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Regulation of trypsin activity by Cu^{2+} chelation of the substrate binding site

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Lysine 188 of trypsin was replaced with histidine in order to create a metal chelation site in the substrate binding pocket of this protease, built in a metal binding 'switch,' and to be able to modulate its activity at lower pH. The catalytic properties of wild-type and mutant trypsin were measured with tetrapeptide substrates containing a nitro-anilide leaving group and whole native protein substrate: β -casein. The results obtained reveal that K188H mutation does not affect catalytic efficiency, raising only slightly (from 6 to 8) the arginine/lysine preference of the mutant and increasing 1.8- and 1.2-fold the second-order rate constant k_{cat}/K_m for arginine- and lysine-containing substrates, respectively. Compared with wild-type trypsin, K188H mutant shows, in the absence of Cu^{2+} , a different catalytic activity pattern as a function of pH. The addition of Cu^{2+} to trypsin K188H induces a 30–100-fold increase in K_m , while k_{cat} is scarcely decreased. The hydrolytic activity of this mutant can be fully restored by addition of EDTA. In contrast to a chelating active site, a novel mode of metal-dependent inhibition activity of trypsin with copper is presented. As suggested by molecular modelling studies, the substrate binding pocket of the protease is considerably perturbed by vicinal chelation. More generally, this type of transition metal chelate may present wider possibilities of trypsin activity and specificity modulation than the previously accomplished chelation of a histidine moiety from a catalytic triad.

Keywords: β -casein/inhibition/metal chelation/serine protease/site-directed mutagenesis/trypsin

Introduction

A variety of metal ions known to interact with a large number of proteins are required for multiple physiological processes. The specific role of the protein-bound metal ions can vary, but in many metalloproteins, for example, metal ions can function as cofactors. Metal ions associated with proteins can also mediate substrate binding and catalysis and stabilize protein folding. Fundamental principles based on protein–metal interactions of naturally occurring cation-binding proteins can now be used to develop enzymes with specific characteristics. The design of metal ion-dependent regulatory sites in enzymes can be of particular interest, since the ability to control the activity of an enzyme by regulating the concentration of metal ions in the external media may have useful applications.

Recent efforts to tune up hydrolytic reactions by building in regulatory switches in hydrolases have resulted in the design

of a recombinant enzyme whose activity was controlled in a metal-independent fashion by manipulating the redox potential (Matsumura and Matthews, 1989) and in a metal-dependent fashion by the reversible filling of the substrate binding pocket of staphylococcal nuclease (Corey and Schultz, 1989). Additional strategies have been developed conferring novel substrate specificities on serine proteases in order to enable them to carry out substrate-assisted catalysis (Carter and Wells, 1987; Corey *et al.*, 1995; Willett *et al.*, 1995).

Trypsin is a good test system to design metal binding sites since its expression and purification system are relatively well known. Its three-dimensional structure is well determined (Craik *et al.*, 1984) and the kinetic analysis of its catalytic properties is developed. Trypsin catalyses with relative specificity the hydrolysis of ester and amide bonds at the carboxy terminus of the α -carbon of arginyl and lysyl residues. It is a member of a large and well studied family of serine proteases that all have nucleophilic serine in the active sites. In the catalytic mechanism of serine proteases, His of the catalytic triad serves initially as a general catalytic base to increase the nucleophilicity of an essential Ser moiety. In the next round, His acts as general acid donating a proton to the leaving group (Fersht, 1985). Higaki *et al.* (1990) designed a variant of rat trypsin with catalytic activity regulated reversibly by first-row transition metals. Substitution of Arg96 with a His (R96H) places a new imidazole group on the surface of the enzyme near the catalytic His57. Chelation of this site by a metal ion involved the imidazole of catalytic His57 and the introduced His96. Hence His57 could no longer participate as a general base in catalysis. The esterase and amidase activities of trypsin R96H were inhibited. In that way, the activity of this proteinase could be controlled, knocking out by chelation one of the residues of the catalytic triad.

This paper describes an attempt to regulate the activity of trypsin in a metal-dependent fashion by the replacement by oligonucleotide-directed mutagenesis of lysine 188 by histidine (K188H). This method introduces a chelating site in the vicinity of the substrate binding pocket of this proteinase and seals it without involving any residues of the catalytic triad. The use of a histidine side-chain copper system was selected because imidazole easily shares a free pair of electrons with this transition metal and the coordination bonds formed between the imidazole nitrogen and cations are stable and reversible. Previous studies using site-directed mutagenesis strengthened by a genetic selection approach for the isolation of new variants (Evnin *et al.*, 1990; Perona *et al.*, 1993) described well the role of the negatively charged Asp189 residue in substrate binding and catalysis. Asp189 is contiguous to the targeted site. Kinetic characterization of these trypsin mutants, as well as of the variants D189K (Graf *et al.*, 1987) and D189S (Graf *et al.*, 1988), indicates that the presence of a negative charge at the bottom of the binding pocket is essential for efficient cleavage of peptide bonds with N-terminal basic residues by trypsin. In this work, it was expected that negatively charged

Asp189 would stabilize the imidazole-Cu²⁺ chelate by additional electrostatic dentate interaction.

The analysis of the available three-dimensional structures of trypsin by molecular modelling techniques, reveals that side chain of Lys188 (labelled K188a) protrudes outside the substrate binding pocket (Brinen *et al.*, 1996). By substituting lysine 188 with histidine, it was expected that Cu²⁺ chelation would sterically perturb the substrate binding site and additionally neutralize its negative charge and it would strap the substrate binding pocket of trypsin with coordination bonds. It was expected also that in absence of transition ions, the catalytic properties of trypsin could be altered, owing to the different pK_a of mutated residue 188 ($\Delta pK_a \approx 2$), without decreasing the efficiency of this enzyme. The catalytic properties of K188H and wild-type trypsin were compared using synthetic substrates and whole protein substrate. The effect of pH on the activity and the inactivation of mutant K188H by Cu²⁺ was studied. In the latter case, a 3D model of chelation involving H188a residue is proposed.

Materials and methods

Ampicillin, isopropyl β -D-thiogalactopyranoside (IPTG), lysozyme, 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB), *N*-(2-hydroxyethyl)piperazine-*N'*-(3-propanesulfonic acid) (HEPPS), 2-(*N*-morpholino)ethanesulfonic acid (MES) and Luria broth were obtained from Sigma (St Louis, MO). The components used for *in vitro* mutagenesis were purchased from Bio-Rad (Ivry sur Seine, France) and these included *Escherichia coli* strains, T4 DNA polymerase and T4 DNA ligase. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Succinyl-Ala-Ala-Pro-Arg-*pNA* and succinyl-Ala-Ala-Pro-Lys-*pNA* were obtained from Bâle Biochimie (Voisins le Bretonneux, France). CM-Sepharose and Q-Sepharose fast-flow and benzamidine-Sepharose were purchased from Pharmacia (Uppsala, Sweden). Organic solvents used for high-performance liquid chromatography (HPLC) were supplied by Carlo Erba (Milan, Italy). All other reagents were of analytical grade.

Strains and plasmids

E. coli strain HB2151 [K12, *ara*, $\Delta(lac-pro)$, *thi*, *F*, *proA*⁺*B*⁺, *lacZ* Δ 15] was used for subcloning and expression of trypsins. *E. coli* CJ236 (*F'* *CJ105/dut*⁻, *ung*⁻, *relA1*) was purchased from Bio-Rad. The plasmid pZ3 containing the DNA encoding rat anionic trypsin II with a *tac* promoter and the *Salmonella typhimurium hisJ* signal peptide was a kind gift from Charles S. Craik (Department of Pharmaceutical Chemistry, University of California, San Francisco, CA).

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel (1985), using single-stranded, uracil-containing DNA templates. The 30-base oligonucleotide primer 5'-GCCCTGGCAGGAATCGTGGCCTCCCTCTAG-3', directing histidine substitution of Lys188 was synthesized with a Biosearch Cyclone DNA synthesizer (mismatched bases are underlined).

The 0.65 kb fragment from the *EcoRI*-*HindIII* digestion of pZ3 carrying the complete DNA encoding trypsin was subcloned into the *EcoRI*-*HindIII* site of Blue-script pKS-M13⁺ phagemid. Single-stranded, uracil-containing DNA was prepared and used as a template along with the mutagenic primer for the mutagenesis reaction. Putative clones were sequenced

by the dideoxy chain termination method (Sanger *et al.*, 1977). The *EcoRI*-*HpaI* fragment from a mutant was subcloned back into pZ3. The entire trypsin cDNA was sequenced to confirm the absence of any spontaneous mutations.

Protein production and purification

Culture of *E. coli* strain HB2151 was carried out on Luria broth medium overnight at 37°C in the presence of ampicillin (50 mg/l) and 0.5 mM IPTG to induce expression. Wild-type and K188H trypsins were secreted in the periplasm space of cells. Periplasmic extracts were prepared by treatment with lysozyme (Vasquez *et al.*, 1989) and were subsequently dialysed against 10 mM sodium citrate, pH 2.2, at 4°C for 24 h. The resulting solution was then applied to a CM-Sepharose fast-flow column (30×2.6 cm i.d.) in a volume equal to the volume of the column equilibrated with 0.1 M citric acid, 0.1 M trisodium citrate buffer, pH 4.0. Fractions containing trypsin were monitored by the following method. An aliquot of each fraction was mixed with an equal volume of 2× Laemmli buffer (Laemmli, 1970) lacking 2-mercaptoethanol. The sample was then submitted to electrophoresis on 12.5% polyacrylamide, 1% SDS running gel with a 4.75% polyacrylamide stacking gel. After electrophoresis, the separating gel was soaked in 2.5% Triton X-100 for 35 min and then in 50 mM Tris-HCl, 20 mM CaCl₂ solution, pH 8.0. This gel was overlaid with an 8% polyacrylamide gel in 40 mM Tris-HCl, 15 mM CaCl₂, pH 8, containing 0.1% whole bovine casein. The 'gel sandwich' was incubated overnight at 37°C, then dyed with Coomassie blue. Trypsin was therefore detected by digestion of the casein leading to a lack of coloration at a level corresponding to the molecular weight of this protein. The pH of the fractions obtained from chromatography on CM-Sepharose and containing trypsin was adjusted to 6.0 with 6 N NaOH. The solution was applied to a benzamidine-Sepharose column (4.0×1.6 cm i.d.) and chromatography was carried out in 50 mM Tris-HCl, 20 mM CaCl₂, pH 8. After complete elution of unbound proteins as indicated by the return of the absorbance to its initial value, bound trypsin was eluted with 10 mM HCl. All enzyme purifications were carried out at 4°C.

Determination of active-site concentration

The concentration of the active enzyme was determined according to Jameson *et al.* (1973). MUGB, which reacts with serine 195 (Corey and Craik, 1992), was used as a spectrofluorimetric titrant for trypsin. An Aminco SLM 4800C luminescence spectrophotometer was used to measure the change in fluorescence. The spectrophotometer was set to an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Titration was performed at 20°C in 50 mM Tris-HCl, 20 mM CaCl₂, 100 mM NaCl, pH 8.0.

Kinetic measurements

Succinyl-Ala-Ala-Pro-Arg-*pNA* and succinyl-Ala-Ala-Pro-Lys-*pNA* were used for the kinetic studies. Hydrolysis of the substrate was monitored spectrophotometrically at 405 nm ($\epsilon = 13\,500$) in a Varian Cary 13E spectrophotometer. Twelve substrate concentrations were used for determining initial rates. The initial slopes of the reaction curves were measured to yield $\Delta A/\text{min}$. Michaelis-Menten parameters were calculated from Eadie-Hofstee plots. Hydrolysis was performed at 37°C using 1.5 mM Tris-HCl, 1 mM CaCl₂, 10 mM NaCl, 5 mM MES, pH 6, and 1.5 mM Tris-NaOH, 1 mM CaCl₂, 10 mM NaCl, 5 mM HEPPS, pH 8, in the absence and presence of 5 or 200 μM CuCl₂.

β -Casein hydrolysis and peptide assignment

Crude β -casein A1 was prepared as described by Zittle and Custer (1963). It was further purified by chromatography according to the method of Mercier *et al.* (1968) on a Q-Sepharose fast-flow column.

β -Casein was dissolved (1 mg/ml, initial concentration) in the appropriate buffer (0.2 M Tris-HCl, for pH 7.0–9.0; 0.1 M glycine-NaOH, pH 10). The final concentration of β -casein was determined spectrophotometrically using the molecular absorption coefficient $E_{280} = 11\,000\text{ M}^{-1}\text{cm}^{-1}$. Active enzyme was added to β -casein solution at an enzyme/substrate ratio (E/S) of 0.005% (w/w) for wild-type and K188H mutant. The reaction mixture was incubated at 37°C. Aliquots were taken at intervals (30 min, 1, 2, 4, 8, 24 and 48 h) and the hydrolysis was stopped by addition of 0.5 vol. of 0.2 M HCl. Tryptic peptides of β -casein were separated by reversed-phase HPLC on a Nucleosil C₁₈ column (25 cm × 4.6 mm i.d.) (SFCC, Gagny, France) with a linear gradient from solvent A (0.11% TFA in H₂O) to 100% solvent B (60% acetonitrile, 40% H₂O, 0.09% TFA) in 62.5 min (Briand *et al.*, 1994). The peptides produced were identified by electrospray mass spectrometry on a Perkin-Elmer Sciex (Thornhill, Ontario, Canada) API III⁺ mass spectrometer.

Computer modelling method

Molecular modelling studies were carried out on Silicon Graphics computers with Biosym/Molecular Simulation packages. Molecular display and energy minimizations were performed with Insight II and Discover packages, respectively. Two force fields were used at different stages of the calculations. The Amber force field (Weiner *et al.*, 1984) was initially employed for energy measurements and in minimization procedures. For the copper chelation, the ESFF (Extensible Systemic Force Field) developed by Biosym was necessary. As far as we know, this new force field is the only one than can explicitly treat organometallic compounds with a Morse functional form for the bond energy term and specific partial charges for metal and immediate ligands by summing their formal charges to obtain a net fragment charge.

The initial structure of trypsin was extracted from X-ray data of its complex with ecotine (Brinen *et al.*, 1996) accessible from the Protein Data Bank (1SLU). Although two segments are missing in this structure (114–117 and 146–148), this trypsin structure was chosen as the starting geometry because of its high crystallographic resolution (1.8 Å). These missing residues do not significantly perturb the meaning of this molecular modelling study since all energy calculations were performed by keeping fixed the backbone of heavy atoms not involved in the binding pocket contour (essentially residues 182–195 and 213–228).

The mutant model was obtained by replacing Lys188 with His using Insight II facilities. Both wild-type and mutant geometries were optimized with the same protocol as follows: a first minimization for 1000 iterations fixing the backbone heavy atoms followed by a second minimization step for 3000 iterations fixing all heavy atoms except those involved in the substrate binding pocket.

The construction of the copper chelate model was based on biochemical information (see Results). On the one hand, in the absence of copper ions, mutant K188H exhibits similar K_m values to the wild-type. Therefore, it was considered that the general shape of the substrate binding pocket is not altered by the histidine replacement. This is consistent with the

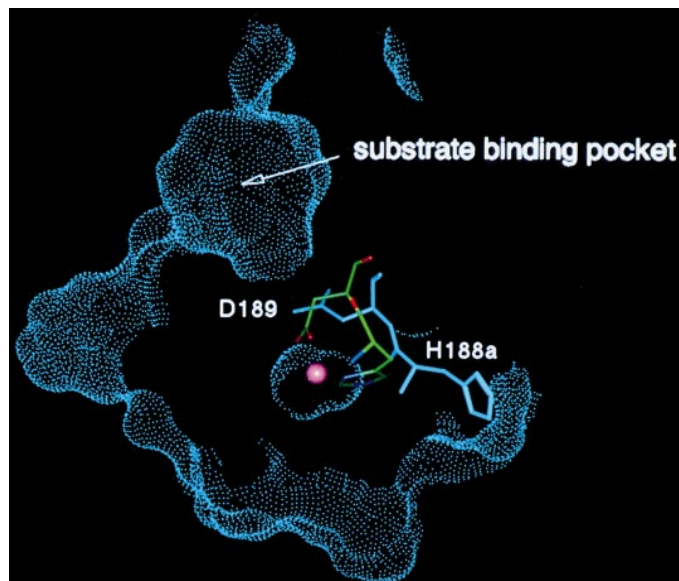


Fig. 1. View (Connolly contour) of a minor cavity in the neighbourhood of the substrate binding site of trypsin, as a potential site of Cu²⁺ positioning in the chelate. His188a side chain pointing out and Asp189 wild-type side chains are coloured in blue. Imidazole of His188a and Asp189 side chain in mutant trypsin are colored according to the atom type code (N, blue; O, red). Cu²⁺ cation is pink.

protruding position of the initial lysine side chain pointing out of this pocket. Consequently, its replacement by a histidyl residue with the same backbone conformation has no significant effect on the substrate binding pocket. On the other hand, only the presence of copper ions perturbs the catalysis of K188H trypsin, in contrast to wild-type trypsin. It was therefore concluded that the chelation site of copper should be located at the vicinity of the binding site but not inside it. Furthermore, the imidazole ring of His188 should be engaged in this chelation.

A careful examination of the binding pocket environment (for both the wild-type and the mutant) revealed another small pocket (Figure 1) well separated from the main one by Asp189, which is well known to play a major role in the substrate positioning. Hence it was postulated that this site was a good candidate for copper ion location. However, even in this position, the copper ion is too far from any nitrogen atom of the imidazole ring concerned for possible chelation. It was therefore necessary to impose preliminarily a distance constraint in order to bring this ring closer to the potential site of the copper ion. This was performed in a specific minimization procedure with respect to the other residues. A chelation involving only the OD2 oxygen atom of Asp189 and the ND1 nitrogen atom of His188 was imposed during the following minimization (1000 iterations). During this stage, three other nitrogen atoms were found to be significantly close to the copper ion, suggesting their involvement in the final chelation system. The final model was obtained by imposing five ligands for the copper ion during the last minimization procedure (1000 iterations).

Results

Comparison of wild-type and K188H trypsins

Wild-type and K188H trypsins were purified to homogeneity. Their kinetics were characterized and compared in order to

Table I. Kinetic parameters of wild-type trypsin measured at pH 6 and 8 with 0, 5 or 200 μM Cu^{2+}

pH	$[\text{Cu}^{2+}]$ (μM)	Substrate ^a	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
pH 6	0	Arg	86 ± 6	67 ± 5	0.78 ± 0.12
	5		115 ± 1	81 ± 11	0.70 ± 0.10
	200		199 ± 16	50 ± 5	0.25 ± 0.005
pH 8	0		41 ± 1	132 ± 5	3.22 ± 0.17
	5		49 ± 2	109 ± 16	2.22 ± 0.43
	200		42 ± 1	79 ± 5	1.88 ± 0.17
pH 6	0	Lys	311 ± 19	44 ± 3	0.14 ± 0.02
	5		355 ± 2	42 ± 2	0.12 ± 0.01
	200		~ 1300	$\sim 29 \pm 2$	0.02 ± 0.001
pH 8	0		75 ± 7	39 ± 2	0.52 ± 0.08
	5		102 ± 4	34 ± 2	0.33 ± 0.03
	200		104 ± 3	26 ± 5	0.25 ± 0.06

^aSubstrates: Suc-Ala-Ala-Pro-Arg-pNA and Suc-Ala-Ala-Pro-Lys-pNA.

Table II. Kinetic parameters of K188H trypsin measured at pH 6 and 8 with 0, 5 or 200 μM Cu^{2+}

pH	$[\text{Cu}^{2+}]$ (μM)	Substrate ^a	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \mu\text{M}^{-1}$)
pH 6	0	Arg	70 ± 4	96 ± 3	1.37 ± 0.13
	5		152 ± 6	86 ± 6	0.57 ± 0.07
	200		~ 1900	$\sim 82 \pm 8$	0.04 ± 0.01
pH 8	0		30 ± 2	169 ± 12	5.63 ± 0.84
	5		118 ± 3	150 ± 11	1.27 ± 0.32
	200		~ 2200	$\sim 131 \pm 10$	0.06 ± 0.13
pH 6	0	Lys	302 ± 15	51 ± 3	0.17 ± 0.01
	5		~ 600	$\sim 54 \pm 5$	0.09 ± 0.01
	200		$\sim 10^4$	$\sim 54 \pm 5$	0.005 ± 0.001
pH 8	0		74 ± 1	48 ± 5	0.65 ± 0.05
	5		409 ± 42	54 ± 3	0.13 ± 0.02
	200		~ 7500	$\sim 54 \pm 5$	0.007 ± 0.001

^aSubstrates: Suc-Ala-Ala-Pro-Arg-pNA and Suc-Ala-Ala-Pro-Lys-pNA.

quantify the effect of Cu^{2+} on enzyme activity. Tables I and II list the kinetic parameters obtained for wild-type and K188H trypsins when measured at pH 6 and 8 using comparable tetrapeptide arginine and lysine substrates containing a *p*-nitroanilide (*pNA*) chromophore leaving group. In the absence of copper ions, mutant K188H exhibits K_m values similar to those obtained with wild-type trypsin. However, K188H presents a slight increase in k_{cat} values determined with both synthetic substrates at pH 6 and 8. Consequently, the second-order rate constant k_{cat}/K_m of K188H trypsin mutant increases 1.8- and 1.2-fold for arginine- and lysine-containing substrates, respectively. Nevertheless, wild-type and K188H trypsins show a similar 6- and 8-fold arginine/lysine preference, respectively, whatever the pH.

Metal inactivation

Influence of Cu^{2+} concentration. The presence of 5 μM Cu^{2+} is sufficient to alter the catalytic activity of K188H trypsin. The efficiency (k_{cat}/K_m) towards both synthetic substrates is reduced 5- and 2-fold at pH 8 and 6, respectively (Tables I and II). This decrease in activity of trypsin K188H is demonstrated by an increase in K_m values for both substrates studied whereas the K_m values of wild-type trypsin are not changed. In contrast to K_m , the k_{cat} values of wild-type and K188H are not influenced at all by the addition of Cu^{2+} .

In the presence of 200 μM Cu^{2+} , the K_m values of mutant K188H reached the millimolar range for both substrates studied and could not be measured precisely. At this concentration of Cu^{2+} , the k_{cat}/K_m values of trypsin K188H are reduced ~ 100 -fold at pH 8 and 30-fold at pH 6 for both substrates. These values are reduced only 2-fold at pH 8 and 3–6-fold at pH 6 with wild-type trypsin.

As was observed with mutant R96H by Higaki *et al.* (1990), the addition of 2 mM EDTA to the aliquots already containing Cu^{2+} restores the full activity of mutant trypsin K188H. Kinetic parameters obtained in the presence of EDTA match closely what is observed in the absence of Cu^{2+} (data not shown).

Influence of pH. In the presence of 5 μM Cu^{2+} , the loss of K188H trypsin activity is more pronounced at pH 8 than at pH 6, since the k_{cat}/K_m values decrease ~ 5 -fold at pH 8 whereas they only decrease ~ 2 -fold at pH 6 for both synthetic substrates. This effect is even more significant in the presence of 200 μM Cu^{2+} , since the k_{cat}/K_m values of K188H mutant decrease ~ 100 - and 30-fold for both substrates when hydrolysis is realized at pH 8 and 6, respectively. Therefore, similar k_{cat}/K_m values of K188H trypsin are obtained at pH 8 and 6 in the presence of 200 μM Cu^{2+} . Such a result is not observed with wild-type trypsin. This evolution of catalytic constants is

principally due to the increase in K_m at pH 6 since k_{cat} is not much influenced at this pH. The differences in the k_{cat}/K_m decreases at pH 8 and 6 can be explained by the increase in the protonation of 188H imidazole, and hence the smaller population of Cu^{2+} chelates produced.

Hydrolysis of β -casein by wild-type and K188H trypsins

Until recently, the effect of trypsin mutations on catalytic activity has been determined mostly using synthetic substrates or small peptides. Corey *et al.* (1995) examined whether trypsin H57A could cleave ornithine decarboxylase from *Leishmania donovani*. Synthetic substrates allow us to calculate rapidly the catalytic constants of an enzyme. However, the major part of medium-induced conformational states and the changes in electrostatic charges of the targeted peptide bond and created steric hindrance have hitherto been neglected. Since these factors are certainly critical, the hydrolysis of a long and well defined natural substrate, such as β -casein, was studied at different pH. The use of a full native protein as substrate allows us to understand better the scope of the results obtained with synthetic substrates.

Bovine β -casein was incubated at pH 7, 8, 9 or 10 with wild-type and K188H trypsins with an identical E/S ratio (0.005%, w/w). Peptide mixtures obtained after 48 h of hydrolysis of β -casein by the trypsins studied were separated on a C_{18} column and identified by mass spectrometry. The major peptides detected were named according to the potential tryptic cleavage sites (Figure 2).

Kinetics of hydrolysis at pH 8 by wild-type trypsin

The peptides obtained by hydrolysis of β -casein with the studied wild-type rat trypsin are identical with those obtained during the hydrolysis of β -casein by bovine trypsin (Ribadeau Dumas *et al.*, 1972; Carles and Ribadeau Dumas, 1986; Léonil *et al.*, 1988; Briand *et al.*, 1994). The kinetic analysis shows (Figure 3) the rapid appearance of the N-terminal phosphopeptide A (R1–R25) followed by the release of the C-terminal peptide L–N (D184–V209). These peptides result from the cleavage of bonds involving an arginyl residue: R25–I26 and R183–D184. The results obtained agree well with those obtained with synthetic substrates, confirming the greater efficiency of wild-type trypsin for a peptide bond containing arginine. However, N peptide (G203–V209), obtained from the cleavage of a bond also containing an arginine (R202–G203) appears after 8 h of hydrolysis only. It is evident that the small size (four residues) of the synthetic substrates used assists their cleavage because of their quicker diffusion and practically non-existent constraints due to substrate conformation. The latter starts to be perceptible when larger substrates are processed. For example, the arginine/lysine preference is observed in the case of small synthetic substrates when, as seen from the kinetic data, it becomes conditional during β -casein cleavage depending on the accessibility of a particular Arg-containing cleavage site.

After the liberation of A and L–N peptides, large quantities of peptides resulting from the hydrolysis of bonds involving lysyl residues appear. This is the case with peptides B (I26–K28), C (K29–K32), F (E100–K105), J (V170–K176) and K (A177–R183). However, some of the peptide bonds split to yield these peptides are difficult to hydrolyse since larger peptides such as Val–Lys–F (V98–K105), G–H (H106–K113), J–K (V170–R183), C–D–E (K29–K97), D–E (F33–K97) and G–H–I (H106–K169) are still observed even after 24 h of hydrolysis. These results indicate the resistance to hydrolysis

of bonds K99–E100, K107–E108, K176–A177 and K32–F33. The peptides D (F33–K48) and H (E108–K113) appear after 24 h of hydrolysis indicating also the resistance of peptide bonds K48–I49 and K113–Y114 to lysis. As can be readily seen, in addition to the structural shielding of some amide bonds, electrostatic factors can also influence the velocity of lysine-containing peptide bonds cleavage. This is particularly clear in the case of 'K–E' sites.

pH dependence of tryptic peptides profiles (Figure 4)

During the hydrolysis of β -casein by the proteases studied, the pH affects their catalytic activity and may also change the conformation and charges of the substrate. In the case of the comparative study of wild-type and mutant trypsin activities at the same pH, the role of the conformation of the substrate may be neglected and the observed differences can be assigned to the effect of mutation only.

The kinetics of β -casein fragmentation by wild-type trypsin demonstrate that a maximum of activity is reached at pH 8 and 9. Lower activity is observed at pH 7 and the lowest efficiency at pH 10. These results are different from those obtained by hydrolysis of synthetic substrates.

The variation in the disappearance of β -casein as a function of pH can be well followed by the obtention of many of the peptides such as B (I26–K28), C (K29–K32), H (E108–K113), I (Y114–K169), Val–Lys–F (V98–K105) and G–H–I (H106–K169). In despite of decreases of catalytic efficiency with pH, the cleavage of peptide bonds leading to the liberation of peptides A (R1–R25) and N (G203–V209), produced by hydrolysis of peptide bonds involving an arginine and of peptides F (E100–K105), J (V170–K176) and K (A177–R183), produced by hydrolysis of peptide bonds containing lysine is unchanged. The yield of some peptides is reduced at pH 10 only. This is the case with peptides E (I49–K97), D–E (F33–K97), G–H (H106–K113) and L–N (D184–V209). At this pH, the hydrolysis of the bond K48–I49 is significantly diminished. As seen in the case of contiguous 'K–E' peptide bonds, these peptides are probably also engaged in formation of salt bridges and only vigorous deprotonation can make these sites accessible to trypsin.

Kinetics of hydrolysis at pH 8 by K188H trypsin

The kinetic study of hydrolysis of bovine β -casein by K188H trypsin with an E/S ratio of 0.005% (w/w) was realized at pH 8 and followed by RP-HPLC (Figure 3). Compared with hydrolysis of β -casein by wild-type trypsin, the kinetics of liberation of peptides are identical. The same is valid for the peptide maps obtained.

Influence of pH on the comparison yield of main tryptic peptides

Surprisingly, the analysis of the kinetics of hydrolysis of β -casein realized at pH 7, 8, 9 and 10 reveals that K188H trypsin, in contrast to wild-type trypsin, displays catalytic activity at pH 7 identical with that observed at pH 8 and 9 (Figure 4). On the other hand, mutant trypsin K188H shows a lower catalytic efficiency at pH 10 than that measured with wild-type trypsin, whatever kind of peptide bond (Lys–X or Arg–X) is aimed at. Kinetic studies revealed that K188H exhibits the same arginine/lysine preference as wild-type enzyme.

Molecular modelling

According to Amber force field, the wild-type and mutant trypsins have comparable energies without any steric conflicts

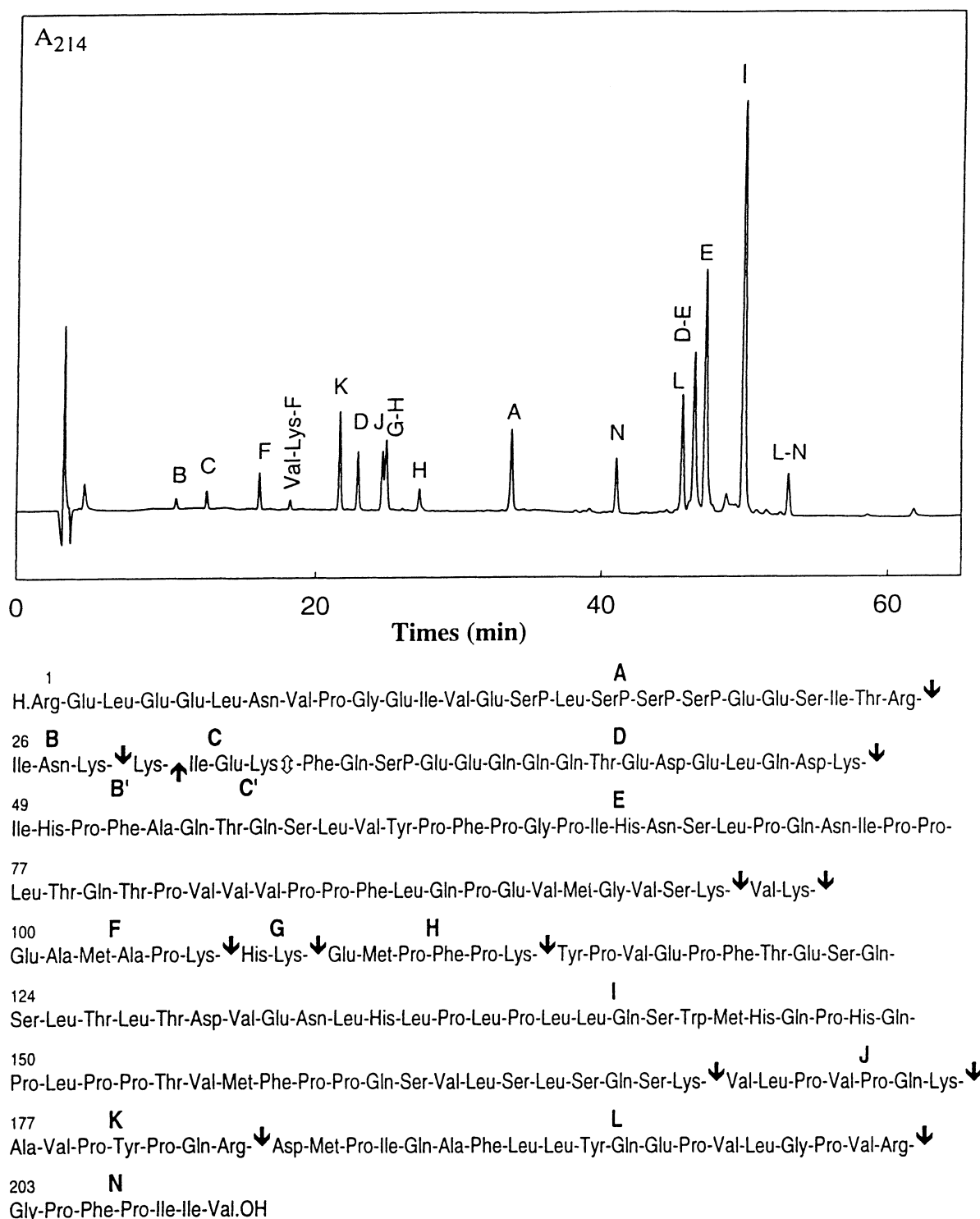


Fig. 2. HPLC profile of tryptic peptides from bovine β -casein and their localization in the sequence of the protein.

in both cases. When superimposing the two backbones, the root mean square deviation is very small (0.03 Å), which tends to confirm that without copper ion the side chain of the substituted histidine has the same orientation as that of lysine, pointing out of the substrate binding pocket where there is sufficient room.

However, in the presence of copper ion, this orientation is

inconsistent with biochemical results, as already explained before. Furthermore, the imidazole ring is too far from the binding pocket to explain the participation of the copper ion in the inhibition of this mutant trypsin. The proposed reorientation of the H188 side chain necessitates only a slight change in the backbone conformation of the two segments encompassing the binding pocket (represented schematically

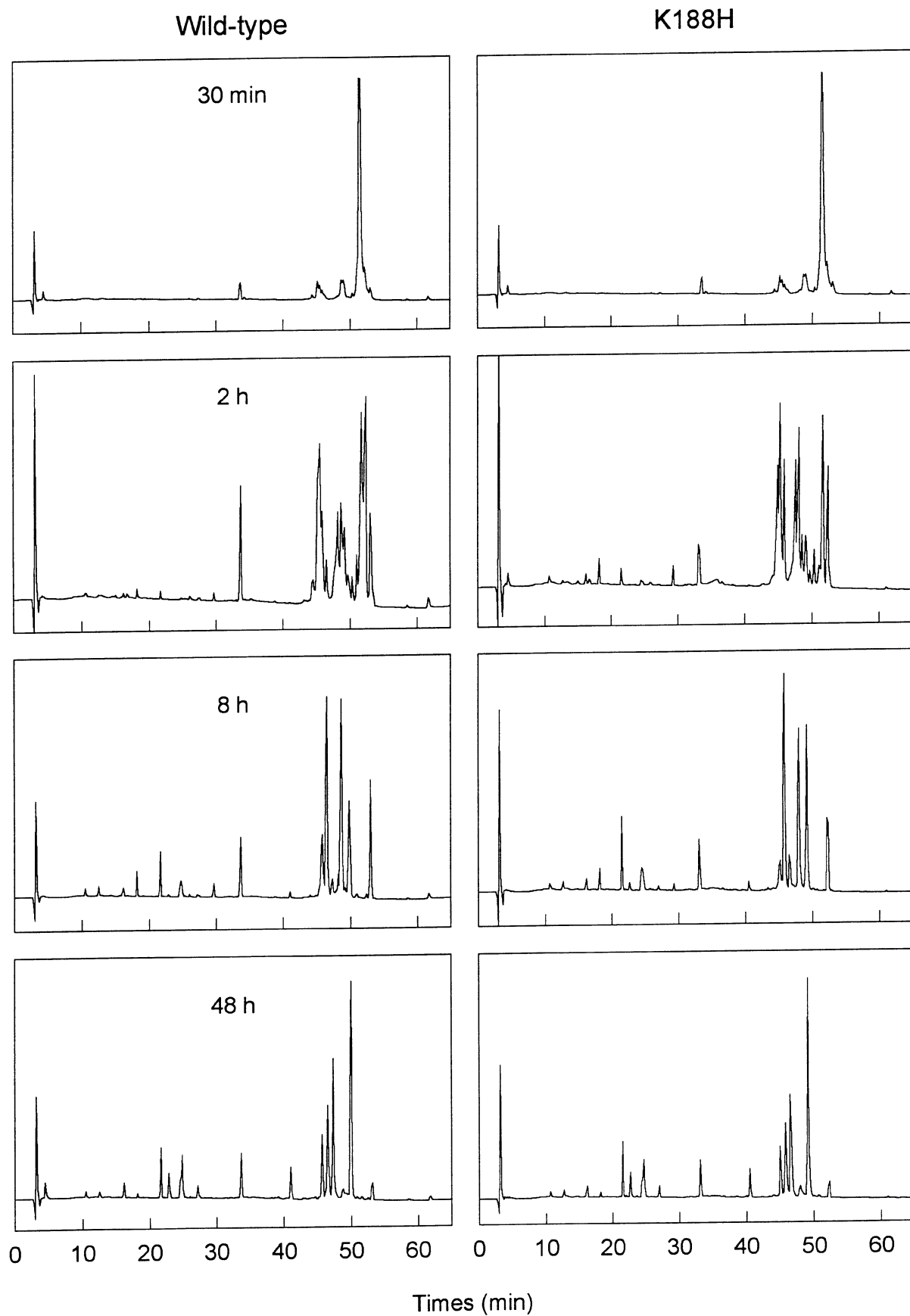


Fig. 3. HPLC profiles of tryptic peptides resulting from hydrolysis at pH 8 of β -casein with wild-type and K188H trypsins.

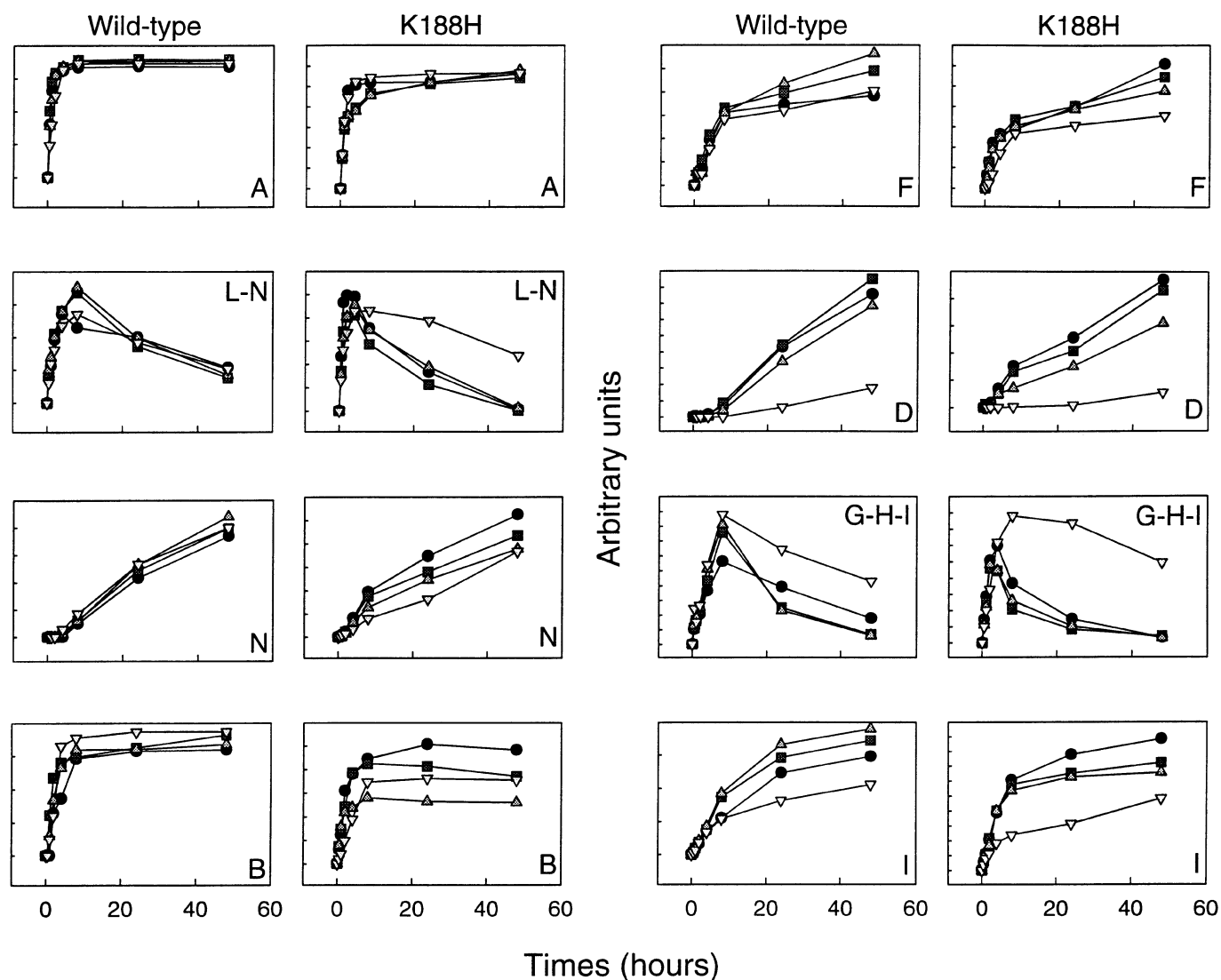


Fig. 4. Evolution of some tryptic peptides resulting from hydrolysis of β -casein at pH 7 (●), 8 (■), 9 (▲) and 10 (▽) with wild-type and K188H trypsins. Peptides are named as in Figure 2.

by a ribbon in Figure 5, left). This conformational change does not significantly modify the conformation of the other residues since no steric clashes were encountered during the minimization stages. As shown in Figure 5 (right), the proposed model of the copper complex has five coordinations involving in particular Asp189 and His188 residues. For the latter, in this new orientation of the imidazole ring, another nitrogen atom belonging to the backbone is involved in the chelation. For the putative copper complex, a six-coordination template with D_{4h} symmetry can be superimposed with only one missing ligand on the square base. As calculated with ESFF, the ligand–copper distances are 2.05, 2.17, 1.94, 1.96 and 1.91 Å for ND1 (His188a), N (His188a), N (Ala221), OD2 (Asp189) and N (Gly188), respectively. This complex model involves three contiguous residues of one segment delimiting the substrate binding pocket plus another residue of the second segment. No oxygen or nitrogen atoms are close enough to be attracted by the copper ion in the direction of the missing ligand, but there is a rather large cavity where a water molecule could be trapped.

Discussion

Transition metal cations can form several types of coordination complexes with lone pairs of electrons on oxygen, nitrogen and sulfur atoms. The potential variety of these complexes defies any attempts at classification. They can range from di- to octacoordinate complexes. Amino acid residues that function as good Cu^{2+} ligands are those containing electron-donor atoms (S, O or N). Although this group also includes amino acids containing non-ionizable groups (Trp, Ser, Met), the strongest interactions typically involve amino acid side chains with ionizable groups such as Asp, Glu, Cys and His. Since it is the greater basicity of the functional group (higher pK_a) that makes it the better ligand, the order of Cu^{2+} binding ability for these amino acids is Cys > His > Asp/Glu. Basic lysyl and arginyl residues rarely participate in metal binding and despite the fact that Cys forms such strong interactions with some ions, the rather scarce occurrence of free sulphydryl groups in proteins makes His the major amino acid residue involved in metal binding (Martell and Smith, 1974) and the one most commonly used in engineering of metal binding sites.

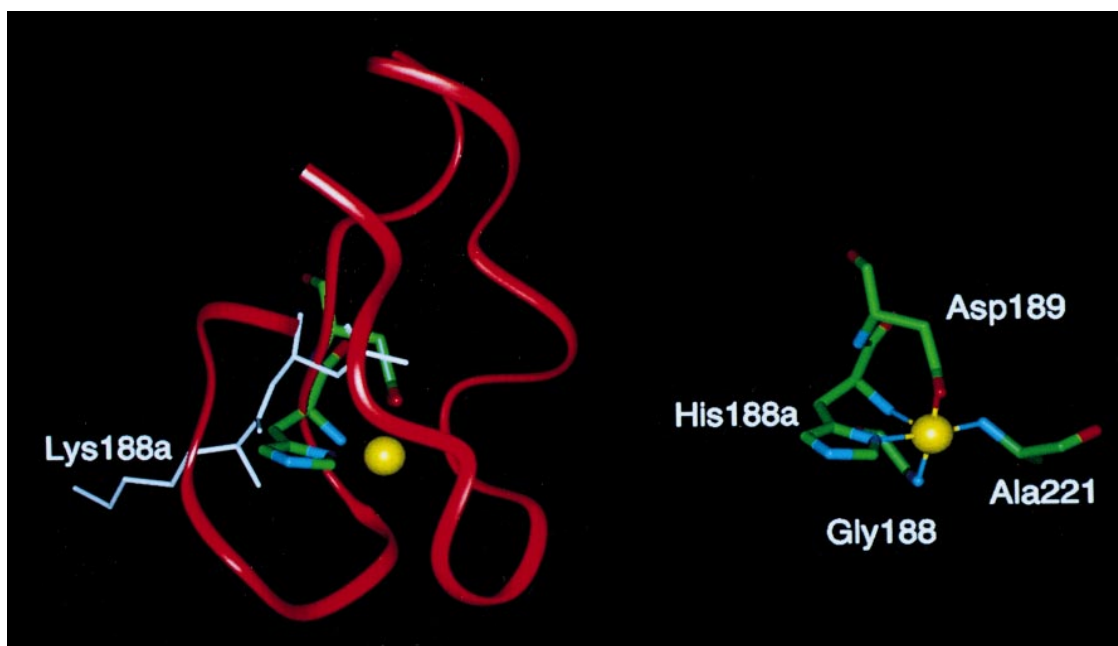


Fig. 5. Left: structure around trypsin substrate binding pocket. Two segments of the binding pocket (182–195 and 213–228) are represented by a ribbon. Lys188a side chain pointing out and Asp189 wild-type side chains are colored in white. Imidazole of His188a and Asp189 side chain in mutant trypsin are coloured according to the atom type code (N, blue; O, red). Cu²⁺ cation is yellow. Right: proposed Cu²⁺ chelation model involving ND1 (His188a), N (His188a), N (Ala221), OD2 (Asp189) and N (Gly188). Cu²⁺ cation is yellow. Amino acid labelling according to the X-ray crystal structure of Brinen *et al.* (1996).

Site-directed mutagenesis was used to engineer a metal-dependent regulatory active site in trypsin (Higaki *et al.*, 1990). The Asp189 residue, located at the bottom of the substrate binding pocket, interacts with the substrate arginine and lysine side chains and is essential for efficient catalysis (Graf *et al.*, 1987, 1988). In this context, a reversible inactivation of trypsin was aimed at by the substitution of the vicinal lysyl residue 188 with histidine. It was expected that $\Delta pK_a \approx 2$ between K/188/H and particular chelating properties of histidine would perturb efficiently the crucial D189 interactions with trypsin substrates.

The catalytic properties measured by hydrolysis of synthetic substrates indicate that the catalytic efficiency of the K188H trypsin mutant produced is even slightly higher than that of wild-type trypsin. The same can be seen for the arginine/lysine preference of the mutant.

Until now, the impact of pH on the catalytic activity of mutant trypsins had never been measured. Since the histidine imidazole side chain has a pK_a (6.5) much lower than that of the lysine side chain (~8.5), it was important to study the hydrolysis of a natural substrate at different pHs. Proteolysis of model substrates revealed that the trypsin mutant, in contrast to wild-type trypsin, has acquired catalytic activity at pH 7 identical with that measured at pH 8 and 9. These observations indicate that the presence of a lysine charge in position 188 plays an important role in ('upwardly') regulating the pH range of catalysis. Unfortunately, the hydrolysis of β -casein could not be followed at pH 6 or 8 in the presence of Cu²⁺ since this substrate precipitated under these conditions.

In contrast to the Cu²⁺-inhibited variant trypsin produced by Higaki *et al.* (1990) which showed, in the presence of Cu²⁺, a decrease in the k_{cat} values, the loss of activity in trypsin K188H is linked to an increase in K_m whereas k_{cat} is not influenced by this mutation. Consequently, it may be supposed that in trypsin K188H, the scissile bond is still in

the same (correct) position with respect to the catalytic triad during its hydrolysis in the presence of Cu²⁺. The structure of binding site produced cannot be compared with naturally occurring Cu²⁺ binding sites such as in azurin, lactoperoxidase or superoxide dismutase involving 2 His + Cys, 4 His and 2 His + Tyr residues, respectively. Also, Cu²⁺ binding to any element of substrate transition state is topologically unlikely. The lack of such interaction is confirmed also by the character of the kinetic data obtained (increase in K_m values signifying a drop in substrate binding specificity; constant k_{cat} values reflecting unchanged triad activity).

The goal of this molecular modelling study was not to search systematically for all possible copper complex conformations in this region but to propose a model (that could not be unique) consistent with the experimental data. In the present case, His188 plays an essential role by initiating a copper chelation which involves Asp189 in a side-chain conformation radically different from the wild-type trypsin one. Thus, this model suggests that the steric (as well as the electrostatic) contours at the bottom of the substrate binding pocket (near Asp189) is sufficiently modified to perturb drastically the catalytic reaction even if the catalytic triad is far from the fixation site of the copper ion. Hence the role of the bound copper cation consists rather in shutting up or sealing the substrate binding site or making it less accessible. Besides imidazole of H188 constituting the primary anchor of Cu²⁺ chelation and D189 carboxyl neutralizing one positive charge of Cu²⁺, only the nitrogens of the backbone appear as possible dentates. At this stage, it is also impossible to exclude water molecules as possible complementary chelation elements. If this was true, the harnessing by His188 of Cu²⁺ could strap the substrate binding site by the system of electrostatic and coordination bonds and it could immobilize one H₂O molecule. This would indicate the possibility of generating new peripherally (with respect to the catalytic site) metal-regulated proteases, still retaining excellent activity in the absence of metal.

The introduction of a coordination complex into the substrate binding pocket of trypsin could probably also be extended to other serine proteinases, esterases and possibly other enzymes of significant therapeutic and biotechnological importance. The combination of coordination chemistry and site-directed mutagenesis could thus be a method to form transitory quaternary structures forcing catalysis towards a halt or oriented activities.

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