Regulation of Proteolytic Activity by Engineered Tridentate Metal Binding Loops

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The amino acids aspartic acid-102, histidine-57, and serine-195 in serine proteases constitute the catalytic triad. Disruption of this catalytic-site triad inactivates these enzymes. We have engineered loops containing histidine residues into trypsin, an archetypal serine protease, so that successful chelation of a metal ion at submicromolar concentrations reversibly inhibits proteolytic activity by involving the catalytic histidine in the metal complex. These engineered metal binding sites permit facile, reversible regulation of the activity of a target protease. Analysis of the efficiency of metal-dependent regulation on enzyme activity allows rapid evaluation of the properties of the designed metal−protein sites. In addition, the variant enzymes provide insight regarding the role these sites may play in a class of naturally-occurring metal-regulated serine proteases, the kallikreins.[1]

While trypsin activity is not sensitive to low concentrations of metal ions,[2] the existence of metal-binding signature sequences in the related family of kallikreins suggests that loops containing these sites near the active site are responsible for their metal-dependent activity. In our study, rat anionic trypsin was used as a scaffold to test the metal-binding properties of the histidine-containing kallikrein loops. Metal preference at these sites was explored by variation of the loop size. Amino acid substitutions were designed through computer modeling using the program Insight II (Biosym Technologies, San Diego, CA) in combination with the X-ray crystal structures of rat trypsin[3] and the tonin−zinc(II) complex.[4] Tonin, a kallikrein homologous to trypsin, had previously been crystallized complexed to zinc(II) in an inactive conformation. The structure suggests that the inactive conformation results from rotation of His57 to form the metal chelate, as shown in Figure 1. Sequences of the trypsin variants examined are indicated in Table 1, and were expressed in the methylotrophic yeast *Pichia pastoris*.[5] (see supporting information for details.) K_i determinations of these metal-dependent enzymes in the presence of various metal ions were made, and these results are shown in Tables 2 and 3. Details of the kinetic experiments are given in the supporting information.

As suggested by the modeling experiments, the addition of histidine ligands increases the metal affinity for the designed site, as indicated by inactivation of the protease without significantly affecting k_{cat}/K_m. Each histidine ligand increases copper(II) affinities for this site approximately 100-fold. That this inhibition is not due to more drastic changes in the protein

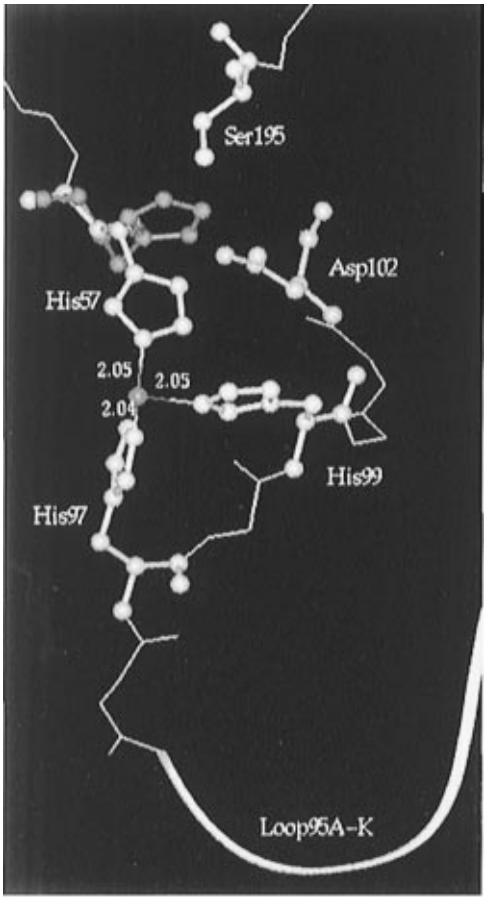

Figure 1. Modeled structure of the trypsin variants TnTo1, TnTo2, and TnTo2Q displayed by Insight II (Biosym Technologies, San Diego, CA). The catalytic residues can be superimposed in the trypsin and tonin X-ray crystal structures, with the exception of His57. In the active conformation (red), this residue is in hydrogen-bonding distance with Asp102 and Ser195, while in the inactive conformation, His57 (yellow) participates in the metal (blue) complex with His97 and His99. The fourth metal site may be either a water or a buffer molecule. The surface loops added to form the TnTo2 and TnTo2Q variants are represented by the solid tube (loop 95A-K).

was shown through the reversibility of this process: EDTA added to the variant−copper(II) complex fully restores activity (data not shown). This enhancement in metal affinity provides a significant improvement over previous work[6] in the ability to regulate proteases with metals. The metal site with three ligands enables tighter regulation of the activity of trypsin than a mutant having two ligands and demonstrates that we can create a high-affinity site in a protease.

We also explored the metal preferences of these variants. These results are presented in Table 3. While all the variants examined were inhibited to the greatest extent by copper(II), the preference for other metal ions differed markedly. It appears that metal-binding to the TnTo1 site is more sensitive to metal−ligand geometry: Metal−ligand angles of 90° (Ni^{2+} and Co^{2+}) are disfavored by this variant, while wider angles (Cu^{2+} and Zn^{2+}) are more easily obtained.

Since the metal site is so sensitive to geometry, we explored the effect of flexibility at this site by adding a loop to yield TnTo2 and TnTo2Q. The TnTo2 loop is identical to the tonin surface loop containing the chelating histidine residues, while

(1) (a) Fiedler, F.; Werle, E. *Eur. J. Biochem.* **1968**, *7*, 27. (b) Watt, K. W. K.; Lee, P. J.; M'Timkulu, T.; Chan, W. P.; Loor, R. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 3166. (c) Bhoola, K. D.; Figueroa, C. D.; Worthy, K. *Pharmacol. Rev.* **1992**, *44*, 1 and references therein.

(2) The relatively weak inhibition of wild-type trypsin by copper(II) may be due to chelation of the metal by the active site residues His-57 and Asp-102. A similar phenomenon is seen with Ag(I), which was demonstrated to bind to these residues by X-ray crystallography. Chambers, J. L.; Cristoph, G. G.; Krieger, M.; Kay, L.; Stroud, R. M. *Biochem. Biophys. Res. Commun.* **1974**, *59*, 70.

(3) Sprang, S.; Standing, T.; Fletterick, R. J.; Stroud, R. M.; Finer-Moore, J.; Xuong, N-H.; Hamlin, R.; Rutter, W. J.; Craik, C. S. *Science* **1987**, *237*, 905.

(4) Fujinaga, M.; James, M. N. G. *J. Mol. Biol.* **1987**, *195*, 373.

(5) Cregg, J. M.; Vedvick, T. S.; Raschke, W. C. *Biotechnology* **1993**, *11*, 905.

(6) Higaki, J. N.; Haymore, B. L.; Chen, S.; Fletterick, R. J.; Craik, C. S. *Biochemistry* **1990**, *29*, 8582.

(7) TnTo2 is glycosylated by this expression system. Cleavage of the sugar moiety by *N*-glycosidase F gave the expected molecular weight (supporting information).

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Table 1. Trypsin Variant Sequences[a]

	92	93	94	95	a	b	c	d	e	f	g	h	i	j	k	96	97	98	99	100	101	102
trypsin	P	N	F	D												R	K	T	L	N	N	D
TnR96H	P	N	F	D												**H**	K	T	L	N	N	D
TnL99H	P	N	F	D												R	K	T	**H**	N	N	D
Tonin	P	D	Y	I	P	L	I	V	T	N	D	T	E	Q	P	V	**H**	D	**H**	S	N	D
TnTo1	P	N	F	I												V	**H**	D	**H**	S	N	D
TnTo2[b]	P	N	F	I	P	L	I	V	T	N	D	T	E	Q	P	V	**H**	D	**H**	S	N	D
TnTo2Q	P	N	F	I	P	L	I	V	T	Q	D	T	E	Q	P	V	**H**	D	**H**	S	N	D

[a]Only the sequence in the region of the metal ligands is shown. All mutants are based on rat trypsin and have identical sequences in the remainder of the protein as verified by DNA sequencing. [b]TnTo2 contains the glycosylation sequence N-D-T and was shown to be glycosylated (supporting information). TnTo2Q eliminates this protein modification.

Table 2. Inhibition of Trypsin Mutants by Cu(II)[a]

enzyme	k_{cat} (min^{-1})	K_m (μm)	k_{cat}/K_m	K_i (μm)
trypsin[b]	3345	14	239	1600
TnR96H[b]	2464	7.5	328	21
TnL99H	507	37	14	70
TnTo1	1210	13	93	0.1
TnTo2	1450	16	91	0.2
TnTo2Q	1170	13	90	0.1

[a] Initial rates were determined at 25 °C from the increase in fluorescence from cleavage of Z-GPR-AMC with 2.5 nM enzyme as determined by active site titrations. Buffer was 10 mM $CaCl_2$, 100 mM NaCl, 1 mM Tris, 5 mM PIPPS, pH 8, with varying amounts of $CuSO_4$. All K_i values are apparent and were determined by Dixon plots. [b] Higaki, J. N.; Haymore, B. L.; Chen, S.; Fletterick, R. J.; Craik, C. S. *Biochemistry* **1990**, *29*, 8582.

TnTo2Q lacks a glycosylation site.[7] For TnTo2Q, the strongest correlation for preferred metal binding to the site appears to be the affinity of the histidine side chain for these metals rather than a preferred geometry. Affinities of imidazole for these transition metals are Cu^{2+}, $10^{4.3}$ M^{-1}; Ni^{2+}, $10^{3.1}$ M^{-1}; Co^{2+}, $10^{2.5}$ M^{-1}; and Zn^{2+}, $10^{2.1}$ M^{-1}.[8] The greater flexibility of the engineered loop makes inherent geometry at the site less important than the ligand affinity for the metal.

These results establish high-affinity metalloregulation of proteolytic activity and lay the foundation for subsequent designs that incorporate higher order ligation at prescribed positions in a protein. We expect that further refinement of the site, in both its geometry and rigidity, will enhance the metal binding ability of the mutants. Inclusion of additional histidine ligands to complete the metal sites is also expected to increase the metal specificity of these variants. This, in turn, will provide us with a powerful method for altering the function of an enzyme in a predictable fashion.

(8)) Chemical Society (Great Britain). *Stability Constants of Metal−Ion Complexes, Supplement 1*; The Chemical Society: London, 1971; p 281.

Table 3. Inhibition by Different Metal Ions[a]

enzyme	metal	k_i (μm)
Tn96H[b]	Cu(II)	21
	Ni(II)	49
	Zn(II)	128
TnTo1	Cu(II)	0.1
	Ni(II)	150
	Co(II)	>500
	Zn(II)	50
TnTo2Q	Cu(II)	0.1
	Ni(II)	4
	Co(II)	120
	Zn(II)	>200

[a] Initial rates were determined at 25 °C from the increase in fluorescence from cleavage of Z-GPR-AMC with 2.5 nM enzyme, as determined by active site titrations. Buffer was 10 mM $CaCl_2$, 100 mM NaCl, 1 mM Tris, 5 mM PIPPS, pH 8, with varying amounts of metal. All K_i values are apparent and were determined by Dixon plots. [b] Higaki, J. N.; Haymore, B. L.; Chen, S.; Fletterick, R. J.; Craik, C. S. *Biochemistry* **1990**, *29*, 8582.

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Supporting Information Available: Listing of the creation, expression, and characterization of trypsin variants in addition to kinetic details (3 pages). This material is contained in many libraries on microfiche, immediately follows this article on the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead for ordering information and Internet access instructions.

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