

Regulation of Serine Protease Activity by an Engineered Metal Switch<sup>†</sup>Jeffrey N. Higaki,<sup>†</sup> Barry L. Haymore,<sup>§</sup> Shell Chen,<sup>||</sup> Robert J. Fletterick,<sup>||</sup> and Charles S. Craik<sup>\*,†,||</sup>

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**ABSTRACT:** A recombinant trypsin was designed whose catalytic activity can be regulated by varying the concentration of Cu<sup>2+</sup> in solution. Substitution of Arg-96 with a His in rat trypsin (trypsin R96H) places a new imidazole group on the surface of the enzyme near the essential active-site His-57. The unique spatial orientation of these His side chains results in the formation of a stable, metal-binding site that chelates divalent first-row transition-metal ions. Occupancy of this site by a metal ion prevents the imidazole group of His-57 from participating as a general base in catalysis. As a consequence, the primary effect of the transition metal ion is to inhibit the esterase and amidase activities of trypsin R96H. The apparent *K<sub>i</sub>* for this inhibition is in the micromolar range for copper, nickel, and zinc, the tightest binding being to Cu<sup>2+</sup> at 21 μM. Trypsin R96H activity can be fully restored by removing the bound Cu<sup>2+</sup> ion with EDTA. Multiple cycles of inhibition by Cu<sup>2+</sup> ions and reactivation by EDTA demonstrate that reversible regulatory control has been introduced into the enzyme. These results describe a novel mode of inhibition of serine protease activity that may also prove applicable to other proteins.

An essential requirement for any system involving a proteolytic enzyme is the regulation of hydrolytic activity. Naturally occurring mechanisms for regulating proteolysis include transcriptional and translational control, zymogen activation, binding of protease inhibitors, and cellular localization (Holzer, 1980). Laboratory methods for manipulating the activity of proteases have used synthetic inhibitors that involve affinity labeling (Schoellman & Shaw, 1963; Shaw et al., 1965) and mechanism-based inactivation (Gelb et al., 1985; Harper et al., 1985). Recent efforts to regulate a hydrolase by engineering a regulatory switch have resulted in the design of recombinant enzymes whose activities can be controlled in a metal-independent fashion by manipulating the redox potential (Matsumura & Matthews, 1989) and in a metal-dependent fashion by reversibly occluding the substrate-binding pocket (Corey & Schultz, 1989). We report here a novel approach to regulating the activity of a protease in a metal-dependent fashion by introducing a neighboring amino acid residue that assists in reversibly repositioning an essential active-site amino acid residue in trypsin.

Trypsin catalyzes the hydrolysis of ester and amide bonds C-terminal to the α-carbon of Arg and Lys amino acid residues. It is a member of a large and well-studied family of enzymes referred to as serine (Ser) proteases which have been named for a nucleophilic Ser amino acid residue at the active site. In trypsin, this essential nucleophile is Ser-195 (Dixon et al., 1955). The requirement for His-57 was also demonstrated by labeling this critical amino acid with the affinity reagent *N*α-(*p*-tosyl)-L-lysine chloromethyl ketone (TLCK) (Shaw et al., 1965). Later, site-directed mutagenesis and X-ray crystallography were used to verify the role of yet a third amino acid, Asp-102, in trypsin-mediated catalysis (Craik et al., 1987; Sprang et al., 1987). The three amino acid residues Asp-102, His-57, and Ser-195 form the catalytic triad found

in all Ser proteases (Kraut, 1977). The catalytic mechanism of Ser proteases involves His-57 serving initially as a general base to increase the nucleophilicity of the essential Ser and subsequently as a general acid to donate a proton to the leaving group (Fersht, 1985).

Since His amino acid residues are known to strongly coordinate transition metals (Martell & Smith, 1974), it was predicted that the coordination of the essential His-57 of trypsin with a transition metal ion would lead to a reversible inactivation of the enzyme. To enhance the interaction between the transition-metal ion and the side chain of His-57, a second neighboring His was strategically introduced by site-directed mutagenesis so that the two His side chains together might form a more stable coordination complex with a transition-metal ion. Such a complex would closely resemble naturally occurring metal coordination sites found in a variety of metalloproteins (Holmes & Matthews, 1982; Ibers & Holm, 1980; Lee et al., 1989). In this report, we describe how a metal-binding site involving His-57 was engineered into trypsin in order to precisely and reversibly control the activity of this protease.

## MATERIALS AND METHODS

## Materials

*Escherichia coli* strain X90 [F' *lac I<sup>q</sup>, lac ZY, pro AB/Δ* (*lac-pro*), *ara, nal A, argEam, thi, rif<sup>r</sup>*] was obtained from Dr. A. Vershon. All restriction enzymes, T<sub>4</sub> DNA ligase, and T<sub>4</sub> DNA polymerase were purchased from New England Biolabs, Inc. Diethylaminoethyl (DEAE)-Sepharose Fast Flow and carboxymethyl (CM)-Sepharose Fast Flow were from Pharmacia. Immobilized *p*-aminobenzamidase-agarose was obtained from Pierce. Isopropyl β-D-thiogalactopyranoside (IPTG), *N*α-(*p*-tosyl)-L-arginine methyl ester (TAME), and CuCl<sub>2</sub>·2H<sub>2</sub>O were purchased from Sigma Chemical Co. *p*-Nitrophenyl *p*-guanidinobenzoate (*p*NPGB) was obtained from Vega Biotechnologies. *N*α-Benzoyloxycarbonyl-L-glycylprolylarginine 7-amino-4-methylcoumarin (Z-GPR-AMC) was from Bachem Biosciences, Inc.

## Methods

**Computer Modeling.** A computer program was developed to search the atomic coordinates of trypsin for pairs of amino

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acid residues that, when substituted with His, could form a potential chelating complex with divalent first-row transition-metal ions. The amino acid pairs had to satisfy four important criteria set by the search algorithm: (1) the side chains had to be solvent-accessible for exogenous metal binding; (2) the distances between the  $\alpha$ -carbons of the unsubstituted side chains had to be less than 13.0 Å since longer distances would prevent the corresponding imidazole side chains from achieving an optimal distance for metal chelation; (3) the His side chains had to be any one of five favorable rotational conformers (Ponder & Richards, 1987) and still coordinate the metal ion; (4) all atoms of the substituted His side chains had to be further than 3.0 Å from other atoms of the protein unless a hydrogen bond could be formed. Subroutines from the program Insight (Biosym Technologies, San Diego, CA) were used to perform the alignments and torsional angle adjustments of the side chains.

**Mutagenesis and Expression.** A 27-base oligonucleotide (5'-CATCCACCTTTGATCATAAGACCCTG-3') containing a three-base mismatch (underlined bases) was used to introduce a His at position 96 of trypsin. This oligonucleotide was synthesized on a 380B DNA synthesizer (Applied Biosystems, Foster City, CA). Oligonucleotide-directed site-specific mutagenesis and subcloning steps were performed as described previously (Evnin & Craik, 1988).

Trypsin (wild type) and trypsin R96H were expressed in *E. coli*, strain X90 (Higaki et al., 1989). Homogenous samples of protein were obtained from the periplasmic fraction of X90 cells by following the procedure described previously (Higaki et al., 1989).

**Assays.** Gel overlay assays for trypsin activity in the presence and in the absence of  $\text{Cu}^{2+}$  ions were performed on crude periplasmic fractions of X90 cells according to Evnin and Craik (1988), except for the following modifications. After the initial electrophoresis was complete, the separating gel was soaked in 2.5% Triton X-100 for 30 min. Following this soaking, the gel was then transferred to a solution of 3 mM Tris, pH 8.0, containing 3 mM  $\text{CaCl}_2$  with or without 3 mM  $\text{CuCl}_2$  for 15 min. The separating gel was then overlaid onto an 8% polyacrylamide gel containing 65 mM concentration of the ester substrate  $N^\alpha$ -*p*-tosyl-L-arginine methyl ester (TAME) and 1 mM phenol red, pH 9.1. The ester substrate diffuses out of the overlay gel and is hydrolyzed by trypsin into acid ( $N^\alpha$ -*p*-tosyl-L-arginine) and alcohol (methanol) moieties. The formation of an acid lowers the pH in the region of the overlay/separating gel sandwich where trypsin is localized. The pH drop is detected as a change in the color of phenol red from magenta to yellow (Evnin & Craik, 1988). To remove all traces of  $\text{Cu}^{2+}$ , the  $\text{Cu}^{2+}$ -soaked separating gel was then soaked for 15 min in 3 mM Tris, pH 8.0, containing 20 mM EDTA before reapplication to a fresh TAME gel for subsequent visualization.

Tryptic activity was measured by using the fluorogenic substrate  $N^\alpha$ -benzyloxycarbonyl-L-glycylprolylarginine 7-amino-4-methyl coumarin (Z-GPR-AMC) in 1 mM Tris, pH 8.0, containing 1 mM  $\text{CaCl}_2$ /10 mM NaCl with and without 0.4 mM  $\text{CuCl}_2$ . Assays were performed by adding 1.25 nM enzyme to a cuvette containing 5–200  $\mu\text{M}$  Z-GPR-AMC at 25 °C. The excitation wavelength was set at 380 nm, and the emission wavelength was set at 460 nm. Initial rates were determined from data obtained for less than 10% of the hydrolysis of substrate. The concentrations of viable active sites in samples of trypsin R96H and trypsin were determined by titration with *p*-nitrophenyl *p*-guanidinobenzoate (*p*NPGb) (Chase & Shaw, 1967). The kinetic parameters for trypsin

R96H and trypsin in the presence of 0.4 mM  $\text{CuCl}_2$  and 2 mM ethylenediaminetetraacetic acid (EDTA) were obtained by adding the appropriate amount of EDTA to the assays already containing 0.4 mM  $\text{CuCl}_2$ . Enzyme activities were determined from the linear portion of each assay following the addition of EDTA. The values obtained for all  $k_{\text{cat}}$ ,  $K_m$ , and  $k_{\text{cat}}/K_m$  parameters were calculated on the basis of a program that performed a linear least-squares regression analysis of data from an Eadie-Hofstee plot (Fersht, 1985).

**Sequence Alignment.** The amino acid sequences of 21 trypsin-like serine proteases from positions 90 to 102 [chymotrypsin numbering system (Hartley et al., 1965)] were arranged by aligning the catalytic Asp at position 102 and the highly conserved His at position 91. The sequences for rat anionic trypsin (rtryp), bovine chymotrypsin (bchy), and porcine elastase (pelas) were aligned according to Craik et al. (1983). The sequences for bovine coagulation factor 10 (bfa10), human coagulation factor 11 (hfa11), human plasminogen activator (hpla), human protease C (hprt), human thrombin (hthrom), human urokinase (hupla), and human kallikrein (hkall) were aligned according to Doolittle and Feng (1987) while the sequences for rat tonin (rton) and porcine kallikrein (pkall) were aligned according to Fujinaga and James (1987). The sequences of all other trypsin homologues listed in Table II were aligned on the basis of similarities with the rat trypsin sequence. All amino acid sequences were selected from the National Biomedical Research Foundation (NBRF) data base, release 21.0 (George, 1986). The amino acid sequence of bovine factor 10 was determined from the mRNA sequence selected from the Genetic Sequence Data Bank (GenBank), release 60.0 (Bilofsky et al., 1986).

## RESULTS AND DISCUSSION

When the coordinates for rat trypsin (file 2RTM, Brookhaven Protein Data Bank) were used, nine pairs of potential His pairs satisfied the four criteria set by the search algorithm; however, only Arg-96, when substituted with His, was found to form a potential chelating site together with the active-site His-57. Computer modeling studies indicated that, in the presence of  $\text{Cu}^{2+}$ , the side chain of His-57 can rotate 90° about the  $\text{C}_\alpha$ - $\text{C}_\beta$  bond, forming a His-96-His-57- $\text{Cu}^{2+}$  coordination complex that superimposes very closely on the natural metal-chelating site of thermolysin (file 2TLN, Brookhaven Protein Data Bank). In this orientation, the side chain of His-57 is in the same trans conformation as that observed when the Asp-102-His-57 hydrogen bond is disrupted (Sprang et al., 1987). Removal of His-57 from the active site in this anchimeric-assisted fashion (Jencks, 1975) would disrupt the catalytic triad and render the enzyme inactive.

Having identified potential coordination complexes involving His-57, we sought to empirically determine the effect of a transition-metal ion on the activity of an appropriately modified trypsin. A 27-base oligonucleotide was used to incorporate the Arg-96  $\rightarrow$  His mutation into the trypsin coding sequence using procedures previously described (Evnin & Craik, 1988). The mutated trypsin sequence was subcloned into an expression plasmid that secreted high levels of the trypsin variant into the periplasmic space of *E. coli* (Higaki et al., 1989; Vásquez et al., 1989).

Crude periplasmic extracts of X90 cells containing trypsin or trypsin R96H were initially screened by using a gel overlay assay (Evnin & Craik, 1988) in the presence and in the absence of  $\text{Cu}^{2+}$  to determine visually the effect of  $\text{Cu}^{2+}$  on trypsin activity (Figure 1). The divalent metal ion initially used was  $\text{Cu}^{2+}$  because this ion forms a stable complex with imidazole-type ligands (Drey & Fruton, 1965; Tang et al.,

Table I: Kinetic Parameters<sup>a</sup> of Trypsin and Trypsin R96H

enzyme	[CuCl <sub>2</sub> ] (mM)	[EDTA] (mM)	<i>k</i> <sub>cat</sub> (min <sup>-1</sup> )	<i>K</i> <sub>m</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>m</sub> (min <sup>-1</sup> μM <sup>-1</sup> )
trypsin	0.0	0.0	3345 ± 301	14.10 ± 1.3	237.3 ± 21
	0.4	0.0	2510 ± 226	16.69 ± 1.5	150.4 ± 14
	0.4	2.0	3263 ± 294	12.32 ± 1.1	264.9 ± 24
trypsin R96H	0.0	0.0	2464 ± 222	7.45 ± 0.7	331 ± 30
	0.4	0.0	495 ± 45	26.0 ± 2.3	19.0 ± 1.7
	0.4	2.0	2942 ± 265	9.96 ± 0.9	295 ± 27

<sup>a</sup>Tryptic activity was measured with the fluorogenic substrate *N*<sup>α</sup>-benzyloxycarbonyl-L-glycylprolylarginine 7-amino-4-methylcoumarin (Z-GPR-AMC) in 1 mM Tris, pH 8.0, containing 1 mM CaCl<sub>2</sub>/10 mM NaCl with and without 0.4 mM CuCl<sub>2</sub>. In order to minimize the interactions between Tris buffer and copper ions (Bai & Martell, 1969), a low concentration of Tris (1 mM) was used in the assays.

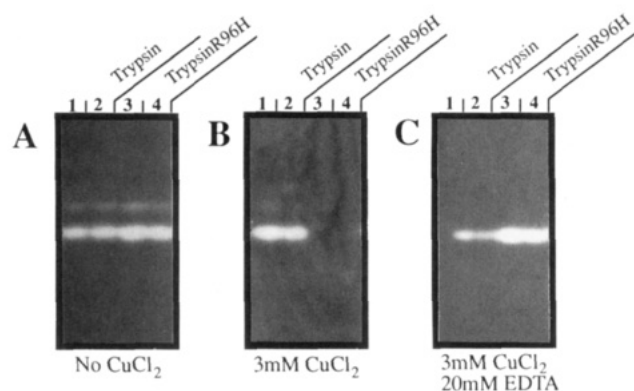


FIGURE 1: Substrate overlay activity gel of trypsin and trypsin R96H in the presence and in the absence of Cu<sup>2+</sup>. Crude periplasmic extracts of X90 containing trypsin or trypsin R96H were subjected to SDS-PAGE under nonreducing conditions. The separating gel was then soaked in a solution of 3 mM Tris, pH 8.0, and 3 mM CaCl<sub>2</sub> with or without 3 mM CuCl<sub>2</sub> before being overlaid onto a substrate gel containing the ester substrate TAME as described under Methods. Panel A is an activity gel that was not treated with CuCl<sub>2</sub>; panel B is an activity gel treated with 3 mM CuCl<sub>2</sub>; panel C is the same gel shown in panel B after subsequently soaking in 20 mM EDTA. Lanes 1 and 2, trypsin; lanes 3 and 4, trypsin R96H.

1978). In the absence of Cu<sup>2+</sup> (panel A), fractions containing trypsin (lanes 1 and 2) and trypsin R96H (lanes 3 and 4) had comparable levels of activity; however, when the separating gel was soaked in a solution containing 3 mM CuCl<sub>2</sub> (panel B), the activity associated with trypsin R96H (lanes 3 and 4) was clearly eliminated while the activity of trypsin (lanes 1 and 2) remained unaltered. Trypsin R96H activity was restored when the CuCl<sub>2</sub>-treated separating gel shown in panel B was subsequently soaked in 20 mM EDTA to remove the Cu<sup>2+</sup> and reapplied to a fresh substrate gel (panel C, lanes 3 and 4). These results dramatically demonstrate that the R96H mutation converts trypsin into an enzyme that is sensitive to exogenous Cu<sup>2+</sup>.

Trypsin and trypsin R96H were purified to homogeneity and kinetically characterized in order to quantitate the effect of Cu<sup>2+</sup> on trypsin activity. Table I lists the kinetic parameters obtained for trypsin and trypsin R96H using the sensitive fluorogenic tripeptide substrate *N*<sup>α</sup>-benzyloxycarbonyl-L-glycylprolylarginine 7-amino-4-methylcoumarin (Z-GPR-AMC) (Higaki et al., 1989). In the absence of copper, the R96H mutation results in a slight increase (40%) in the efficiency (*k*<sub>cat</sub>/*K*<sub>m</sub>) of the enzyme toward this tripeptide substrate relative to trypsin. However, in the presence of 0.4 mM copper, the *k*<sub>cat</sub>/*K*<sub>m</sub> value of trypsin R96H is reduced by 95%. Similar results were obtained by using the ester substrate *N*<sup>α</sup>-*p*-tosyl-L-arginine methyl ester. Relative to other inactive trypsin variants produced by site-directed mutagenesis (Craik et al., 1987; Higaki et al., 1989), copper-inhibited trypsin R96H shows only a moderate decrease in activity. The observed effect may reflect a change in the free energy of activation required to rotate His-57 into its catalytically com-

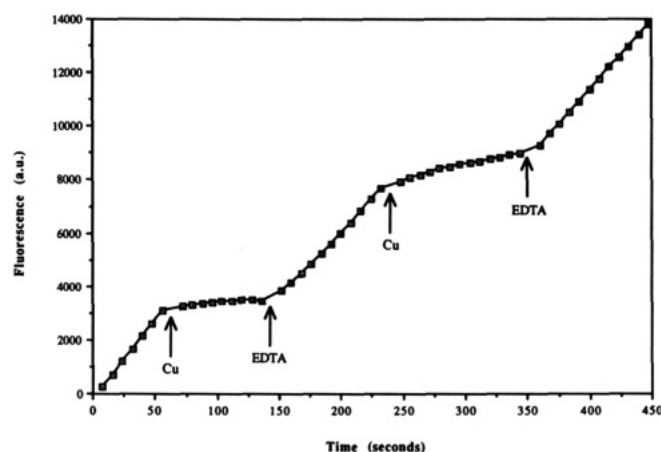


FIGURE 2: Regulating the activity of trypsin R96H by manipulating the Cu<sup>2+</sup> ion concentration. Trypsin R96H was assayed in the absence of Cu<sup>2+</sup> ions as described in Table I. After 60 s, the concentration of Cu<sup>2+</sup> ions was adjusted to 200 μM by adding a 5-μL aliquot of 40 mM CuCl<sub>2</sub> in 50 mM Tris, pH 8.0. The assay was then continued. After 140 s, the activity was restored by adding a 5-μL aliquot of 50 mM ethylenediaminetetraacetic acid (EDTA) to the mixture. The activity of trypsin R96H was again inhibited by the addition of a second (10 μL) aliquot of Cu<sup>2+</sup> ions, and after the activity was monitored for a short period of time, the inhibition was again relieved by chelation with a 20-μL aliquot of 50 mM EDTA.

petent configuration in the active site (Warshel et al., 1989). Likewise, it is possible that a bound metal cation in the vicinity of His-57 could destabilize the positive charge developed on this amino acid residue in the transition state. The partial loss of activity in trypsin at high Cu<sup>2+</sup> concentrations might reflect the ability of copper to weakly coordinate the active-site residues His-57 and Asp-102 as demonstrated in Ag<sup>+</sup>-bound trypsin (Chambers et al., 1974).

In the presence of Cu<sup>2+</sup> ions, the R96H mutation has a slight effect on substrate binding as evident from the slightly higher *K*<sub>m</sub> value. His-57-substrate interactions are likely to exist since the N<sup>ε</sup> atom of the His-57 imidazole interacts with the substrate when it protonates the leaving group of the scissile bond (Kraut, 1977).

The addition of 2 mM ethylenediaminetetraacetic acid (EDTA) restores trypsin R96H to its fully active state since the kinetic parameters obtained in the presence of EDTA closely resemble those observed for the variant in the absence of Cu<sup>2+</sup>. Although the activity of trypsin R96H was not completely eliminated by the addition of Cu<sup>2+</sup>, this design is significant since the mechanism-based inhibition provides universality. No prior knowledge of substrate specificity is required. Furthermore, the restoration of enzyme activity is facile and complete with the use of chelating agents. Other variants are currently being designed and analyzed to more effectively render trypsin inactive in the presence of transition metals.

The plot of velocity of catalysis versus substrate concentration for trypsin R96H (data not shown) shows that the

Table II: Partial Amino Acid Sequence Alignment<sup>a</sup> of Trypsin-like Ser Proteases

	90	91	92	93	94	95	A	B	C	D	E	F	G	H	I	J	K	96	97	98	99	L	M	100	101	102
rtryp	K	H	P	N	F	D	-	-	-	-	-	-	-	-	-	-	-	R	K	T	L	-	-	N	N	D
bchy	K	N	P	K	F	S	-	-	-	-	-	-	-	-	-	-	-	I	L	T	V	-	-	R	N	D
bfa10	K	H	S	R	F	V	-	-	-	-	-	-	-	-	-	-	-	K	E	T	Y	-	-	D	F	D
hfa11	I	H	D	Q	Y	K	-	-	-	-	-	-	-	-	-	-	-	M	A	E	S	-	-	G	Y	D
hfa12	L	H	E	A	F	S	-	-	-	-	-	-	-	-	-	-	-	P	V	S	Y	-	-	Q	H	D
hadpsn	P	H	P	G	S	R	-	-	-	-	-	-	-	-	-	-	-	P	D	S	L	-	-	E	D	D
hcompd	P	H	P	D	S	Q	-	-	-	-	-	-	-	-	-	-	-	P	D	T	I	-	-	D	H	D
htcellpr	P	H	P	D	Y	N	-	-	-	-	-	-	-	-	-	-	-	P	K	T	F	-	-	S	N	D
hmcellpr	I	H	E	S	Y	N	-	-	-	-	-	-	-	-	-	-	-	S	V	P	N	-	-	L	H	D
hpla	V	H	K	E	F	D	-	-	-	-	-	-	-	-	-	-	-	D	D	T	Y	-	-	D	N	D
hcatg	R	H	P	Q	Y	N	-	-	-	-	-	-	-	-	-	-	-	Q	R	T	I	-	-	Q	N	D
hprtc	V	H	P	N	Y	S	-	-	-	-	-	-	-	-	-	-	-	K	S	T	T	-	-	D	N	D
hkall	I	H	Q	N	Y	K	-	-	-	-	-	-	-	-	-	-	-	V	S	E	G	-	-	N	H	D
hthrom	I	H	P	R	Y	N	-	-	-	-	-	-	-	-	-	-	-	W	K	E	N	-	L	D	R	D
pelas	V	H	P	Y	W	N	-	-	-	-	-	-	-	-	-	-	-	T	D	D	V	A	A	G	Y	D
hupla	L	H	K	D	Y	S	-	-	-	-	-	-	-	-	-	-	-	A	D	T	L	A	H	H	N	D
hprte	V	H	P	L	W	N	-	-	-	-	-	-	-	-	-	-	-	R	S	C	V	A	C	G	N	D
pkall	P	H	P	G	F	N	L	S	-	-	-	-	-	-	-	-	-	A	D	G	K	D	Y	S	H	D
rton	R	H	P	D	Y	I	P	L	I	V	T	N	D	T	E	Q	P	V	H	D	H	-	-	S	N	D
mngfg	P	H	P	G	F	N	M	S	L	M	R	K	H	I	R	F	L	E	Y	D	Y	-	-	S	N	D
hprospr	P	H	P	L	Y	D	M	S	L	L	K	N	R	F	L	R	P	G	D	D	S	-	-	S	H	D

<sup>a</sup>The amino acid sequences of 20 trypsin-like Ser proteases from positions 90 to 102 (chymotrypsin number system; Hartley et al., 1965) were arranged by aligning the catalytic Asp at position 102 and the highly conserved His at position 91. Abbreviations: rat anionic trypsin, rtryp; bovine chymotrypsin, bchy; bovine factor 10, bfa10; human factor 11, hfa11; human factor 12, hfa12; human adipocyte serine protease, hadpsn; human complement factor D, hcompd; human t-cell protease I, htcellpr; human mas cell protease II, hmcellpr; human tissue plasminogen activator, hpla; human cathepsin G, hcatg; human protease C, hprtc; human pancreatic kallikrein, hkall; human thrombin, hthrom; porcine pancreatic elastase I, pelas; human urokinase plasminogen activator, hupla; human protein E, hprte; porcine pancreatic kallikrein, pkall; rat submaxillary tonin, rton; mouse submaxillary 7S nerve growth factor (NGF)  $\gamma$ , mngfg; human prostate serine protease, hprospr.

inhibition of this variant follows Michaelis-Menten saturation kinetics. The velocity of trypsin R96H at saturating concentrations of Z-GPR-AMC ( $V_{\max}$ ) was then determined at a variety of total enzyme concentrations to demonstrate that  $\text{Cu}^{2+}$  ions do not inhibit trypsin R96H in an irreversible fashion. Once this was established, a plot of the reciprocal velocity of trypsin R96H activity as a function of the copper concentration at varying concentrations of Z-GPR-AMC (Dixon plot) was used to determine the enzyme-inhibitor equilibrium constant ( $K_i$ ) (Segel, 1975). Although the curves indicated a very slight deviation from linearity (data not shown), they were treated as a representation of a linear, mixed-type inhibition system. From these plots, an apparent  $K_i$  of 21  $\mu\text{M}$ , corresponding to the point of intersection of the plots, was obtained for copper inhibition. This is an apparent  $K_i$  since the effective copper concentration is significantly less than its total concentration due to the competitive binding of  $\text{Cu}^{2+}$  ions by Tris buffer (Bai & Martell, 1969) and trypsin R96H. Regardless, values of  $K_i$  in the micromolar range indicate strong binding of the enzyme to the metal ion. Trypsin

was also assayed in a similar fashion, yielding a  $K_i$  of 1.6 mM. The introduction of the R96H mutation in trypsin strengthens the binding of  $\text{Cu}^{2+}$  by a factor of 76, making trypsin R96H more susceptible to inhibition by  $\text{Cu}^{2+}$ . Since the plot at saturating substrate concentrations has a slope greater than zero (data not shown), copper ions do not inhibit the activity of trypsin R96H in a competitive fashion (Segel, 1975).

In addition to  $\text{Cu}^{2+}$ , other transition-metal ions such as  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  were also expected to have similar effects on trypsin R96H based on similarities in their coordination chemistries. These metal ions behaved as predicted. A comparison of the metal-trypsin R96H  $K_i$ s obtained for these three metal ions indicates that the strength of association with trypsin R96H is in the order  $\text{Cu}^{2+}$  (21  $\mu\text{M}$ ) >  $\text{Ni}^{2+}$  (49  $\mu\text{M}$ ) >  $\text{Zn}^{2+}$  (128  $\mu\text{M}$ ). This is the same order predicted based on experimentally determined association constants ( $K_a$ ) for metal binding to imidazole (Martell & Smith, 1974).

To demonstrate the switchable nature of this inhibition under turnover conditions, trypsin R96H was initially assayed in the absence of copper (Figure 2). Shortly after the assay

was initiated,  $\text{Cu}^{2+}$  was added to a final concentration of 0.2 mM. Immediately upon the addition of this first aliquot of  $\text{Cu}^{2+}$ , the activity was reduced to 6% of the initial value. The assay was continued for 1.5 min, and then an aliquot of EDTA was added to the assay mixture in order to chelate the copper. Within a few seconds after the addition of EDTA, the activity was restored to a level comparable to that observed prior to the addition to  $\text{Cu}^{2+}$ , showing that trypsin R96H activity was fully reversible through one round of inhibition by  $\text{Cu}^{2+}$  and reactivation by EDTA. The assay was then taken through a second cycle of inhibition/reactivation to show that the activity of trypsin R96H can be repetitively removed and restored by adjusting the concentration of  $\text{Cu}^{2+}$ .

Since trypsin belongs to a large family of Ser proteases with a high degree of sequence and structural similarities, it should be possible to engineer similar metal-chelating complexes into a variety of other trypsin-like Ser proteases. Table II is a partial amino acid sequence alignment of a variety of trypsin-like Ser proteases. The sequences were aligned based on a highly conserved His at position 91 [chymotrypsin numbering (Hartley et al., 1965)] and the catalytic Asp at position 102. A large number of Ser proteases have the same number of amino acid residues in this region of the sequence such that the substitution of a His for the wild-type amino acid at position 96 in this set of enzymes may lead to  $\text{Cu}^{2+}$  binding and reversible disruption of the active site.

The trypsin homologues porcine elastase, human urokinase, human protein E, porcine kallikrein, and a large number of other elastases not shown also have a high degree of sequence similarity to trypsin in this region, but contain two to four extra amino acid residues. Likewise, rat tonin, rat 7S NGF- $\gamma$ , and human prostate Ser protease as well as a number of kallikreins not listed contain an 11 amino acid insertion spanning positions 95–96. The placement of a His in the vicinity of position 96 in these enzymes might also create a stable metal-binding site, involving the catalytic His-57, that could inactivate the enzyme in the presence of a metal ion. Indeed, the sequence alignment of rat tonin shows two naturally occurring His residues at positions 97 and 99. These His side chains, along with the active-site His-57, are known to coordinate a single  $\text{Zn}^{2+}$  ion and, in so doing, inactivate the enzyme (Fujinaga & James, 1987). The three-dimensional structure of Zn-bound tonin shows that, when His-97, His-99, and His-57 coordinate a  $\text{Zn}^{2+}$  ion, the imidazole side chain of His-57 is distorted such that the catalytic triad is no longer functional (Fujinaga & James, 1987).

We propose that the inhibition of trypsin R96H by divalent metal ions occurs via a similar mechanism as that observed for tonin, but uses only two His side chains (His-96 and His-57). The introduction of a coordination complex into trypsin involving the active-site His-57 illustrates how an anchimeric-assisted metal switch can be engineered into a protease for the purpose of regulating its function. This is a useful step in protein design which may not be limited to proteases since histidine is one of the most common amino acids at the active site of a protein. It may be feasible to introduce a metal-dependent regulatory switch into many histidine-dependent proteins, several of which are of significant therapeutic and biotechnological importance.

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