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Metal-binding and active-site structure of di-zinc *Streptomyces griseus* aminopeptidase

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Abstract *Streptomyces griseus* aminopeptidase has been characterized to have a dinuclear active site and to follow a dinuclear hydrolytic mechanism by means of activity assay, optical, and NMR spectroscopy. A sequential binding of Co^{2+} to the dinuclear sites in 20 mM Mes buffer at pH 6.1 has also been established. The results from these studies suggest that the two metal sites have a five-coordination sphere, with at least one coordinated His each. A di-Cu^{2+}-substituted derivative of the enzyme has been prepared which exhibits a ^{1}H NMR spectrum with sharp hyperfine-shifted signals, again indicating the presence of a dinuclear active site. This ^{1}H NMR spectrum with sharp hyperfine-shifted features represents a first of its kind for a di-Cu^{2+} center in metalloproteins.

Key words Aminopeptidase · *Streptomyces* · Dinuclear · NMR · Co^{2+} · Cu^{2+}

Introduction

Aminopeptidases (APs) are a family of widely distributed proteases which participate in many significant biological processes, such as protein maturation, hormone production, and peptide digestion [1–3]. While several Zn peptidases are known to contain a single Zn^{2+} ion in their active site [4–6], a few metallo-APs, including those from bovine lens (bAP) [7–10], *Escherichia coli* (eAP) [11], *Aeromonas proteolytica* (aAP) [12], and *Streptomyces griseus* (sAP)[1] have been proved by means of X-ray crystallography to contain a dinuclear metal active site. However, diverse structural features and mechanistic properties have been revealed in these dinuclear metallo-APs [14, 15]; including their different tertiary and quaternary structures, the lack of a conserved geometry and coordinated ligand type in the active site, and the observation of dinuclear catalysis in some APs and mononuclear catalysis in others.

Despite the presence of a dinuclear site in aAP [12] and presumably in porcine kidney AP (pAP) [16], these two APs have been observed to exhibit a "mononuclear" catalysis. These two enzymes have been previously shown to exhibit a selective metal binding property, where a full hydrolytic activity was detected when one metal ion was bound [16, 17]. A modulation of the activity was observed when a second metal ion was introduced, indicating a regulatory role [18, 19]. Nevertheless, this mononuclear catalysis was not observed in bAP action [7–10]. Further studies of other dinuclear APs are therefore necessary to provide more mechanistic information about this mononuclear and dinuclear discrepancy in AP action.

The AP isolated from the culture medium of *Streptomyces griseus* (sAP, MW ~30 kDa) has been characterized to contain two Zn^{2+} ions per molecule [20, 21]. A previous metal-activation study using the slow substrate Ala-*p*-nitroanilide revealed that Mn^{2+}, Co^{2+}, or Zn^{2+} was bound simultaneously to the two metal binding sites of the enzyme at pH 8 in the presence of 1 mM Ca^{2+}; i.e., the activity of the enzyme was parallel to the amount of metal ion bound to the enzyme and reached a plateau with two equivalent metal ions introduced [20]. Since the two metal sites could not be selectively filled with metal ions in the previous study, identification of each metal site was not possible, and the role of each metal ion in catalysis could not be easily revealed. It is important to find out the conditions for a selective metal binding to the metal sites, which is an inevitable step to providing structural information and the catalytic role for each individual metal site by means of physical methods.

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[1] While this manuscript was in preparation, the crystal structure of this enzyme was published [13]

We report here our study of the metal-binding properties, hydrolytic activity, and active-site structure of sAP by means of activity assay and spectroscopic methods. A sequential Co^{2+} binding to this enzyme in Mes buffer at pH 6.1 has been concluded on the basis of optical study, isotropically shifted ^1H NMR features, and activity assay. Moreover, we have documented in this report a very rare case in which Cu^{2+} ion, which is better known as an NMR "relaxation probe", can be utilized as a "shift probe" for the study of metal-binding sites in metalloproteins and affords *sharp* hyperfine-shifted ^1H NMR signals. The presence of a dinuclear metal-active site in sAP has been established by means of ^1H NMR using Cu^{2+} as a probe. This study also demonstrates that although sAP and aAP have nearly identical active sites on the basis of their crystal structures [14, 15] (however, with a low sequence homology ~30% [22]), they exhibit quite different mechanisms in that sAP shows a dinuclear hydrolytic catalysis whereas aAP shows a mononuclear catalysis.

Experimental

Streptomyces griseus aminopeptidase and its apo form were prepared according to established procedures [20, 21], and the protein fractions designated as AP-I in the literature (the first eluted AP from the final DEAE column) were used. The enzyme concentration was determined according to the literature value $E_{280}^{1\%} = 15$ [20, 21], which was consistent with that determined by metal ion titration using standard Zn^{2+} or Co^{2+} solutions where a full enzyme activity was reached after 2 equivalents of metal ion was introduced. In all the preparations, apo-sAP samples of negligible residual AP activities could be easily obtained (e.g., <0.02%, on the basis of the initial rate in the assay). The activity of AP was assayed at an enzyme concentration of ~0.02 μM using 0.8 mM substrate Leu-*p*-nitroanalide (Leu-pNA, Sigma Chemical Co.) in 60 mM NaCl and 10 mM Tris buffer at pH 8 on a Varian Cary 3 spectrophotometer at 25 °C. The initial rate of the hydrolysis of the substrate was obtained by monitoring the release of the chromophore pNA at 405 nm ($\varepsilon = 10600\ M^{-1}\ cm^{-1}$ under the assay conditions) [20, 21]. Since Ca^{2+} was known to affect the enzyme activity [20, 21], it was excluded from all the activity assays in this report, unless otherwise indicated.

In the metal-activation studies of the enzyme, an increasing amount of 0.25 mM Co^{2+} solution (10 μl each time) was added to an apo-sAP solution (~20–30 μM, 1 ml) and incubated at room temperature for a few minutes, then a 10-μl aliquot was taken each time for AP assay. Stock Co^{2+} and Zn^{2+} solutions were prepared from atomic absorption standard solutions or from their nitrate or chloride salts and standardized against a standard EDTA solution. The use of enzyme solutions with relatively high concentrations (>10 μM) in the metal-binding studies in this report has afforded consistent results. All the solutions were prepared from deionized water of >18 MΩ from a MilliQ system (Millipore, Bedford, Mass.). All the glassware and plastic wares were treated with EDTA solution and rinsed with deionized water prior to use.

The electronic spectra of the Co^{2+} derivatives of sAP in 20 mM MES buffer at pH 6.1 were obtained on the Varian spectrophotometer. The sAP samples with 1 and 2 equivalents Co^{2+} bound that were used in the optical study were subsequently concentrated by the use of a Centricon-10 microconcentrator (Amicon, Beverly, MA) to ~1 mM for the detection of hyperfine-shifted ^1H NMR signals. The ^1H NMR spectra of the Co^{2+}-substituted derivatives (20 mM MES buffer at pH 6.1) were obtained at 303 K on a Bruker AMX360 operating at 360.13 MHz using a 90° acquisition pulse (~7.5 μs) and a presaturation pulse for water suppression, 8 K data points, a repetition time of ~200 ms, with a total of 8000 transients. A 10%-shifted Gaussian window function with a line-broadening factor of –50 Hz was applied to the FIDs (free induction decays) prior to Fourier transformation to improve spectral resolution in the near-diamagnetic region. An NOE (nuclear Overhauser effect) difference spectrum was obtained by subtracting the FID in which the signal of interest was irradiated from the FID in which the irradiation was placed at a reference position for every 400 scans until a difference spectrum with a sufficient signal-to-noise ratio was obtained.

Results and discussion

Metal activation

Several factors such as pH and buffer are known to affect the selectivity of metal binding to multi-metal binding sites in metalloproteins. Under appropriate conditions, metal ions can be selectively introduced into a dinuclear metal-binding site, such as that observed in Cu,Zn-superoxide dismutase (CuZnSOD) [23–25], in which a lower pH shows a better selective binding. While selective metal binding in CuZnSOD can be achieved owing to the different geometries of the two metal-binding sites, a selective metal binding to two nearly identical coordination sites in a dinuclear metalloprotein cannot always be expected. However, a selective metal binding to the dinuclear metal active site in aAP was observed [17] despite the fact that the two metal-binding sites have been shown to have almost identical coordination environments, i.e., both tetrahedral with 1 His and 1 Glu in one site and 1 His and 1 Asp in the other site plus the bridging Asp and water [12]. In order to study separately the two metal-binding sites in sAP, it is important to find out the conditions where a selective metal binding can be achieved. On the basis of the observations in the study of Cu,Zn-SOD, it seems that the discrimination of metal binding in a multinuclear metalloprotein may become more pronounced at lower pHs.

The di-Co^{2+}-substituted derivative of this enzyme exhibits a full activity; thus, Co^{2+} ion with rich spectroscopic properties can be used as an ideal substitute for the native Zn^{2+} ion in mechanistic studies of the native enzyme. Several activity profiles of apo-sAP upon Co^{2+} titration under different conditions are shown in Fig. 1. A selective Co^{2+} binding to sAP is observed in 20 mM Mes buffer at pH 6.1 (trace 6 ○). This profile shows that a very low activity is detected with less than 1 equivalent Co^{2+} introduced, and a breaking point is observed when metal/enzyme ratio reaches 1 (to form a "one-Co derivative", CoE-sAP, with E being an empty site), which is then followed by a dramatic increase of the activity thereafter. A plateau of the metal activation is reached at a metal/enzyme ratio greater than 2, affording a fully active "two-Co derivative" CoCo-sAP. Virtually identical results were obtained when a number of apo-sAP samples containing different amounts of metal ions (0.2–4 equivalents)

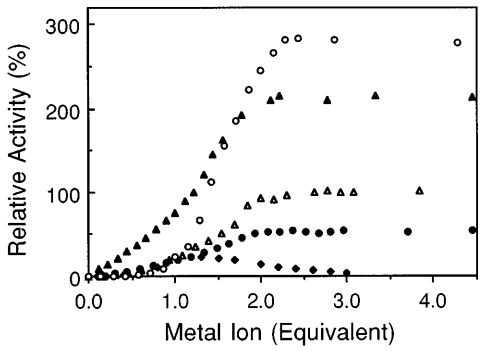

Fig. 1 The activity profile of *Streptomyces* AP upon Co^{2+} binding without Ca^{2+} in 20 mM MES buffer at pH 6.1 (trace ○) and in the presence of 5 mM Ca^{2+} at pH 8.5 in 20 mM Tris buffer (trace ●), and upon Zn^{2+} binding without Ca^{2+} at pH 6.1 (△) and with 5 mM Ca^{2+} at pH 8.5 (▲). The binding of Cu^{2+} to CoE-sAP under the same conditions as in trace (●) is shown as trace (⧫). An amount of 60 mM NaCl is present in all the solutions. The corresponding electronic and ^1H NMR spectra of the two derivatives with 1 and 2 Co^{2+} bound, CoE-sAP and CoCo-sAP, respectively, are shown in Figs. 2 and 3

were prepared separately and incubated overnight at room temperature. A similar pattern was also obtained in the presence of the regulator Ca^{2+} at pH 6.1 with a slightly lower selectivity (data not shown). The binding of Zn^{2+} to apo-sAP in 20 mM Mes buffer at pH 6.1 (trace △) shows much less selectivity than in the case of Co^{2+} binding under the same conditions. Full activity of the enzyme can be achieved *only* after 2 equivalents of Zn^{2+} have been introduced.

A pronounced increase in the activity after the introduction of >0.75 equivalents of Co^{2+} to apo-sAP is possibly due to the competition of the second site for the binding of the metal ion. This is evident in an experiment where subsequent addition of the "inhibitory" Cu^{2+} (which activates sAP toward Leu-pNA hydrolysis to ~0.2% of the native enzyme) to CoE-sAP abolishes the residual activity, presumably due to the binding of Cu^{2+} to the second site and replaces the Co^{2+} in that site (Fig. 1, trace ⧫). These metal-binding studies suggest that the second metal-binding site is crucial for sAP activity. This is based on the observation that only the second equivalent Zn^{2+} or Co^{2+} ion can restore the activity, while the second equivalent Cu^{2+} abolishes the activity. Hence, *Streptomyces* AP can be concluded to exhibit a dinuclear catalysis similar to bAP [7–10], but different from pAP [16] and aAP [17], which exhibit mononuclear catalysis.

This selective metal binding to apo-sAP is seemingly contradictory to what was reported previously [20] where a simultaneous metal binding to the two metal sites was observed. We have investigated the possible causes of this discrepancy, including the different buffers and pHs as well as the influence of Ca^{2+}. The enzyme still shows a clear but less selective Co^{2+} binding in Tris buffer at pH 8.5 in the absence of Ca^{2+} using the specific substrate Leu-pNA. This selectivity is significantly diminished in the presence of 5 mM Ca^{2+} (Fig. 1, trace ●). A similar result is also observed for Zn^{2+} binding to this enzyme, where a less selective binding is revealed at pH 8.5 in the presence of 5 mM Ca^{2+} (Fig. 1, trace ▲). These observations imply that metal ions bind less selectively to the two metal-binding sites at high pHs in the presence of Ca^{2+}, consistent with previous observations under similar conditions using the slow Ala-pNA substrate [20]. The influence on transition metal binding by Ca^{2+} indicates that this ion plays an important role in sAP action as shown in the activity profiles. However, the Ca^{2+} modulation mechanism that activates the native enzyme while deactivating the Co^{2+} derivative cannot be revealed at this stage. The mechanism by which a Ca^{2+} found at 22 and 25 Å away from the two metal sites [13] affects the enzyme activity and metal-binding properties deserves further investigation.

Optical studies

The determination of the conditions for selective metal binding described above allows us to investigate each of the metal-binding sites by the use of electronic and NMR spectroscopies. The introduction of one equivalent Co^{2+} to apo-AP in 20 mM Mes buffer at pH 6.1 affords the mono-Co^{2+}-substituted derivative (CoE-sAP) with a very low activity (<10%, presumably due to Co^{2+} binding to the second site, see above) whose electronic spectrum is shown in Fig. 2 (Spectrum A, $\lambda_{max} \approx 540$ nm with $\varepsilon = 88\ M^{-1}\ cm^{-1}$ and shoulders at 474 and 583 nm). The subsequent addition of the second equivalent of Co^{2+} (to give CoCo-sAP with a fully active enzyme) causes a noticeable perturbation in the electronic absorptions of CoE-sAP and exhibits a spectrum with λ_{max} at 540 nm ($158\ M^{-1}\ cm^{-1}$) and shoulders at 474, 498, and ~590 nm (Fig. 2B). The differences between the spectra of the two derivatives can be more clearly seen with a difference spectrum of B minus A (Fig. 2C), where the absorptions due to the Co^{2+} in the

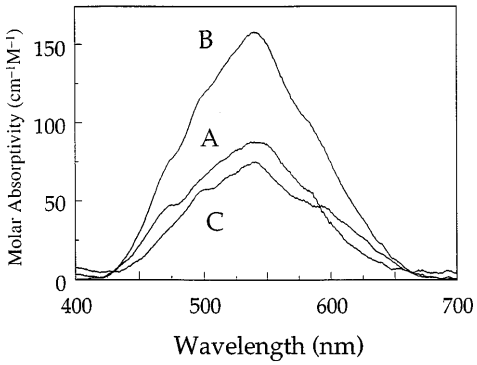

Fig. 2 The electronic spectra of *Streptomyces* AP (~0.3 mM) with the binding of (**A**) 1 and (**B**) 2 equivalents Co^{2+} in 60 mM NaCl and 20 mM Mes buffer at pH 6.1 at 25 °C. The spectrum **C** is the difference spectrum of **B** minus **A**, representing the absorptions of the Co^{2+} in the second site in CoCo-sAP

empty site of CoE-AP are revealed (λ_{max} = 540 nm with $\varepsilon = 80\ M^{-1}\ cm^{-1}$, and shoulders at 498 and 595 nm) which are quite distinct from those of the first site. The two derivatives CoE-sAP and CoCo-sAP show different spectral features, suggesting that Co^{2+} binds to the two metal sites in a sequential manner under the experimental conditions. This corroborates with the activity profiles (Fig. 1). Whether or not the two metal sites are structurally correlated as a dinuclear site cannot be concluded based on these spectra, as the change in the electronic absorptions is not significant. Nevertheless, the similar absorption features, e.g., λ_{max} and ε values, of the Co^{2+} in the two metal-binding sites indicate that the two metal-binding sites may have a similar geometry and structure.

The intensity of the electronic transitions in Co^{2+}-substituted metalloproteins and complexes can reflect the geometry about the Co^{2+} in the order of their Laporte-allowed transition probabilities, i.e., tetrahedral > five-coordinated > octahedral geometry in a range of >200 to <50 $M^{-1}\ cm^{-1}$ in most cases [26, 27]. The intensity of the electronic absorptions for the Co^{2+} (80–90 $M^{-1}\ cm^{-1}$ per Co^{2+}) in these two derivatives of sAP is best accounted for by a five-coordinated geometry. This suggests that the weaker Zn-$O_{\delta 2}$ bond (2.6 Å) for Asp160 in one Zn site and Zn-$O_{\varepsilon 2}$ (2.7 Å) for Glu132 in the other Zn site) observed in the crystal structure[1] have to be taken into account to afford two five-coordinated Zn ions in the active site.

NMR studies

The binding status of Co^{2+}, the ligand identity, and the structure of the active site in Co^{2+}-substituted sAP can be revealed by the use of NMR spectroscopy via the studies of hyperfine-shifted ^1H NMR signals, even though the large paramagnetism of the Co^{2+} center is generally considered not favorable for NMR studies [28, 29]. A simultaneous Co^{2+} binding to the two sites of sAP should give rise to identical ^1H NMR spectra with 1 and 2 equivalents of Co^{2+} introduced, in which the latter should afford double the signal intensity. On the contrary, we observed two completely different ^1H NMR spectra for sAP with 1 and 2 equivalents of Co^{2+} bound in 20 mM MES at pH 6.1 (Fig. 3A,B), indicating the formation of two different derivatives, CoE-sAP and CoCo-sAP, respectively. This is corroborative with the conclusion derived from the optical and activity studies discussed above.

When the ^1H NMR spectrum of the derivative CoE-sAP is acquired in a D_2O buffer of 20 mM Mes at pD 6, the isotropically shifted signal at 62.8 ppm disappears (asterisked signal in spectrum A, Fig. 3). The chemical shift of this signal is in the range for the ring NH proton of a coordinated His ligand in the metal-binding site of several Co^{2+}-substituted metalloproteins [30]. This suggests that there is one coordinated His in the first metal-binding site. Similarly, the detection of two solvent-exchangeable signals at 81.3 and 51.0 ppm in the spectrum of CoCo-sAP (Fig. 3B) reflects the presence of two coordinated His residues in the active site. When a solvent-exchangeable His ring NH proton exchanges fast with water, its signal may become broader and possibly undetectable. Thus, the number of solvent-exchangeable His ring NH signals reflects the minimum number of coordinated His residues.

The isotropically shifted signals, particularly the less shifted features, in the derivative CoCo-sAP become

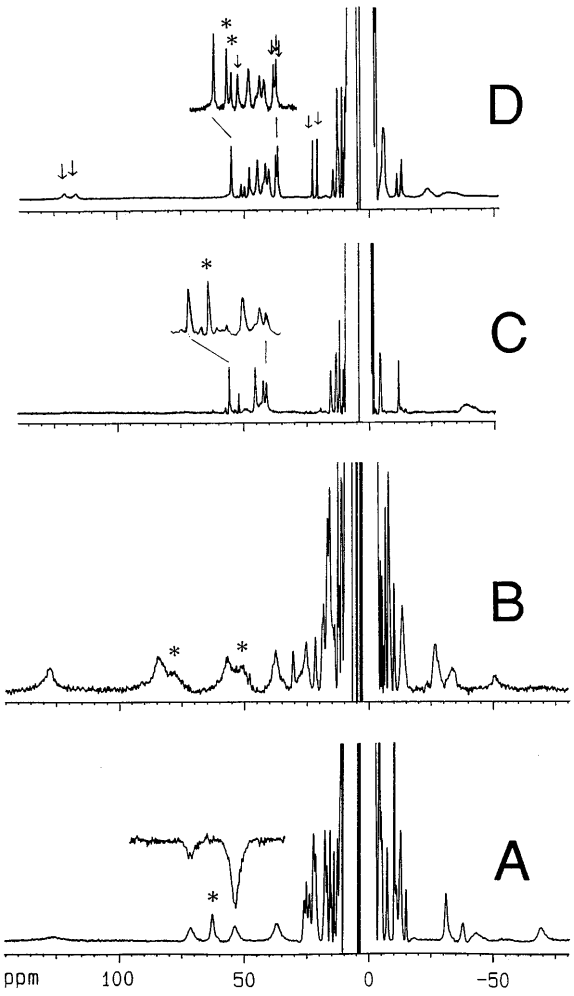

Fig. 3A–D The ^1H NMR spectra (360.13 MHz and 303 K) of Co^{2+}-substituted derivatives of *Streptomyces* AP in 60 mM NaCl and 20 mM MES buffer at pH 6.1. **A** CoE-sAP (~1 mM), **B** CoCo-sAP (0.8 mM), **C** CuCu-sAP (1.5 mM) under the same conditions as in **A** and **B**, and **D** CuCu-sAP from **C** in the presence of 1 equivalent 1,10-phenanthroline. The smaller signal-to-noise ratio in spectrum **B** is attributable to the lower concentration of the sample and broader features of the spectrum. The *inset* in **A** is an NOE difference spectrum of CoE-sAP in which the signal at 53.5 ppm is irradiated for 30 ms, and a cross relaxation on the signal at 71.5 ppm is clearly revealed. The *insets* in **C** and **D** are the spectra acquired using the selective excitation hard pulse sequence 1-3-3-1 to reveal full intensities for those fast-exchanging solvent-exchangeable signals. The solvent-exchangeable signals (marked with *asterisks*) were identified by acquiring the spectra of the derivatives in D_2O buffer solutions. The signals marked with *arrows* in **D** are due to the bound 1,10-phenanthroline

broader at a concentration $\gtrsim 1$ mM, which may be attributable to the formation of coagulates. This is noticeable even at ~ 0.5 mM, although to a much lesser extent. A 0.8 mM sample shows a spectrum in which most of the signals near the edges of the diamagnetic envelope are resolved (Fig. 3B). Although the signal broadening can be attributable to protein coagulation, it is also likely to be due to a loosely bound Co^{2+} in the second metal site. (The loosely bound Co^{2+} in the second site can be removed simply by ultrafiltration.) This makes the whole active site fluxional, which may result in the broadening of the isotropically shifted signals. Upon addition of one Co^{2+} to apo-sAP, room temperature magnetic susceptibility was estimated to be 3.1 Bohr magnetons (BM) using the Evans method, suggesting a mononuclear center [28]. The introduction of the second Co^{2+} did not allow us to see separated two reference signals. This might be attributable to a virtually diamagnetic state (however, similar chemical shifts are observed in both CoE- and CoCo-sAP) or an extensive broadening of the reference signal due to a fluxional second Co^{2+}-binding site. The magnetic properties of these Co^{2+} derivatives cannot be described in detail at this moment, and await further studies. Nevertheless, the conclusion that two different Co^{2+} derivatives can be selectively prepared and that sAP is likely to have a dinuclear center is still valid at this stage.

A 1D NOE difference spectrum of CoE-sAP acquired with the signal at 53.5 ppm irradiated for 30 ms shows a cross relaxation to the signal at 71.5 ppm (ca. –20% NOE, Fig. 3A, inset). This intense NOE can be generated only from a geminal pair, such as the $C_{\beta/\gamma}H_2$ geminal pair of a coordinated Asp or Glu, which can be closer to the metal and thus show broader signals compared with the coordinated His ring NH signal [31]. The broadness of these signals has prevented us from obtaining useful coherence transfer information to establish a spin system.

The lack of other isotropically shifted signals in the >20 ppm region with comparable linewidths as the signal of the His ring NH proton (at a *meta* position to the metal) in the CoE-sAP spectrum suggests that the His residue is coordinated to the metal via its N_ε nitrogen, leaving the two ring CH protons close to the metal (at *ortho* positions to the metal) and not able to show sharp signals. When the ring NH signal at 62.8 ppm is irradiated for 30 ms, there is no NOE detected on any solvent non-exchangeable signal. This is consistent with an N_ε-binding mode for this coordinated His residue; otherwise, an NOE between the $N_\varepsilon H$ and $C_\delta H$ protons can be revealed for a N_δ-coordinated His residue [32–35]. This His coordination mode is the same as that found in the recently determined crystal structure.[1] The detection of a large number of isotropically shifted ^1H NMR signals in the upfield region of the two Co^{2+}-substituted derivatives is due largely to the presence of the dipolar shift mechanism. This is suggestive of a distorted five-coordination sphere about the Co^{2+}, which often exhibits a large magnetic anisotropy to afford a significant dipolar shift [28–30], consistent with the conclusion based on the optical study described above.

The isotropically shifted ^1H NMR features of CoE-sAP are not reproduced in the spectrum of CoCo-sAP. This indicates that the two metal sites are structurally correlated with each other, likely a "dinuclear center", where the binding of Co^{2+} to the empty second metal-binding site in CoE-AP causes a significant perturbation of the first site, and thus a change in its spectral features. The presence of a dinuclear center can be further proved by using Cu^{2+} ion as a probe, where the introduction of 2 equivalents of Cu^{2+} to apo-sAP at pH 6.1 produces a derivative that gives rise to several well-defined isotropically shifted ^1H NMR features in the 40–60 ppm region (Fig. 3, spectrum C). Mononuclear Cu^{2+} centers only show featureless or poorly defined broad ^1H NMR signals that are attributable to the coordinated ligands [26, 27, 36]. The detection of the relatively sharp isotropically shifted signals for the derivative CuCu-sAP reflects that the two Cu^{2+} ions are magnetically coupled, mediated by the bridging Asp97 and the water/hydroxide revealed in the crystal structure [13], as observed in several dinuclear iron proteins [37]. The presence of a dinuclear site in sAP can thus be concluded as observed in the crystal structure[1], and a dinuclear catalysis in sAP action can be established based on our metal activation and spectroscopic studies.[1]

There is only one solvent-exchangeable signal with a low intensity observed in the ^1H NMR spectrum of CuCu-sAP (asterisked signal in spectrum C, Fig. 3). This is inconsistent with the observation in the study of CoCo-sAP, in which two isotropically shifted solvent exchangeable signals are detected. Upon the addition of one equivalent of the inhibitor 1,10-phenanthroline to CuCu-sAP, a different spectrum is detected with all eight protons of the inhibitor shown in a pattern of pairs (marked with arrows in Spectrum D, Fig. 3). Based on the linewidths of the signals starting from the far shifted pair at ~ 140 ppm, these signals can be assigned to the 2/9, 3/8, 4/7, 5/6 protons, respectively, of the bound 1,10-phenanthroline. In this inhibitor-bound complex, two isotropically shifted solvent-exchangeable signals with low intensities are detected (asterisked). The low intensity of these two His ring NH signals in the di-Cu^{2+} derivative is due to the application of a presaturation pulse for water suppression where saturation transfer from the saturated water to the solvent-exchangeable signals occurs. This problem can be eliminated by using a selective excitation 1-3-3-1 pulse sequence for spectrum acquisition, in which the two NH signals exhibit normal intensities (inset, Spectrum D). Since there is no solvent-exchangeable proton in 1,10-phenanthroline, the two solvent-exchangeable signals in the enzyme-inhibitor complex are indicative of the presence of two coordinated His residues in the active site of sAP. This is consistent with the observation in the di-Co^{2+} derivative discussed above. These ^1H NMR spectra with sharp isotropically shifted signals

that are due to a di-Cu^{2+} center represent a first of its kind in NMR studies of metalloproteins [38–42][2], and are expected to stimulate future use of Cu^{2+} as an NMR probe for the study of other dinuclear metalloproteins.

Concluding remarks

In summary, we have proved by the use of optical and ^1H NMR techniques and activity assay that *Streptomyces* AP contains a dinuclear active site, and have revealed one His residue in each five-coordinated metal site and one Asp/Glu ligand in one metal-binding site. Although crystallographic studies showed that the backbone folding and the active-site structure of sAP and aAP are virtually identical [12, 13][1], we have demonstrated in this report that the actions of these two APs in solution are *completely different*; i.e., while aAP shows mononuclear activity [17], sAP exhibits a dinuclear catalysis. In addition, both optical and ^1H NMR spectra of the Co^{2+} and Cu^{2+} derivatives of these two enzymes are quite different,[2] reflecting their different active-site structures and actions *in solution*. Since the conditions for a selective Co^{2+} binding to sAP have been established in our study, which was impossible according to previous studies [20, 21], the structural features and the role of each metal ion in the dinuclear active site can be studied selectively in solution by using optical and magnetic spectroscopic techniques. Particularly, the usefulness of *Cu^{2+}as an NMR probe* for the study of dinuclear centers in metalloproteins via hyperfine-shifted ^1H NMR signals has been demonstrated in this report. The presence of a multinuclear metal active site in several functionally diverse hydrolytic enzymes indicates the significance of multinuclear centers for hydrolytic reactions [14, 15]. Detailed structural and mechanistic studies of dinuclear sAP are complementary to the studies of other dinuclear APs that exhibit different mechanisms [16, 17], and can provide more insight into the mechanism of dinuclear hydrolytic reaction.

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[2] Although dinuclear Cu^{2+} sites in small metal complexes have been studied in some detail by using NMR spectroscopy [38–42], only preliminary studies in metalloproteins using Cu^{2+} as an NMR probe have been discussed, such as the trinuclear site in *Bacillus* phosphotidylcholin-specific phospholipase C [Epperson J, Ming L-J, unpublished spectra cited in Ming L-J (1996) Chemistry (Chin Chem Soc Taipei) 54:69–79] and *Aeromonas* AP [Holz RC, Bennett B, Chen G, D'Souza V (1996) poster presented at the 212th National ACS meeting]

References

1. Taylor A (1993) FASEB J 7:290–298
2. Taylor A (1993) TIBS 18:167–172
3. Gonzales T, Robert-Baudouy J (1996) FEMS Microbiol Rev 18:319–344
4. Christianson DW, Lipscomb WN (1989) Acc Chem Res 22:62–69
5. Mangani S, Carloni P, Orioli P (1992) Coord Chem Rev 120:309–324
6. Matthews BW (1988) Acc Chem Res 21:333–340
7. Burley SK, David PR, Taylor A, Lipscomb WN (1990) Proc Natl Acad Sci USA 87:6878–6882
8. Burley SK, David PR, Sweet RM, Taylor A, Lipscomb WN (1992) J Mol Biol 224:113–140
9. Sträter N, Lipscomb WN (1995) Biochemistry 34:9200–9210
10. Kim H, Lipscomb WN (1994) Adv Enzymol 68:153–213
11. Roderick SL, Matthews BW (1993) Biochemistry 32:907–3912
12. Chevrier B, Schalk C, D'Orchymont H, Rondeau J-M, Moras D, Tarnus C (1994) Structure 2:283–291
13. Greenblatt HM, Almog O, Maras B, Spungin-Bialik A, Barra D, Blumberg S, Shoham G (1997) J Mol Biol 265:620–636
14. Sträter N, Lipscomb WN, Klabunde T, Krebs B (1996) Angew Chem Int Ed Engl 35:2024–2055
15. Lipscomb WN, Sträter N (1996) Chem Rev 96:2375–2433
16. Van Wart HE, Lin SH (1981) Biochemistry 20:5682–5689
17. Prescott JM, Wagner FW, Holmquist B, Vallee BL (1985) Biochemistry 24:5350–5356
18. Vallee BL, Auld DS (1993) Proc Natl Acad Sci USA 90:2715–2718
19. Vallee BL, Auld DS (1993) Biochemistry 32:6493–6500
20. Ben-Meir D, Spungin A, Ashkenazi R, Blumberg S (1993) Eur J Biochem 212:107–112
21. Spungin A, Blumberg S (1989) Eur J Biochem 183:471–477
22. Maras B, Greenblatt HM, Shoham G, Spungin-Bialik A, Blumberg S, Barra D (1996) Eur J Biochem 236:843–846
23. Valentine JS, Pantoliano MW (1981) In: Spiro TG (ed) Copper Proteins Vol. 3, Chapter 8, Wiley, NY
24. Ming L-J, Valentine JS (1987) J Am Chem Soc 109:4426–4428
25. Ming L-J, Valentine JS (1990) J Am Chem Soc 112:6374–6383
26. Bertini I, Luchinat C (1984) Adv Inorg Biochem 6:71–111
27. Lever ABP(1986) Inorganic Electronic Spectroscopy, 2nd Rev. Ed.; Elservier, NY
28. Bertini I, Luchinat C (1986) NMR of Paramagnetic Molecules in Biological Systems, Benjamin/Cumming, Menlo Park, CA
29. La Mar GN, Horrocks WDeW Jr, Holm RH (1973) NMR of Paramagnetic Molecules; Academic, NY
30. Bertini I, Turano P, Vila A (1993) J Chem Rev 93:2833–2932
31. Banci L, Bertini I, Luchinat C, Viezzoli MS (1990) Inorg Chem 29:1438–1440
32. Banci L, Dugad LB, La Mar GN, Keeting KA, Luchinat C, Pierattelli R (1992) Biophys J 63:530–543
33. Banci L, Bertini I, Luchinat C, Piccioli M, Scozzafava A, Turano P, Inorg Chem 28:4650–4656
34. Bertini I, Luchinat C, Ming L-J, Piccioli M, Sola M, Valentine JS (1992) Inorg Chem 28:4433–4435
35. Sette M, Paci M, Desideri A, Rotilio G (1993) Eur J. Biochem 213:391–397
36. Kalverda AP, Salgado J, Dennison C, Canters GW (1996) Biochemistry 35:3085–3092
37. Que L Jr, True AE (1990) Prog Inorg Chem 38:97–220
38. Maekawa M, Kitagawa S, Munakata M, Masuda H (1989) Inorg Chem 28:1904–1909
39. Wang S, Pang Z, Zheng J-C, Wagner MJ (1993) Inorg Chem 32:5975–5980
40. Holz RC, Brink JM (1994) Inorg Chem 33:4609–4610
41. Satcher JH Jr, Balch AL (1995) Inorg Chem 34:3371–3373
42. Murthy NN, Karlin KD, Bertini I, Luchinat C (1997) J Am Chem Soc 119:2156–2162.