Structure of an Engineered, Metal-Actuated Switch in Trypsin†

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ABSTRACT: The X-ray crystal structure of the copper complex of the rat trypsin mutant Arg96 to His96 (trypsin R96H) has been determined in order to ascertain the nature of the engineered metal-binding site and to understand the structural basis for the metal-induced enzymatic inhibition. In the structure, the catalytically essential His57 residue is reoriented out of the active-site pocket and forms a chelating, metal-binding site with residue His96. The copper is bound to the Nε2 atoms of both histidine residues with Cu-Nε2 = 2.2 Å and Nε2-Cu-Nε2 = 89°. The metal is clearly bound to a third ligand leading to a distorted square planar geometry at Cu. The X-ray results do not unambiguously yield the identity of this third ligand, but chemical data suggest that it is a deprotonated, chelating Tris molecule which was used as a carrier to solubilize the copper in alkaline solution (pH 8.0). Upon reorientation of His57, a unique water molecule moves into the active site and engages in hydrogen-bonding with Asp102-Oδ2 and His57-Nδ1. Except for small movements of the peptide backbone near His96, the remainder of the trypsin molecule is isostructural with the native enzyme. These data support the notion that the effective inhibition of catalytic activity by metal ions observed in trypsin R96H is indeed caused by a specific and reversible reorganization of the active site in the enzyme.

Metal ions frequently mediate important biological processes, and nearly two-fifths of all known proteins contain one or more metal atoms for binding small molecules, for facilitating electron transfer, for cross-linking and maintaining protein structure, for effecting catalysis, or for controlling important allosteric interactions (Ibers & Holm, 1980; Lipscomb, 1980). Although metalloproteins have been known and studied for decades, the molecular details of metal recognition and complexation by proteins are just now being understood to the extent that synthetic metal-binding sites can be successfully engineered into known protein frameworks. In designing new metalloproteins from existing proteins several important goals must be achieved. A sufficiently tight and metal-selective binding site must be constructed in such a manner that the metal-binding event will impart the desired new property or function to the protein.

The reversible disruption of an integral part of a catalytic site in an enzyme can lead to useful modulation of catalytic activity; this was the premise upon which this work was initiated. The design concept was to build into an enzyme a chelating metal-binding site (Higaki et al., 1990) which included two critical metal-binding residues (often histidine) so as to render the enzyme temporarily dysfunctional in the presence of the metal. The variant enzyme in the absence of metal was to be catalytically competent. The use of a chelating site, as opposed to a less avid monodentate one, was essential in order to attain metal-binding selectivity at a single site. This also allows for rapidly reversible "switching" at low metal ion concentrations, thus avoiding the potentially deleterious effects of metals at high concentrations. Recent work has discussed the control of enzymatic activity by the steric blocking action of a thiol or mercury(II) ion bound to a cysteine residue which was engineered into the substrate binding pocket of an enzyme (Corey & Schultz, 1989; Matsumura & Matthews, 1989). Our initial modeling studies were designed to find pairs or triplets of residues which when changed to histidine could lead to a multisite chelating interaction of the protein with a single metal. There was no need to mimic natural metal-binding sites but rather to find a site which satisfied the stereochemical demands of the target metal without suffering any unfavorable steric interactions. It was clear from studies on small ligands and peptides that the structural framework to which the histidine side chains were to be attached needed to possess a significant degree of conformational rigidity; otherwise, chelation was not likely to occur.

In an earlier work (Higaki et al., 1990), we reported the production of a variant of rat trypsin, trypsin R96H, whose catalytic activity was reversibly regulated by first row transition metals but which retained excellent activity in the absence of available metal. Many cycles of rapid "on and off switching" were carried out using copper(II)–Tris complexes to inhibit the enzyme and slight excesses of EDTA to reactivate the enzyme via complete sequestration of the copper. The apparent inhibition constant (K_i) of trypsin R96H by copper was found to be 21 μM. Our original intent was to disrupt the trypsin catalytic triad (Ser195, His57, Asp102) by metal-induced reorientation of His57. The present study was undertaken in order to understand the structural basis for the observed metal inhibition and to ascertain the nature of the His96, His57, and Cu interactions. We report here the X-ray crystal structure of trypsin R96H at pH 8.0 in the presence of $CuCl_2$ and small amounts of Tris carrier/ligand, which was needed to solubilize the copper.

EXPERIMENTAL PROCEDURES

Trypsin R96H was expressed and purified as described previously (Higaki et al., 1990) and crystallized by hanging drop vapor diffusion using 22% (w/v) $MgSO_4$ as the

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precipitating agent buffered with 20 mM Tris-HCl at pH 8.0 (McGrath et al., 1989). With mixed results, we attempted to soak copper (as $CuCl_2$) into trypsin R96H crystals at various concentrations and under different conditions. The relatively high concentration of Tris in these crystals caused experimental problems stemming from the high affinity of this buffer for copper ion. Using known and reliably estimated equilibrium constants, species distribution calculations were performed which simulated the conditions of crystallization, copper-soaking, and enzyme assay (Bai & Martell, 1969; Bologni et al., 1983; Fischer et al., 1979; Hall et al., 1962; Hanlon et al., 1966). The results indicated that 1:1 $CuCl_2$–Tris mixtures near 1 mM in concentration should provide ample copper for complexation with trypsin R96H. The best X-ray diffraction data were obtained on crystals in which Tris was soaked out of the crystal and replaced with the buffer *N*-(2-hydroxyethyl)-piperazine-*N'*-3-propanesulfonic acid (HEPPS), which has a lower binding affinity for copper ion and is an effective buffer at pH 8.0. The crystals were subsequently soaked to a final concentration of 1 mM $CuCl_2$, 5 mM HEPPS, and 1 mM Tris.

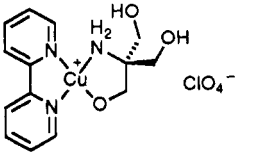
Data were reduced using the Buddha (Blum et al., 1987) crystallographic data processing package. The initial model against which the data were scaled was a modified refined structure of trypsin S214K at 2.2 Å (McGrath et al., 1992). The side chains of the catalytic triad residues (Asp102, His57, Ser195) as well as that of His96 (and Lys214) were replaced with Ala, and the solvent molecules and pseudosubstrate benzamidine were removed prior to stereochemically restrained least-squares refinement with PROLSQ (Hendrickson & Konnert, 1980) in order to minimize phase bias. After several cycles of refinement and electron density map calculation, missing side chains were fitted, and the structure was refined. The starting atomic coordinates for Tris were retrieved from the Fine Chemicals Database (#4397). After some initial refinement, the process was continued with the XPLOR (Brunger et al., 1987) refinement package, which allowed us to exploit the features of simulated annealing with restraints on the Cu-ligand moiety.

A good copper force field should retain the ligation state of the metal during minimization and annealing steps without strongly biasing the ligation geometry. XPLOR uses a harmonic force field which includes bond stretching, angle bending, torsion, van der Waals, and electrostatic terms. This presents two options for copper force field development, one using only van der Waals and electrostatic terms and the other including bond and angle connectivity as well. The simplicity of the first is appealing but gives rise to two difficulties. First, the balance between electrostatic terms in the force field is important but the effect of d orbital delocalization on the partial charge of the copper is unknown. Second, only two copper ligands are contributed by the protein: the rest are solvent molecules. Without connectivity terms, the copper position may drift during early stages of the refinement prior to the incorporation of solvent (as initially observed).

Parameters were generated using seven X-ray structures for copper-binding proteins (one copper/molecule) from the Brookhaven Protein Data Bank (Bernstein et al., 1977) [PDB IDs: azurin, 2AZA (Baker, 1988); pseudoazurin, 1PAZ (Petrakos et al., 1988) and 2PAZ (Adman et al., 1989); plastocyanin, 1PCY (Guss & Freeman, 1983), 4PCY (Garrett et al., 1984), 5PCY (Guss et al., 1986), and 7PCY (Collyer et al., 1990)]. The bond and angle reference values for the metal–ligand assembly were set equal to the mean geometries found in the crystal structures. The harmonic force constants were selected such that a displacement from the reference value equal to twice the standard deviation (σ = 0.5 Å for bonds, σ = 10° for angles) would require 1.0 kcal/mol for bonds and 0.5 kcal/mol for angles. The relative bond–angle weights are consistent with the XPLOR polar hydrogen force field for proteins. The copper charge was set to +1, and the copper van der Waals radius was set to 1.4 Å (Weast, 1971). Refinement sensitivity to the values chosen for partial charges is reduced because the electrostatic term is only calculated for atoms separated by three or more bonds. Molecular parameters for Tris were calculated analogously. Reference bond lengths and bond angles were taken from crystallographic data (Ivarsson, 1984); corresponding force constants were calculated so that 0.1 Å and 10° deformations require 1.0 and 0.5 kcal/mol, respectively. Since no dihedral terms containing both Tris and protein atoms were defined, no assumptions are made about the orientation of the Tris with respect to the protein. (The XPLOR parameter set is available from the authors.)

RESULTS AND DISCUSSION

Copper Species in Solution. Preliminary experiments showed the need for an effective carrier for copper, one which would form soluble, stable complexes of copper in a slightly alkaline solution (pH 8.0), yet which would interefere minimally with the metal–protein interactions that are necessary for the "switching" functions. Free copper(II) as $CuCl_2$ (10–1000 μM) at pH 8.0 exists almost exclusively as the copper hydroxy species, of which most are insoluble in water. Several potential carrier ligands were tested; the common pH buffers, Tris [tris(hydroxymethyl)aminomethane] and serinol, satisfied the necessary requirements and performed well in our system. In order to understand the nature of the copper–enzyme complexes in solution, we studied the mixed-ligand complexes of Tris and the chelating nitrogen ligand 2,2'-bipyridyl and found the principal species, Cu(Bipyridyl)-(Tris-H)$^{1+}$, to be monomeric, stable, and well-behaved in aqueous solution at pH 8.0 (unpublished results).



Cu(Bipyridyl)(Tris-H)$^{1+}$

To evaluate how well the engineered metal-binding site in trypsin binds copper ions and to be able to judiciously plan experimental procedures, it was useful to have semiquantitative information regarding the amounts of various copper species in solution. Using known values or reliable estimates of binding constants (K_f) for Cu^{2+} and H^+ reacting with Tris, OH^-, and HEPPS buffer, species distribution of copper complexes in solution were calculated. The principal copper species in solution is $Cu(OH_2)_2$(Tris-H)$^{1+}$ under the conditions of the enzyme assay (1 mM Tris, 2–200 μM Cu^{2+}, pH 8.0). Under conditions of incipient crystallization (450 μM trypsin R96H, 1 mM Cu^{2+}, 1 mM Tris, and 5 mM HEPPS), similar calculations showed that almost all of the enzyme is metallated (~99%) and that the principal species is Cu(enzyme)(Tris-H)$^{1+}$ (81%) (unpublished results).

Modeling and Design Concepts. Simple molecular modeling calculations indicated that a chelating dihistidine site could be constructed involving the active site His57 residue and produced reasonable models of the actual metal-binding site

Table I: Modeling Results for His57-His96 Site with 2.0 Å Cu-Nε2 Distances

region	His57 χ1	His 57 χ2	His96 χ1	His96 χ2	av angle Nε2-Cu-Nε2
1	−157 ± 1	−66 ± 4	−182 ± 1	−134 ± 6	94
2	−159 ± 1	−75 ± 1	−155 ± 1	−238 ± 1	75
3	−147 ± 12	−183 ± 49	−181 ± 2	−57 ± 11	87

Table II: Crystal and Diffraction Data for Trypsin R96H

space group	*I*23
cell dimensions (Å)	*a* = 124.4
molecules per asymmetric unit	1
data measurement	Xentronics
resolution (Å)	2.3
total observations	26 500
unique reflections	13 200
R_{symm}[a]	0.07
Hendrickson–Konnert refinement	32 cycles
XPLOR refinement	Powell minimization temperature factor simulated annealing
R_{cryst}[b]	0.161
resolution (Å)	7.0–2.3
rms differences	
bond (Å)	0.013
angle (degrees)	2.9
av temperature factor (Å²)	15.0
copper occupancy	96%
no. of putative solvent molecules	141

[a] $R_{symm} = \sum_h \sum_i |\langle F_h \rangle - F_{hi}| / \sum_h F_h$ where $\langle F_h \rangle$ is the mean structure factor amplitude of the i observations of reflections that are related to the Bragg index h. [b] $R_{cryst} = \sum ||F_o| - |F_c|| / \sum |F_o|$ where F_o and F_c are the observed and calculated structure factor amplitudes, respectively.

in the mutant trypsin enzymes. Using the structure of trypsin D102N (Sprang et al., 1987) as a starting point, conformational searches were carried out on pairs of histidine residues in which active-site His57 was one member of the pair. Preliminary calculations indicated His57 to be within chelating distance of a histidine residue at positions 96 or 99. Detailed calculations with histidine at position 96 showed that it satisfied the requirements of cis-chelation to a first row transition metal, such as copper. The torsional angles χ1 and χ2 for His57 and His96 were systematically varied while steric overlap and metal geometry were evaluated. Three conformational regions were found in the χ1-χ2 maps where both Nε2 nitrogens could simultaneously bind to copper (Table I). Chelation involving Nδ1 of either histidine was found to be excluded for steric reasons. The modeling results showed that trypsin R96H could accommodate a ligated copper ion with two equal Cu-Nε2 distances of 2.0–2.1 Å and reasonable His57Nε2-Cu-His96Nε2 angles (80–100°). Comparison with the X-ray data shows that region 3 of the modeling results (Table I) yielded a reasonable picture of the metal-bound His57 residue; χ1 and χ2 of His96 were also in agreement but slightly out of range owing to the movement of the peptide backbone upon metal binding.

Crystallographic Results. Details of the X-ray data set are provided in Table II. Early attempts at soaking $CuCl_2$ into crystals of trypsin R96H met with mixed results and limited success. In a background of 35 mM Tris (pH 8.0), 20 mM $CuCl_2$ led to crystals with noticeably increased mosaicity. A total of 10 mM $CuCl_2$ seemed to give better results while 5 mM $CuCl_2$ led to incomplete metal occupancy. Model calculations of species distributions on trypsin R96H/$CuCl_2$/Tris/OH^- system showed that lower Tris concentrations (0.5–2.0 mM, pH 8.0) would adequately keep the copper in solution while minimally interfering with the copper–enzyme interactions. When the original buffer was soaked out of the crystals and replaced with 5 mM HEPPS and 1 mM Tris, it was possible to fully occupy the copper binding site with only 1 mM $CuCl_2$. The X-ray data statistics as well as the initial electron density map indicated that this was the best data set. Consequently, these data were used for structure determination. The electron density map (Figure 1a) showed strong density for the omitted His57 and His96 side chains and a distorted sphere of density, corresponding to a copper ion, equidistant between the His57 and His96 imidazolyl groups. The His57 side chain had clearly rotated about the Cα-Cβ bond to a position out of the active site. The

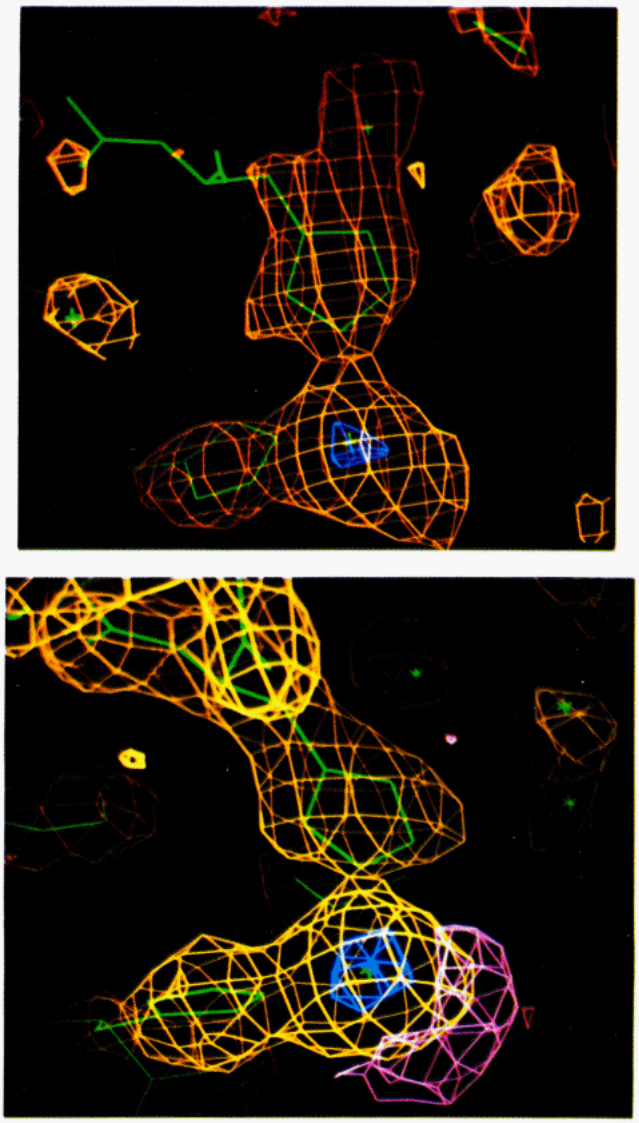


FIGURE 1: (a, top) An electron density map ($F_o - F_c$) was calculated from a starting model for trypsin R96H where His57 and His96 were replaced with alanines and subjected to four cycles of Hendrickson–Konnert refinement prior to placement of the omitted residues. To facilitate viewing, the contours are shown along with the residues (in green) which were later fitted to this density. His57 is shown at the top center and His96 at the lower left. The copper atom is shown as a cross at the bottom center of the photo. The contour levels used to fit these residues were 2σ, shown in orange, and 9σ, shown in blue. At the top center of the photo is the density corresponding to the water molecule which replaces the His57 side chain in the active site. The position of the fitted water molecule is shown as a green cross. (b, bottom) The coordinates for the refined structure of the copper-binding site in trypsin R96H are shown in green. The view is almost identical to that in (a). The electron density ($2F_o - F_c$) is contoured at 1.3σ (orange) and also at 6.5σ (blue) to show the copper atom. The new water molecule at the active site, described above, is faintly visible at the top center of the figure. Electron density which is not accounted for in this model of trypsin R96H ($F_o - F_c$), and which corresponds to the third copper ligand, is shown in pink, countoured at 2σ. FRODO was used to produce this figure.

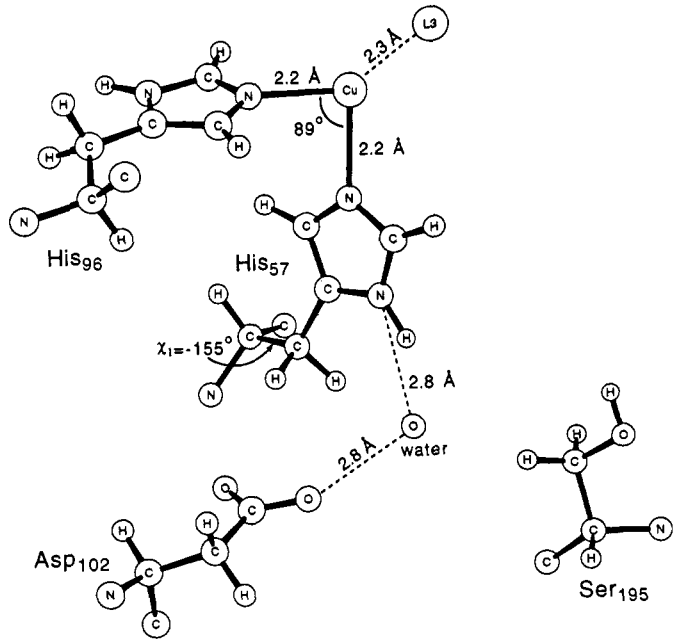


FIGURE 2: Geometry at the copper-binding site in trypsin R96H. The view differs from Figure 1. The third ligand is represented by L3, which lies 0.7 Å above the His57Nε2-Cu-His96Nε2 plane. The water molecule at the active site is shown in relation to the catalytic triad.

side chain of His96 was shifted several angstroms closer to the active site and to the reoriented His57. Although the electron density map was contoured at 2σ to fit the histidine side chains, a contour level of 9σ was used to place the heavier copper ion. Discrete, spherical density (Figure 1a) corresponding to one water molecule was seen between Asp102 and reoriented His57. Excess electron density was also clearly evident near the copper ion across from the two histidine ligands and suggested the presence of a third ligand (Figure 1a). Refinement commenced with only His57 and His96 as ligands. After positional and temperature factor refinement with PROLSQ (Hendrickson & Konnert, 1980) followed by simulated annealing, positional and temperature factor refinement with XPLOR, density for the putative third ligand had improved (Figure 1b) and a chelating Tris molecule was fitted to this site and refined with XPLOR.

Description of the Structure. The principal structural differences between trypsin R96H in the presence of 1 mM copper and native rat trypsin (unpublished results) are $\chi 1$ and $\chi 2$ angles of the metal-binding residues His57 and His96 and the main chain in the loop region containing His96. When the two structures are superimposed using positions of Cα atoms for the entire 223 residues of the protein, small deviations are observed in Cα positions for residues 94–98. This deviation in the position of the main chain at residue 96 amounts to 0.6 Å and serves to bring His96 closer to His57, which facilitates copper binding.

A single, localized, and fully occupied (approximately 96%) copper-binding site is clearly evident in the metallated enzyme. No other fully or partially occupied sites are seen. The copper in trypsin R96H is attached to imidazolyl groups at His57 and His96 (Figure 1b). His57 is rotated about Cα-Cβ from a gauche conformation ($\chi 1 = 79°$, $\chi 2 = 83°$) with the imidazolyl group in the active site between Asp102 and Ser195 to a trans conformation ($\chi 1 = -155°$, $\chi 2 = -163°$) with the imidazolyl group out of the active site and bound to copper through Nε2. The rotation of His57 allows the entry of a water molecule, which engages in hydrogen-bonding to both Nδ1 of His57 (2.8 Å) and to Oδ2 of Asp102 (2.8 Å) (Figure 2). It is uncertain if or how this hydrogen-bonding network might stabilize the observed conformation in the metallated enzyme. His57 occupies its normal position (the less common gauche conformer) in the active-site pocket in most structures of serine proteases, but is displaced to the trans conformer in rat trypsin D102N at pH 6.0 (Sprang et al., 1987), in rat tonin with zinc present (Fujinaga & James, 1987), and in a *Streptomyces griseus* protease A complex with an aldehyde inhibitor (James et al., 1980). In the native enzyme, the surface Arg residue at position 96, though partly disordered in crystal structures, is seen to extend out into the solvent. However, in metallated trypsin R96H, His96 reaches toward the copper ion and His57.

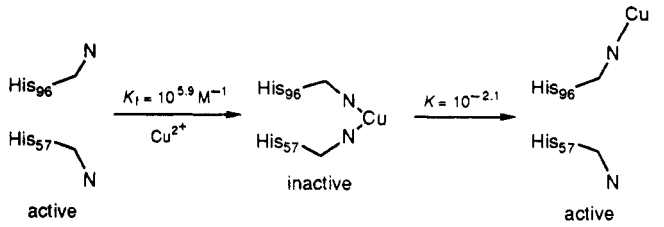
In general, the metal ion in trypsin R96H displays a geometry typical for copper(II) with cis coordination (His57Nε2-Cu-His96Nε2 = 89°) of the two histidine ligands which are ligated through Nε2 nitrogen atoms (Figure 2). Copper–nitrogen distances at 2.2 Å (His96) and 2.2 Å (His57) though longer than the expected distances (2.0–2.1 Å) in small complexes are not atypical of copper–imidazole distances in Cu–Zn superoxide dismutase, plastocyanin, and azurin (Chakrabarti, 1990). The orientation of the imidazole rings with respect to the copper ion shows that the metal is situated in the imidazole planes and near the expected positions of the lone pairs of electrons on the Nε2 atoms. Copper is out of the His57 and His96 planes by 4° and 9°, respectively ($90–\theta$) (Chakrabarti, 1990), and deviates from ideality within the imidazole planes by 1° and 8°, respectively (ϕ) (Chakrabarti, 1990). The $\chi 1$ angles for both histidines are in the normal range for trans conformers (His57 = –155°; His96 = –165°). However, the $\chi 2$ angles are less common (His57 = –163°; His96 = 152°), and the imidazole planes of residues 57 and 96 are 17° and 28° away from being coplanar with the respective Cα-Cβ-Cγ planes. These "pseudoparallel" orientations are slightly less favored than the pseudoperpendicular arrangement owing to minor steric hindrance between ring atoms and hydrogen atoms on Cβ.

"Banana-shaped" density corresponding to a third copper ligand is clearly evident from the electron density maps (Figure 2b) and is not an artifact of Fourier ripples from the copper ion. Refinement of this ligand as a water molecule produced a distorted trigonal planar geometry, but one water molecule accounted for neither the amount nor the shape of the electron

density. Species distribution calculations for copper complexes strongly suggested the possibility of a metal-bound, deprotonated Tris molecule which is known to form stable *N,O*-bidentate chelating complexes with copper(II) (Ivarsson, 1984). The refinement of the third ligand as a chelating Tris molecule was attempted with the two possible orientations as initial configurations. Tris was placed 0.7 Å above the His57Nϵ2-Cu-His96Nϵ2 plane resulting in distorted square planar geometry for the copper. After refinement, the resultant models had high temperature factors (B = 45–60 Å^2) for individual atoms of the Tris molecule and less than satisfactory electron density ($2F_o - F_c$) in this region. The Tris model was better than the water representation, but no completely satisfactory structural model was found for the third ligand. Although it is possible that different species could be attached to the copper at this site, on chemical grounds we believe this is unlikely and that partial occupancy, orientational disorder, and excessive mobility have resulted in poor electron density for the Tris ligand.

Our engineered metal-binding site is attached to the protein by only two residues and is more solvent accessible than known copper and zinc binding sites in metalloproteins in which the metal is attached to the protein through three or more residues (Adman et al., 1989; Baker, 1988; Collyer et al., 1990; Fujinaga & James, 1987; Guss & Freeman, 1983; Holmes & Matthews, 1982; Kannan et al., 1984; Rees et al., 1983; Tainer et al., 1982). Based on structures of natural metalloproteins, various workers have speculated that extended secondary stabilization of a metal-binding site is possible (1) by interdomain cross-linking via hydrogen bonds (Roberts et al., 1990), (2) by surrounding the metal-binding site with a shell of hydrophobic side chains (Yamashita et al., 1990), and (3) by specific carboxylate (Asp, Glu) hydrogen-bonding to a metal-bound histidine (Christianson & Alexander, 1990; Nakagawa et al., 1981). Other than the serendipitous H-bonded water molecule linking His57 and Asp102, there is no secondary stabilization in the present structure, and yet the metal switch in the enzyme functions well. Future, more sophisticated designs of synthetic metal-binding sites in proteins might include such additional features of secondary stabilization.

How well designed is the His57-His96 chelating site from the standpoint of metal-binding strength? Several useful observations can be made. (1) Trypsin R96H chelates to the bound copper with His57 and His96 which reorient themselves to accommodate the metal. (2) Any other potential metal-binding site in trypsin R96H is likely to be at least 2 orders of magnitude weaker in binding, and no additional copper site was observed in the crystal structure. (3) The estimated binding constant of aquated Cu^{2+} to trypsin R96H of $10^{5.9}$ M^{-1} indicates good metal–protein binding with both histidine residues interacting with the copper. However, this binding constant is about 3 orders of magnitude weaker than what might be expected for an analogous small chelate [negative chelate effect (Fauso da Silva, 1983; Hancock & Martell, 1988)]. The reduced copper–trypsin binding strength is partly caused by the net loss of hydrogen bonding when His57 rotates out of the active site; whatever energy cost there is to disrupt the hydrogen bond to Asp102 and to a lesser extent, the bond to Ser195, in order to reorient His57 for metal binding reduces the overall binding constant for copper at this site. (4) A consequence of the negative chelate effect is that small but measurable amounts of a fully metallated yet catalytically active species can exist in solution: the copper would be bound only to His96 in a monodentate fashion, and His57 is in its normal catalytically competent position.



If the binding of Cu to His96 is estimated to be about $10^{3.8}$ M^{-1}, then a bidentate-to-monodentate equilibrium constant of $10^{-2.1}$ would result (above), and about 1% of the metallated protein would remain an active catalyst. These considerations place a fundamental limitation on how completely enzymatic activity can be "switched off". As part of the general design problem, it is not only important to create a tight metal-binding site which does not adversely effect catalytic activity but also one which reduces the dissociation of critical residues. A tighter metal-binding site (smaller K_i) does not necessarily mean that enzymatic activity can be "switched off" to a greater degree under conditions of full metal saturation. (5) High concentrations of copper (~2mM) can partially inhibit the enzymatic activity of native rat trypsin. It is possible that copper can bind to His57 or to His57 plus Asp102 in native rat trypsin; however, it is uncertain whether the loss of enzymatic activity results from specific metal binding to His57 or from global, metal-induced denaturation of the protein.

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