52 to 24 $mM^{-1}\,s^{-1}$; however, the k_{cat}/K_m for the P_4 Ser substrate remained about the same. The P_2 Asp-substituted substrate showed similar specificity constants for the wild-type and the mutant enzyme. The P_4 Asp-containing peptide, on the other hand, resulted in a threefold decrease in the k_{cat}/K_m for the mutant (15 to 5.5 $mM^{-1}\,s^{-1}$). Interestingly, both the P_4 Lys- and P_2 Lys-containing substrates were cleaved almost three times more efficiently by the Ile287Glu mutant. The P_4 Lys substrate, however, showed the most dramatic change in kinetic parameters with a 2.3-fold decrease in K_m. The Ile287Glu mutant had an increase in K_m for the P_2 Lys substrate similar to the rest of the P_2-substituted peptide series. The improved specificity constant was due to a higher k_{cat}.

Kinetic characterization of the double mutant, Met13Glu/Ile287Glu

The enzyme with both Met13 and Ile287 changed to Glu was also constructed and analyzed with all three sets of peptide substrates. For the P_3-varied substrates in Table 5, the double mutant exhibits roughly the same values as seen for the single Met13Glu mutant. Thus, the interaction at P_3/S_3 is isolated and not influenced by the second substitution.

A different picture emerges when evaluating the substrates with changes in P_2 or P_4. For most substitutions (Table 6), the double mutant showed activity at nearly the wild-type levels. For the P_2Lys or P_4Lys substrates, the double mutant has values of k_{cat}/K_m that are higher than either single mutant alone. This suggests that binding in the C-terminal domain or catalysis may be influenced by the substitution at Met13 in the N-terminal domain.

Table 5. *Comparison of the kinetic properties of wild-type plasmepsin II, the Met13Glu mutant, and the double mutant Met13Glu/Ile287Glu using substrates with variations in the P_3 position*[a]

	Wild-type			Met13Glu			Met13Glu/Ile287Glu		
P3 substitution	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Phe	2.4 ± 0.3	24 ± 3	100 ± 16	3.1 ± 0.4	70 ± 13	43 ± 9	2.9 ± 0.6	64 ± 13	45 ± 14
Ile	2.0 ± 0.2	23 ± 3	87 ± 17	2.0 ± 0.2	55 ± 8	37 ± 6	3.5 ± 0.5	70 ± 14	50 ± 12
Ala	0.50 ± 0.1	37 ± 8	14 ± 4	0.53 ± 0.07	46 ± 8	12 ± 2	0.5 ± 0.06	53 ± 10	9 ± 2
Ser	0.32 ± 0.04	92 ± 9	3.4 ± 0.5	0.27 ± 0.03	71 ± 11	3.8 ± 0.7	0.20 ± 0.02	56 ± 9	4 ± 0.7
Asp	0.40 ± 0.09	221 ± 57	1.8 ± 0.6	0.32 ± 0.03	88 ± 11	3.7 ± 0.6	0.30 ± 0.04	87 ± 15	3 ± 0.6
Lys	0.10 ± 0.02	250 ± 60	0.4 ± 0.1	0.08 ± 0.01	35 ± 4	2.2 ± 0.3	0.05 ± 0.01	37 ± 3	1.4 ± 0.3

[a]Substrate = Lys-Pro-**P₃**-Glu-Phe*Nph-Arg-Leu, where Nph = *p*-nitrophenylalanine.

Table 6. *Comparison of the kinetic properties of wild-type plasmepsin II, the Ile287Glu mutant, and the double mutant Met13Glu/Ile287Glu using substrates with variations in the P2 and P4 positions*[a]

	Wild-type			Ile287Glu			Met13Glu/Ile287Glu		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
P_2									
Glu	2.0 ± 0.2	23 ± 3	87 ± 17	1.7 ± 0.1	70 ± 8	24 ± 3	3.5 ± 0.5	70 ± 14	50 ± 12
Ala	0.73 ± 0.08	6 ± 1	117 ± 22	0.89 ± 0.05	37 ± 3	24 ± 3	1.8 ± 0.4	41 ± 3	45 ± 9
Ser	0.91 ± 0.11	17 ± 1	52 ± 5	0.73 ± 0.01	31 ± 5	24 ± 4	1.1 ± 0.12	39 ± 6	28 ± 5
Asp	0.25 ± 0.03	36 ± 6	8.3 ± 1.5	0.86 ± 0.07	76 ± 10	12 ± 2	0.44 ± 0.05	70 ± 12	6.3 ± 1.3
Lys	0.01 ± 0.002	24 ± 3	0.4 ± 0.1	0.06 ± 0.01	50 ± 10	1.1 ± 0.3	0.03 ± 0.006	4 ± 0.3	7.3 ± 1.6
P_4									
Phe	1.1 ± 0.2	21 ± 4	50 ± 10	1.0 ± 0.2	45 ± 9	21 ± 5	2.6 ± 0.6	85 ± 17	30 ± 9
Ala	2.0 ± 0.3	58 ± 11	33 ± 8	0.8 ± 0.1	69 ± 8	12 ± 2	0.9 ± 0.1	30 ± 5	30 ± 6
Ser	1.3 ± 0.2	110 ± 18	12 ± 2	1.0 ± 0.2	100 ± 18	10 ± 2	1.1 ± 0.1	49 ± 6	23 ± 4
Asp	1.1 ± 0.2	73 ± 12	15 ± 4	0.6 ± 0.1	109 ± 19	6 ± 1	0.9 ± 0.1	55 ± 10	16 ± 4
Lys	0.17 ± 0.03	117 ± 18	1.5 ± 0.3	0.19 ± 0.01	51 ± 6	3.8 ± 0.5	0.4 ± 0.09	75 ± 14	5.3 ± 1.5

[a]The substrate is Lys-**P_4**-Ile-**P_2**-Phe*Nph-Arg-Leu, where Nph = *p*-nitrophenylalanine. For the P_2 variants, P_4 was fixed as Pro; for the P_4 variants, P_2 was fixed as Glu.

Kinetic characterization of the Ser77Asp mutant

One position that maintains a similar residue in all the mammalian aspartic proteinases but varies in rhizopuspepsin, as well as other fungal aspartic proteinases, is residue 77 (residue 79; Silva et al., 1996). Most aspartic proteinases have either a Ser or Thr at this position, while there is an Asp in rhizopuspepsin. This residue lies on the flap region of the enzymes, and is thought to be involved in determining P_1 specificity (Lowther et al., 1995) (Fig. 1). Reports have shown that if the Asp77 in rhizopuspepsin is mutated to a Thr, the S_1 specificity is modified by eliminating the ability of the enzyme to cleave a peptide with a positive charge in the P_1 position (Lowther et al., 1995).

A recent report by Shintani et al. (1997) described an approach to convert the S_1 specificity of porcine pepsin to that of rhizopuspepsin by mutating the Thr77 to an Asp. This mutant cleaved the substrate Ac-Ala-Ala-Lys*Nph-Ala-Ala-NH_2 between the Nph-Ala bond rather than between the Lys*Nph. The authors argued that the pepsin Thr77Asp mutant could still not tolerate a substrate with a Lys in the P_1 position. The mutant cleaved another substrate tested, however, between a Lys*Nph bond (in Pro-Thr-Glu-Lys*Nph-Arg-Leu). They concluded that this Thr77Asp mutant hydrolyzed the Lys*Nph bond, not because it was able to recognize the P_1 Lys, but instead, because of electrostatic repulsion between the Asp77 and the P_2 Glu in the substrate. It was concluded that for pepsin to acquire P_1 Lys specificity, it required an insertion of a Ser, where most mammalian enzymes have a deletion, in addition to the Asp substitution (Table 4). This pepsin double mutant was successful at cleaving both substrates with a P_1 Lys residue. As seen from Table 4, PfPMII contains a Ser at position 77 where rhizopuspepsin has an Asp. In addition, PfPMII is similar to pepsin and other mammalian aspartic proteinases in the deletion of a Ser between residues 78 and 79. The Ser77 was, therefore, mutated to an Asp to see if it alone could change the P_1 specificity to that of rhizopuspepsin.

Cleavage of the hemoglobin-based substrate was slightly reduced relative to the wild-type enzyme (Table 3).

To analyze the ability of the Ser77Asp mutant to cleave after a positively charged residue, a new substrate was designed that contained a series of hydrophobic residues and a Lys at the P_1 position. For solubility purposes, an "extra" Lys residue was attached to the C-terminal end of the peptide. This new P_1 Lys peptide was analyzed with the mutant and wild-type enzymes. In addition, a substrate having a P_2 Lys residue was tested, as well as the parent substrate (Table 7).

Table 7. *Comparison of the kinetic properties of wild-type plasmepsin II and the Ser77Asp mutant*

	Wild type			Ser77Asp		
P5-P4-P3-P2-P1*IP1′-P2′-P3′-P4′	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Lys-Glu-Phe-Val-Lys*Nph[a]-Ala-Leu-Lys	—	Not cleaved	—	0.84 ± 0.07	46 ± 6	18 ± 3
Lys-Pro-Ile-Glu-Phe*Nph-Arg-Leu	2.0 ± 0.2	23 ± 3	87 ± 17	2.0 ± 0.2	52 ± 7	38 ± 6
Lys-Pro=Ile-Lys-Phe*Nph-Arg-Leu	0.01 ± 0.002	24 ± 3	0.4 ± 0.1	0.08 ± 0.01	40 ± 7	2.0 ± 0.4

[a]Nph = *p*-nitrophenylalanine.

Interestingly, by changing only one amino acid on the flap of PfPMII, the enzyme was now able to cleave a substrate with a positive charge in P_1 (18 $mM^{-1} s^{-1}$). The wild-type enzyme could not cleave this P_1 Lys peptide even after an overnight incubation at 37 °C with 5 μg of enzyme. Furthermore, the Ser77Asp mutant cleaved a substrate with a Lys in P_2 more efficiently (0.4 to 2.0 $mM^{-1} s^{-1}$). The parent peptide, which contains a P_2 Glu and P_1 Phe, gave a moderate k_{cat}/K_m for the mutant enzyme (38 $mM^{-1} s^{-1}$). This specificity constant was lower than for the wild-type enzyme (87 $mM^{-1} s^{-1}$), which was expected because of electrostatic repulsion between the Asp77 and the P_2 Glu in the substrate.

Discussion

The redesigned substrates

By analyzing chromogenic octapeptides with variations in different P-positions, preferential amino acids were discovered for each of those sites. This led to the synthesis of two new peptides that proved to be better substrates for PfPMII. The approximate eightfold increase in the specificity constants for these substrates with PfPMII was mainly due to an improvement in k_{cat}. This is probably due to the replacement of the Arg in P_2' as presented in the analysis of the substrates in Table 1.

Site-specific mutants

The objective of replacing amino acids within the binding cleft of PfPMII was to evaluate the importance of interactions between enzyme and substrate at specific sites. Mutations that compromise folding or catalytic activity are of limited value in such studies. Reassuringly, the cleavage of the hemoglobin-based substrate (Table 3) by the mutants was observed to be on a par with that obtained for the wild-type recombinant enzyme. This indicates that mutations in the binding site have not altered the natural function of the enzyme.

Met13Glu mutant

It was found (Table 1) that the wild-type PfPMII prefers large hydrophobic residues within the S_3 pocket, while Lys substitution in P_3 is discriminated against. In contrast, pepsin and rhizopuspepsin can tolerate substrates in this series with Lys in P_3. Structural analyses show each of these latter enzymes contains a Glu within the S_3 subsite, which may aid in the binding of the Lys side chain through electrostatic pairing. Therefore, Met13 in the S_3 subsite of PfPMII was replaced with a Glu, the homologous residue found in pepsin, rhizopuspepsin, and also cathepsin E.

By replacing the hydrophobic Met with a negatively charged Glu, the specificity constants for the peptides containing large hydrophobic residues (Phe and Ile) decreased, most likely due to the loss of stabilizing (i.e., van der Waals) interactions between P_3 and Met13. The hydrophobic Ala side chain is probably short enough to avoid a destabilizing interaction with the hydrophilic carboxyl group of the Glu. Thus, cleavage of this small hydrophobic substrate remains unchanged in k_{cat}/K_m. The fact that the Ser-containing substrate also does not change supports this observation.

With the incorporation of a Glu in the S_3 pocket, substrates containing negatively charged P_3 residues are expected to bind less favorably. Paradoxically, the Asp-containing peptide shows a 2.5-fold decrease in K_m, thereby increasing the k_{cat}/K_m for the mutant enzyme. This increase can be explained if, at the pH of the assay (4.4), the Glu residue of the enzyme and the Asp residue of the substrate are hydrogen bonding partners.

The most significant changes in the kinetic parameters for the Met13Glu mutant are seen with the Lys-containing peptide. The Michaelis constant decreased from the highest value in this series for the wild-type PfPMII (250 μM, Table 1) to the lowest value for the mutant enzyme (35 μM, Table 5). These changes resulted in a 5.5-fold increase in the specificity constant.

These results show that the Met13 residue contributes to the S_3 subsite specificity of PfPMII. In addition, the Met13Glu mutant data support the theory that the presence of a Glu at this position in aspartic proteinases enhances the binding of substrates/inhibitors with Lys in P_3. Furthermore, because wild-type PfPMII varies at position 13 from the human enzymes, cathepsin E and pepsin, specific inhibitors may be rationally designed to take advantage of this specificity distinction.

We must point out, however, that the hemoglobin-based substrate (Table 3) contains an Arg in the P_3 position. The mutants containing Glu13 process this peptide with nearly equal efficiency as the wild-type enzyme. Thus, the effects observed with our Lys-containing peptides may be specific for that amino acid.

Ile287Glu mutant

The Ile287 lies within the interface of the S_2 and S_4 subsites of PfPMII. Therefore, it is possible that it plays a role in determining both S_2 and S_4 subsite preferences. PfPMII prefers hydrophobic over charged residues in both the P_2 and P_4 positions (Table 1). In contrast, porcine pepsin can tolerate positively charged substituents in P_2 (Rao, 1994). Glu is the residue found in porcine pepsin at the structurally equivalent position 287. To test whether the Ile in PfPMII contributes significantly to these subsite distinctions, it was mutated to a Glu and changes in both S_2 and S_4 specificity were analyzed.

In both the P_2 and P_4 substrate series, there is a decrease in k_{cat}/K_m for the hydrophobic containing peptides due to an increase in the K_m values (Table 6). This suggests that the Ile287 may contribute to the hydrophobicity of both the S_2 and S_4 binding pockets. On the other hand, changes in the specificity constants with the negatively charged Asp substitutions were only observed for the P_4 varied peptide. Here, there was a 2.5-fold decrease in the k_{cat}/K_m. This may be due to electrostatic repulsion between the Asp and the Glu residue in the Ile287Glu mutant. The P_2 and P_4 Lys peptides both showed almost threefold increases in k_{cat}/K_m. The increase in the specificity constant for the P_4 Lys substrate was due to improved binding of the Lys in the P_4 subsite. This is likely a result of an electrostatic interaction with the Glu at 287. The P_2 Lys substrate, on the other hand, showed an increase in the K_m but an improved k_{cat}. Therefore, amino acid 287 does not affect the binding of the substrate within the S_2 subsite, but rather the enzyme turnover.

In conclusion, a mutation was made from an Ile in PfPMII to the homologous residue found in porcine pepsin, Glu. The resulting changes in specificity were seen in the S_2 and S_4 subsites. This position appears to determine binding of residues in S_4, whereas the k_{cat} is affected by interactions in S_2. The mutant enzyme was thus engineered to have "pepsin-like" specificity by showing an increase in the specificity constant for a Lys-containing peptide.

Therefore, the Ile287 is a structural determinant of the S_2 and S_4 specificity of PfPMII.

Double mutant, Met13Glu/Ile287Glu

Positions 13 and 287 are on opposite sides of the active site cleft. Thus, they interact with different parts of a substrate (or inhibitor) bound in an extended β-strand conformation. Analysis of the double mutant Met13Glu/Ile287Glu in comparison to the Met13Glu single mutant with P3-varied substrates showed that the P_3/S_3 interaction is isolated, as no further change in kinetic parameters was seen. This reflects the inaccessibility of the P_3/S_3 region position in a complex (Fig. 1). In contrast, analysis of the double mutant with P_2- or P_4-varied substrates showed that an additive effect over the Ile287/Glu single mutant in binding of Lys-containing substrates. This observation may reflect the alteration in the electrostatic environment achieved by substituting two hydrophobic residues by hydrophilic Glu residues.

Ser77Asp mutant

Fungal aspartic proteinases are unique compared to the mammalian aspartic proteinases in that they can cleave substrates with a P_1 Lys residue (Balbaa et al., 1993). Crystallographic data and site-directed mutagenesis experiments on penicillopepsin, aspergillopepsin I, and rhizopuspepsin have shown the importance of Asp77 for this activity (James et al., 1985; Shintani & Ichishima, 1994; Lowther et al., 1995). Shintani et al. (1997) suggested that for pepsin to cleave a substrate with a P_1 Lys residue it requires the substitution of Thr77 to Asp as well as an insertion of a Ser between residues 78 and 79. The results shown here with PfPMII suggest that the replacement of Ser77 to Asp is sufficient for this aspartic proteinase to recognize a substrate having a P_1 Lys (Table 7). Furthermore, the PfPMII Ser77Asp mutant efficiently cleaved the peptide having a Glu in P_2. Rhizopuspepsin is also capable of hydrolyzing this peptide (3,840 $mM^{-1} s^{-1}$; Lowther et al., 1995), suggesting that an Asp at 77 does not cause electrostatic clashes with a Glu in P_2. This contradicts previous reports that claim that the porcine pepsin Thr77Asp mutant cleaves Pro-Thr-Glu-Lys*Nph-Arg-Leu after the Lys only because of electrostatic repulsion between the P_2 Glu and Asp77. X-ray crystallographic structures of these mutants are needed to help resolve these contradicting data about the involvement of the flap residues in determining S_1 specificity.

Materials and methods

Site-directed mutagenesis

Mutations in the wild-type PfPMII gene were generated using a four primer, overlap extension strategy similar to Ho et al. (1989). This procedure has been previously described in detail (Scarborough & Dunn, 1994). Polymerase chain reaction (PCR) was performed using a Gene Machine II Programmable Thermal Controller (USA Scientific Plastics, Ocala, Florida). Vent polymerase was purchased from New England Biolabs (Beverly, Massachusetts). The products were purified on a 1% NuSieve GTG low melting agarose gel. The correct size bands were excised and further purified using the Bio-Rad DNA purification kit. These products and the wild-type plasmepsin II gene were then restriction digested and ligated together. Last, the entire coding regions were dideoxy sequenced.

Expression and refolding

One liter of Luria Broth media containing 100 μg/mL ampicillin was inoculated with 20 mL of an overnight culture of *E. coli* BL21 (DE3) pLysS cells containing the recombinant pET3a plasmid. These cells were grown at 37 °C with 300 rpm shaking to an OD_{600} of 0.6. Protein expression was then induced by addition of IPTG to a final concentration of 0.5 mM. The OD of the cells was monitored at 0 and 3 h after induction, and 1 mL of culture was removed for analyzing expression amounts by SDS-PAGE. After 3 h, the cells were harvested by centrifugation (5,000 $\times$ *g*, 15 min, 4 °C). The cell pellet was re-suspended in cold Tris buffer (4.2 mL/g of cells, 0.01 M Tris, pH 8.0, 0.02 M $MgCl_2$, 0.005 M CaCl, and 2 Kunitz U/mL of DNase I) and passed through a French Press cell two times. The lysed cell suspension was layered over 10 mL of 27% sucrose and centrifuged at 12,000 $\times$ *g* for 30 min to isolate the inclusion body pellet (Taylor et al., 1986). The inclusion bodies were washed by resuspension in 20 mL of 0.01 M Tris, pH 8.0, 0.001 M EDTA, 0.002 M β-mercaptoethanol, and 0.1 M NaCl, and pelleted through a sucrose cushion again. Next, the pellet was resuspended in 20 mL of 0.05 M Tris, pH 8.0, 0.005 M EDTA, 0.005 M β-mercaptoethanol, and 0.5% Triton X-100 and recentrifuged (12,000 $\times$ *g*, 15 min, 4 °C). Finally, the inclusion bodies were washed one last time in another 20 mL (0.05 M Tris, pH 8.0, 0.005 M EDTA, 0.005 M β-mercaptoethanol) and pelleted as before. The inclusion bodies were weighed and stored at −20 °C.

The inclusion bodies were solubilized and refolded. Solubilization was done at a concentration of 1 mg (wet)/mL in freshly deionized 8 M urea, 0.05 M CAPS, pH 10.5, 0.005 M EDTA, and 0.2 M 2-mercaptoethanol. The solution was stirred slowly at 25 °C for 45 min. Any undissolved material was removed by centrifugation at 24,000 $\times$ *g* for 30 min. The supernatant was dialyzed for 4 h against five times the original volume in Spectrapor dialysis tubing (MWCO 12–14 kDa) and 20 mM Tris pH 8.0 buffer. The buffer was replaced with 20 mM Tris pH 8.0 buffer at 4 °C and allowed to dialyze for 6 h at 4 °C. Under these conditions, the dialysis buffer was changed two more times. The resulting solution was centrifuged to remove any precipitate (24,000 $\times$ *g*, 4 °C, 30 min).

Purification

Anion-exchange chromatography was used to purify the correctly folded protein to homogeneity. A Pharmacia Resource Q column (5 mL) was equilibrated in 20 mM Tris pH 8.0 buffer at 4 °C using a Pharmacia FPLC. Next, 40 mg of the refolded material was loaded on the column. The enzyme was eluted by running a step gradient profile using 20 mM Tris, 1 M NaCl pH 8.0 buffer. Three peaks were eluted at 0.10, 0.18, and 1 M NaCl concentrations. These peak fractions were pooled and stored in 0.5 mL aliquots at 4 °C.

Spectrophotometric analyses

Substrate hydrolysis

Stock peptide solutions were made in 10% formic acid and 90% distilled water and quantitated by amino acid analysis using a Beckman System 6300 High Performance Amino Acid Analyzer. Plasmepsin catalyzed hydrolysis of the octapeptide substrate, where cleavage occurs between the Phe*Nph (Nph = para-nitrophenylalanine, * = point of cleavage), was performed at 37 °C, pH 4.4 in 0.1 M sodium formate buffer using a 5 min

preincubation time. Assays using cathepsin D were in 0.1 M sodium formate buffer pH 3.7 with a 3 min preincubation time. The preincubation times allow complete conversion of the zymogen enzymes to active mature forms as verified by N-terminal sequencing and SDS-PAGE analyses. The substrate cleavage reaction was monitored by the decrease in the average absorbance from 284–324 nm on a Hewlett Packard 8452A diode array spectrophotometer (Scarborough et al., 1993; Dunn et al., 1994). The K_m and k_{cat} values were calculated from the initial rates of at least six substrate concentrations using Marquardt analysis (Marquardt, 1963) and the equation $v = V_{max}[S]/(K_m + [S])$. The observed rates in AU $*$ s^{-1} were converted to M $*$ s^{-1} by dividing by the total change in absorbance for complete hydrolysis of a known concentration of each substrate used. To ensure cleavage occurred between the Phe and Nph, cleavage products were separated by high-performance liquid chromatography and then identified by amino acid analysis.

Active site titration and k_{cat}

Inhibitor stock solutions were made in DMSO and quantified by amino acid analysis. Inhibitor working dilutions were then diluted into distilled water. The amount of active enzyme in each assay was determined by competitive inhibition with Pepstatin A (Sigma, St. Louis, Missouri). The resulting curve was fitted with the Henderson equation on the Enzfitter program (Henderson, 1972; Leatherbarrow, 1985).

The k_{cat} values were then calculated by the equation $k_{cat} = V_{max}$ (AU/s)/$(\Delta A/[S] * [E])$, where ΔA = total change in absorbance observed with a specific substrate concentration $[S]$, and $[E]$ = total enzyme concentration (molar). The standard deviations of the k_{cat} and k_{cat}/K_m were propagated using equations derived by standard procedures for independent errors as outlined by Meyer (1975).

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