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The mechanistic role of the coordinated tyrosine in astacin

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Abstract

Crayfish astacin belongs to the *only* Zn protein family containing a coordinated Tyr ligand in the active site, and is a rare example of Zn enzymes whose activity can be significantly restored by Cu^{2+}. The highly active Co^{2+} and Cu^{2+} derivatives of astacin can serve as good models of the native enzyme for structural and mechanistic studies by means of optical and magnetic resonance techniques. Upon the introduction of the inhibitor Tyr-hydroxamate to these two metal derivatives of astacin, the coordinated Tyr in the active site is detached based on our optical and NMR studies in solution. The significance of the detachment of the coordinated Tyr in the action of astacin is fourfold: (1) to enhance the Lewis acidity of the active site metal, (2) to balance the negative charge of the transition state *gem*-diolate-enzyme complex, (3) to relieve the steric crowding upon substrate binding, and (4) to stabilize the enzyme-substrate complex by way of a H-bonding.

Keywords: Astacin; Metalloprotease; NMR; Co(II); Cu(II)

1. Introduction

Crayfish astacin is a unique small Zn^{2+} endopeptidase (MW = 22 kDa) found in the digestive fluid of crustaceans, and belongs to the *only* Zn protein family that contain a coordinated Tyr under physiological conditions [1]. However, the presence of a negatively charged phenolate, i.e., the coordinated Tyr, is known to decrease the Lewis acidity of the metal ion in metal complexes [2,3]. As a result, a decrease in water activation by ~2 orders in terms of the pK_a of the coordinated water has been observed in metal complexes. This decrease in Lewis acidity of the metal by a coordinated phenolate should presumably result in a significant decrease in metal-centered hydrolytic activity. On the contrary, this is not the case in phenolate(Tyr)-coordinated astacin which still exhibits high hydrolytic activity under neutral conditions. Hence, the coordinated Tyr may not act to lower the Lewis acidity of the active-site metal in astacin *during its catalysis*, but may play some unique roles in the action of astacin that have not yet been observed in other metalloproteases. A better understanding of the roles of this coordinated Tyr is essential to provide further insight into the catalysis of astacin family.

Several zinc proteins [4–6], such as bone morphogenetic protein-1, hydra metalloprotease 1, the embryonic hatching proteins from medakafish and sea urchin, and the alkaline endopeptidases from *Serratia* and *Pseudomonas* species have been revealed by means of sequence alignment to contain conserved metal binding residues as in astacin, i.e., three-His residues found in the consensus sequence **HEXXHXXGXXH** and 1 Tyr in a loop containing Met-His(or Ser)-**Tyr**. The three-His sequence is also found to be the consensus sequence in snake venom proteases and collagenases [4–6]. Since astacin is the simplest member in this Zn protease family, it may serve as a prototype which may offer us the opportunity to study and understand the mechanism of this new protease family.

Despite the high Lewis acidity of Cu^{2+} ion [2,3,7], almost all Cu^{2+}-substituted derivatives of Zn^{2+} hydrolytic enzymes are inactive [7]. A few Cu^{2+}-substituted derivatives of *Aeromonas* aminopeptidase were determined to exhibit "super-activities" compared with the native enzyme toward some poor substrates, although these activities are still far below the activity of the native enzyme toward the most efficient substrate [8]. Cu^{2+}-substituted astacin exhibits surprisingly high activity against the tripeptide substrate succinyl-tri-Ala-nitroanalide (37% that of the native enzyme in terms of k_{cat}/K_m [9]), thus deserves a thorough study to shed light on this unique Cu^{2+} activation of this hydrolytic

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enzyme. Conversely, full activities are usually observed in the case of Co^{2+}-substituted derivatives of Zn enzymes, such as astacin [9], and are occasionally higher than those of the native enzymes [7,9]. Due to the presence of unpaired electrons in Cu^{2+} and Co^{2+} ions, various spectroscopic and magnetic techniques can be applied to the study of their binding environments in proteins. Thus, Cu^{2+}- and Co^{2+}-substituted derivatives of astacin can serve as very good model systems for the study of astacin action by means of spectroscopic and magnetic techniques.

Amino acid and peptide hydroxamates are very potent inhibitors for Zn proteases [10–12], including astacin [13,14], and have utilized as probes for mechanistic studies of these proteins. In this report, we present our studies of the highly active Cu^{2+} and Co^{2+} derivatives of astacin from Louisiana crayfish (*Procambarus* spp.) and their binding with the inhibitor Tyr-hydroxamate in solution by means of electronic and magnetic resonance spectroscopies. Based on the results from these studies, displacement of the coordinated Tyr upon the inhibitor binding is concluded. The implication of this ligand displacement during astacin action is discussed.

2. Experimental section

The isolation of astacin from Louisiana crayfish (*Procambarus* spp.) followed the procedures for the preparation of astacin from European crayfish (*Astacus astacus*) with some minor modifications [1]. Briefly, about 60 ml digestive fluid could be extracted from ~4 kg *Procambarus* crayfish, from which astacin was first absorbed by DEAE-cellulose that was added directly into the digestive fluid; then the enzyme was eluded with 2M NaCl. After further concentrating, passing through a 1-m size-exclusion column (Sephadex G50) and then a DEAE-Sephacel column with a 0.2–2 M salt gradient, about 20 mg astacin was obtained. The purity of the enzyme was determined to be >95% by SDS-PAGE. Metal derivatives of *Procambarus* astacin were prepared by first removing the native Zn from the enzyme (~50 μM) with 10-mM 1,10-phenanthroline in 50 mM HEPES buffer at pH 7.5 containing 0.5 M NaCl, removing the chelator by thorough dialysis against the buffer, then followed by dialysis of the apo enzyme against 1 mM metal ion or by direct addition of stoichiometric amount of metal ion. The Co^{2+} derivative is sensitive to the air, and loses its activity in about two months when exposed to the air. A sample in degassed buffer solution under argon can retain its activity for at least six months. The activities of astacin and its metal-substituted derivatives were measured against 4-mM succinyl-tri-Ala-p-nitroanalide (STANA, Sigma Chemical) in 0.1 M HEPES buffer at pH 8.0 [1].

Optical measurements of Cu^{2+}-astacin and its titration with the inhibitor Tyr-hydroxamate (Tyr-NHOH, Sigma Chemical) were carried out on a Varian Cary 3 spectrophotometer at 30°C. X-band EPR spectra were acquired on a Bruker ER200 spectrometer at UCLA. The ligand (2-hydroxybenzyl)bis(2-pyridylmethyl) amine (HBPA), which contains a phenolate moiety that mimics the coordinated Tyr in astacin, was synthesized according to the literature [15]. All the chemicals used were purchased from Sigma, Aldrich, or Acros.

The Co^{2+}-substituted astacin samples for NMR studies were further concentrated to ~1 mM by ultrafiltration, then transfer to NMR tubes, capped with rubber septa, degassed and stored under argon. These samples did not show noticeable change in their NMR spectra in six months. All the ^1H NMR spectra were acquired on a Bruker AMX360 at 360.13 MHz and 298 K. A 90°-pulse was used for data acquisition (8 K data points and a spectral window of ~250 ppm) with a presaturation pulse for solvent saturation and a total recycle time of ~100 ms. The selective hard-pulse sequence 1-*3*-3-*1* [16] was used for data acquisition to reveal fast solvent exchangeable signals. A Gaussian window function with a line-broadening factor around −50 Hz and 5–10% shift was applied to the FIDs prior to Fourier transformation.

3. Results and discussion

The formation of the Cu^{2+}-substituted derivative of *Procambarus* astacin can be monitored by both electronic and EPR spectroscopies. The EPR spectrum of this derivative (Fig. 1(A)), with $g_{\|} = 2.29$, $g_{\perp} = 2.07$, and $A_{\|} = 11.2 \times 10^{-3}$ cm^{-1} for the major species (see later), is similar to that of the Cu^{2+} derivative of *Astacus* astacin [9]. The g values of $g_{\|} > g_{\perp}$ reflect that the Cu^{2+} center has a tetragonally distorted octahedral geometry with the unpaired electron residing in the $d_{x^2-y^2}$ orbital, although the $A_{\|}$ value is in the lower margin for such a geometry [17]. The crystal structure of the Cu^{2+} derivative of *Astacus* astacin reveals a trigonal bipyramidal

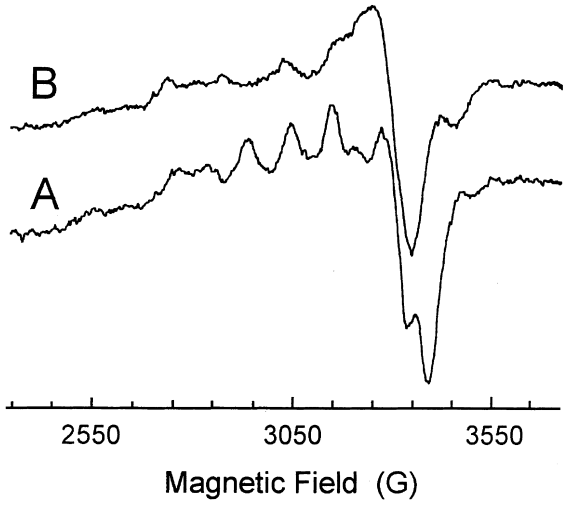

Fig. 1. X-band EPR spectrum (9.55 GHz and 90 K) of Cu^{2+}-substituted *Procambarus* astacin (A) and in the presence of ~4 equivalents Tyr-NHOH (B) in 0.1 M MES buffer at pH 6.1.

Cu^{2+} coordination sphere in which the unpaired electron resides in the d_{z^2} orbital. This geometry and electron configuration would show an EPR spectrum with $g_{\|} < g_{\perp}$ as observed in several trigonal bipyramidal Cu^{2+} complexes [18,19]. The EPR spectrum of Cu^{2+}-astacin suggests that the active site Cu^{2+} may have a distorted octahedral coordination sphere in solution. Upon addition of the inhibitor Tyr-NHOH, the EPR spectrum is changed, with $g_{\|} = 2.31$, $g_{\perp} = 2.07$, and $A_{\|} = 15.1 \times 10^{-3}$ cm^{-1} (Fig. 1(B)). The change of the spectrum indicates that the inhibitor is directly bound to the Cu^{2+} in the active site. All the EPR parameters of this enzyme-inhibitor complex fall in the normal region for a tetragonally distorted octahedral Cu^{2+} center [17].

The EPR spectrum of *Procambarus* Cu^{2+}-astacin reveals a mixture of two different species, similar to that observed in *Astacus* Cu^{2+}-astacin [9]. This cannot be rationalized by the purity of the enzyme based on SDS-PAGE and NMR spectrum, but may be possibly attributable to two slightly different active site configurations that are under equilibrium, such as protonation–deprotonation of the coordinated Tyr. The presence of a pH-dependent "mixture" in the EPR spectrum of a Cu^{2+} center in proteins has been observed previously, such as in that of Cu^{2+}-substituted isopenicillin N synthase [20]. However, the inhomogeneity is seemingly removed upon inhibitor binding (Fig. 1(B)), suggesting that the mixture is due to the presence of an equilibrium. More thorough EPR studies of Cu^{2+}-substituted derivatives of astacin and the endopeptidase from *Serratia* are in progress, which should provide more information about these unusual EPR features of astacin.

The presence of a coordinated Tyr in Cu^{2+}-substituted *Procambarus* astacin is demonstrated by intense Tyr-to-Cu^{2+} charge transfer transitions at 445 and 325 nm (Fig. 2), similar to those observed in *Astacus* Cu^{2+}-astacin [9]. These transitions can serve as fingerprints for the investigation of the binding status of the coordinated Tyr. Upon the addition of the chelating inhibitor Tyr-NHOH to *Procambarus* Cu^{2+}-astacin, these two absorptions are bleached away (Fig. 2). This result reflects that the coordinated Tyr is detached from the Cu^{2+} coordination sphere upon Tyr-NHOH binding to the metal. The gradual decrease of the intensity of the charge transfer transitions upon Tyr-NHOH (I) titration can be described by the simple equilibrium shown below (Eq. (1)),

$$\text{Astacin(bound Tyr)} + \text{I} \rightleftharpoons \text{I-Astacin(unbound Tyr)} \quad (1)$$

in which the binding of one inhibitor molecule to the Cu^{2+} results in a concomitant detachment of the coordinated Tyr (Inset, Fig. 2). The disappearance of the charge transfer transition is due to the binding of this one particular inhibitor that replaces the coordinated Tyr. Any other bound inhibitors, if existed, that do not displace the Tyr would not be seen via the charge transfer band. The fitting of the decrease in the intensity of the charge transition band with respect to the concentration of the inhibitor according to the equilibrium above gives an apparent association constant of 2.0×10^4 M^{-1}. The value is bigger than that for this inhibitor binding to native *Astacus* astacin, as expected to be due to the presence of larger ligand field stability energy in Cu^{2+} complexes. The charge transfer transitions and the activity of the inhibited Cu^{2+}-astacin can be fully recovered when the inhibitor is removed by passing the enzyme-inhibitor complex through a desalting column. This confirms that the disappearance of these transitions upon inhibitor binding is not due to removal of the active site metal by the chelating hydroxamate inhibitor, but is attributable to the detachment of the coordinated Tyr.

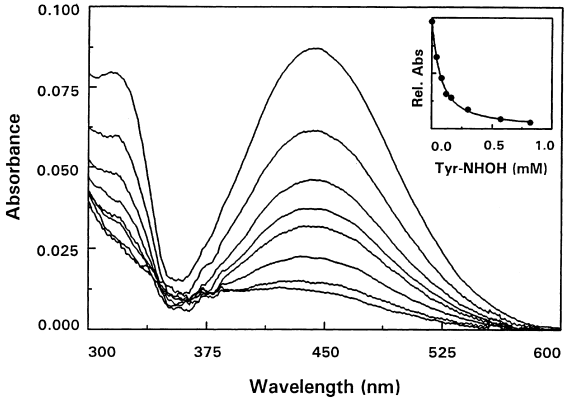

Fig. 2. Titration of Tyr-NHOH (from top to bottom, 0, 0.8, 1.6, 2.4, 3.2, 6.4, 12.8, 19.2 equivalents) to Cu^{2+}-substituted *Procambarus* astacin (46 μM in 0.1 M MES buffer at pH 6.1 and ambient temperature) results in the disappearance of the intense Tyr → Cu^{2+} charge transfer transitions at 325 and 445 nm. The inset shows the decrease of the absorption at 445 nm upon Tyr-NHOH binding, which gives an apparent affinity constant 2.0×10^4 M^{-1} upon fitting to the simple equilibrium in Eq. (1).

The metal active site of astacin and the binding of inhibitors to astacin can be further studied with an NMR spectrometer using Co^{2+} as a probe, which afford a fully active metal-substituted derivative. The short electronic relaxation times of Co^{2+} result in the appearance of relatively sharp hyperfine-shifted ^1H NMR signals attributable to the coordinated ligands in Co^{2+} complexes and Co^{2+}-substituted metalloproteins [21]. The ^1H NMR spectrum of a ~1 mM Co^{2+}-substituted *Procambarus* astacin sample in H_2O (10 mM MES buffer at pH 6.1) is shown in Fig. 3(A), where a few signals can be clearly detected in the downfield regions <30 ppm and ~70 ppm. The far downfield shifted signals ~70 ppm disappear when the protein is in D_2O buffer solution, indicating that these three signals at 76.2 and 68.9 (overlapped) ppm are solvent exchangeable. The chemical shifts of these signals are in the range for the ring NH proton of a coordinated His in several Co^{2+}-substituted metalloproteins [22], and can be assigned to the three coordinated His residues revealed in the crystal structure of the *Astacus* enzyme [23,24].

The intensity of the signal at 76.2 ppm is highly suppressed in the spectrum acquired with a presaturation pulse, indicating that it has a relatively higher

solvent exchangeable rate than the signal at 68.9 ppm. The full intensity of this signal can be revealed by the use of the selective excitation 1-*3*-3-*1* pulse sequence (inset, Fig. 3(A)). The fast solvent exchange rate of this signal suggests that it can be assigned to a labile His ring NH proton, like the $N_\delta H$ proton of His102 (*Astacus* astacin numbering) that is the only coordinated His whose $N_\delta H$ proton is H-bonded with a water molecule [23,24].

When His residues are bound to a paramagnetic Co^{2+} ion via the N_ε nitrogen, the only signal on the His imidazole ring that is sharp enough to be detected is the $N_\delta H$ proton; whereas the $C_\delta H$ and $C_\varepsilon H$ protons are expected to be broad beyond detection. In the case of N_δ-coordinated His residues, two relatively sharp signals can be detected that are due to the $N_\varepsilon H$ and the $C_\delta H$ protons [21,22]. Since there are no hyperfine-shifted solvent non-exchangeable signals (i.e., CH signals) in the region that can be assigned to coordinated His ring protons, the three coordinated His residues in *Procambarus* Co^{2+}-astacin must be coordinated to the metal via the N_ε nitrogen, consistent with that observed in the crystal structure of *Astacus* astacin [23,24]. This result demonstrate that NMR can serve as a useful and convenient tool for the study of the metal binding site of astacin family when the paramagnetic Co^{2+} ion is substituted for the diamagnetic Zn^{2+} ion in the native enzyme, as also shown in the studies of several other metalloproteins [21,22]. Combining the NMR studies with the optical and EPR studies described above, the active site of *Procambarus* astacin is revealed to contain a coordinated Tyr and 3 His residues in a tetragonally distorted environment, same as that of *Astacus* astacin.

The only solvent non-exchangeable signal >20 ppm in Co^{2+}-substituted astacin is a broad signal ($\Delta\nu_{1/2} = 280$ Hz) at 22.3 ppm which can be a good candidate of a ring proton of the coordinated Tyr. This Tyr is weakly bound to the metal with a long metal-O_η bond in *Astacus* Zn-astacin (2.54 Å [23,24]) and Co-astacin (2.3 Å [9]), and would be expected to show a small through-bond ^1H NMR hyperfine shift.

To further assist the assignment of the hyperfine-shifted signals due to the coordinated Tyr, we have prepared a 1:1 Co^{2+} complex of HBPA and assigned the hyperfine-shifted signals of the phenolate moiety of the ligand in the complex. A Co^{2+}-coordinated phenolate in a few different complexes shows quite different chemical shifts in a large range from ~−20 to 70 ppm for the *ortho* protons and about 6–55 ppm for the *meta* protons, with clear indication of the presence of both spin delocalization and spin polarization mechanisms for the isotropic shifts [25]. The large range of these isotropic shifts presents a practical difficulty using these values as criteria for signal assignment of other Co^{2+}-bound phenolate. Nevertheless, the chemical shifts of the signals attributable to the phenol group should exhibit significant pH-dependent changes associated with the deprotonation of the phenol proton. The *o*-CH proton of the phenolate in the complex Co^{2+}-HBPA is detected at <20 ppm at pH 4, and shifts to 37 ppm at pH 8 where the phenolate is fully bound to the metal (Fig. 4). The change of the chemical shift with respect to pH can be fitted to give $pK_a = 5.3$ for deprotonation of the phenol moiety with concomitant metal binding upon the deprotonation (Fig. 4). The results from the studies of the complex suggest the 22.3-ppm hyperfine-shifted signal in Co^{2+}-astacin is likely to be due to one $C_\varepsilon H$ proton of the coordinated Tyr.

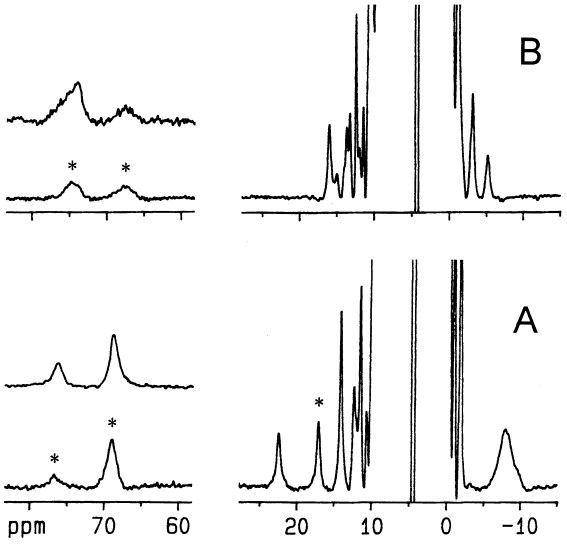

Fig. 3. The ^1H NMR spectra (360.13 MHz, 298 K, 8 K data points, and 10,000 scans) of (A) Co^{2+}-astacin (~1 mM) and (B) Co^{2+}-astacin-Tyr-NHOH complex. The solvent exchangeable signals are marked with asterisks. There are no signals detected in the range 30–60 ppm. Full intensities of the far downfield solvent exchangeable signals (insets) can be recovered by using the selective excitation 1-*3*-3-*1* pulse.

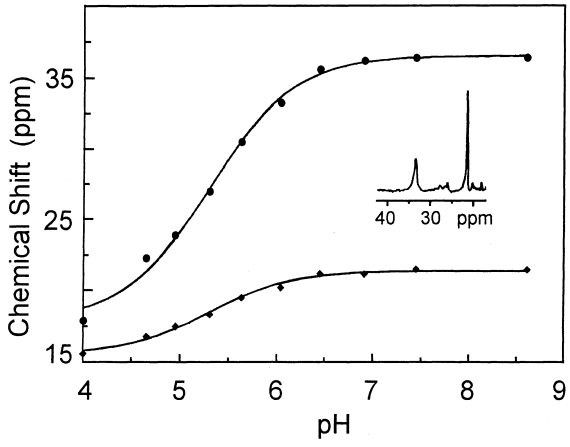

Fig. 4. Plot of ^1H NMR chemical shift of hyperfine-shifted phenolate ring signals in Co^{2+}-HBPA in aqueous solution. A single pK_a value can be revealed by fitting the data according to deprotonation of the phenolate with concomitant metal binding upon the deprotonation. The inset shows the signals due to the *o*-CH and *m*-CH protons of the coordinated phenolate at pH 8.

The binding of Tyr-NHOH to Co^{2+}-astacin can also be studied by means of NMR spectroscopy, where influence on the hyperfine-shifted ^1H NMR signals attributable to the coordinated ligands and the surroundings upon inhibitor binding can be observed and analyzed.

Upon the addition of Tyr-NHOH to Co^{2+}-astacin (Fig. 3(B)), one His ring NH signal is significantly affected which moves from 68.9 to 76.2 ppm and overlaps with the His102 $N_\delta H$ signal. In the mean time, the solvent non-exchangeable signal at 22.3 ppm is wiped out. The disappearance of a hyperfine-shifted signal can reflect the detachment of a coordinated ligand associated with the signal as observed in a few other metalloproteins [26–29]. This observation also indicates the 22.3 ppm signal is best assigned to a proton of the coordinated Tyr that is detached upon inhibitor binding to the active-site metal of astacin, as shown in Cu^{2+}-astacin.

In a recent crystallographic study of a phosphonate inhibitor-bound native (Zn) *Astacus* astacin [30], the coordinated Tyr was found detached from the active site Zn^{2+}. Moreover, the crystal structures of *Serratia* and *Pseudomonas* proteases and their inhibitor-bound forms also revealed a similar change of the binding status of the coordinated Tyr [31–34]. The results from our studies of Co^{2+}- and Cu^{2+}-substituted *Procambarus* astacin further indicate that the coordinated Tyr is detached upon a hydroxamate inhibitor binding to the enzyme *in solution*, in which hydrolytic reactions take place. The detachment of the coordinated Tyr in these enzymes must be a key step in their actions. A working model for astacin action is proposed below on the basis of the studies of *Procambarus* astacin in solution presented here, the crystallographic studies of a few enzymes in the astacin family [30–34], and chemical modeling studies [2,3] (Fig. 5): First, the detachment of the coordinated Tyr can significantly increase the Lewis acidity of the metal ion which results in lowering the pK_a of the coordinated water to facilitate its nucleophilic attack of the substrate. In addition, the steric crowding upon substrate binding can be accommodated by ligand detachment, and the negative charge on the transition state *gem*-diolate can be better balanced by the removal of the negative Tyr from the metal coordination sphere. Moreover, the detached Tyr can form a H-bond with the substrate in the transition state enzyme-substrate complex as suggested in the crystallographic study of the enzyme-inhibitor complex [30]. From the above discussion, the "Tyr-switch" mechanism [35] that was implied in previous crystallographic studies [30–34] can thus be further established in the astacin family.

Taken together, the detachment of the coordinated Tyr is considered to be a key step in the action of the astacin family based on our solution spectroscopic studies of metal-substituted derivatives and previous crystallographic studies of the native enzyme. The endogenous ligands of *Procambarus* astacin are also revealed to be the same as those of *Astacus* astacin. Moreover, the similar inhibitor-binding behavior and binding status of the coordinated Tyr between Co^{2+}- and Cu^{2+}-substituted derivatives of *Procambarus* astacin *in solution* and the native (Zn) *Astacus* astacin *in crystal forms* may also partially account for the significantly high activity of Cu^{2+}-astacin.

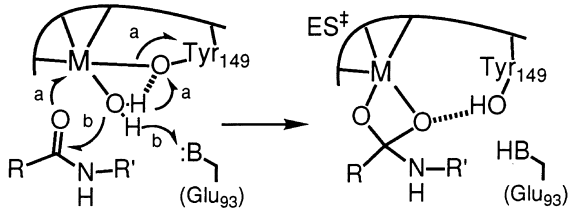

Fig. 5. A working model for the action of astacin based on the results from our studies of Co^{2+}- and Cu^{2+}-substituted derivatives of *Procambarus* astacin in solution, from crystallographic studies of native *Astacus* astacin, and from chemical modeling studies. Detachment of the coordinated Tyr is considered an important initial step in astacin action, which occurs upon substrate binding (step a). Step a enhances the Lewis acidity of the active site metal, thus lowers the pK_a of the coordinated water molecule for the generation of a coordinated hydroxide that is more effective in nucleophilic attack on the substrate (step b). This step may be assisted by a base, like Glu93 that may remain H-bonded to the *gem*-diolate intermediate in the transition state enzyme-substrate complex ($ES^‡$). The detached Tyr is also suggested to be H-bonded to the substrate in the $ES^‡$ complex.

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