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Cobalt(II) and copper(II) binding of *Bacillus cereus* trinuclear phospholipase C: a novel ^{1}H NMR spectrum of a 'Tri-Cu(II)' center in protein

Jon D. Epperson, Li-June Ming*

Department of Chemistry and Institute for Biomolecular Science, University of South Florida, 4202 Fowler Ave., CHE305, Tampa, FL 33620-5250, USA

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Abstract

The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PC-PLC$_{Bc}$) is a tri-Zn enzyme with two 'tight binding' and one 'loose binding' sites. The Zn^{2+} ions can be replaced with Co^{2+} and Cu^{2+} to afford metal-substituted derivatives. Two Cu^{2+}-substituted derivatives are detected by means of ^{1}H NMR spectroscopy, a 'transient' derivative and a 'stable' derivative. The detection of sharp hyperfine-shifted ^{1}H NMR signals in the 'transient' derivative indicates the formation of a magnetically coupled di-Cu^{2+} center, which concludes that the Zn^{2+} ions in the dinuclear (Zn1 and Zn3) sites are more easily replaced by Cu^{2+} than that in the Zn2 site. This might possibly be the case for Co^{2+} binding. Complete replacement of the three Zn^{2+} ions can be achieved by extensive dialysis of the enzyme against excess Cu^{2+} to yield the final 'stable' derivative. This derivative has been determined to have five-coordinated His residues and an overall $S'=1/2$ spin state with NMR and EPR, consistent with the formation of a tri-Cu^{2+} center (i.e. a di-Cu^{2+}/mono-Cu^{2+} center) in this enzyme. The binding of substrate to the inert tri-Cu^{2+} center to form an enzyme–substrate (ES) complex is clearly seen in the ^{1}H NMR spectrum, which is not obtainable in the case of the native enzyme. The change in the spectral features indicates that the substrate binds directly to the trinuclear metal center. The studies reported here suggest that ^{1}H NMR spectroscopy can be a valuable tool for the characterization of di- and multi-nuclear metalloproteins using the 'NMR friendly' magnetically coupled Cu^{2+} as a probe.

Keywords: Bacillus; Co^{2+}; Cu^{2+}; NMR; Phospholipase C; Trinuclear

1. Introduction

The phosphatidylcholine-preferring phospholipase C from *Bacillus cereus* (PC-PLC$_{Bc}$; EC 3.1.4.3; M_r=28 520) catalyzes the hydrolysis of phospholipids to yield a diacylglycerol and a phosphate ester with high specificity toward a positive polar group, such as choline, ethanolamine, and serine [1,2]. Unlike the mammalian phosphatidylinositol-PLC that plays an important role in Ca^{2+} regulation and signal transduction [3–6] the function of PC-PLC$_{Bc}$in vivo is not fully known other than its involvement in membrane metabolism. It has been suggested that this enzyme may replenish lost supplies of phosphate in the bacterial cell via a phosphate retrieval mechanism [7]. Since PC-PLC$_{Bc}$ is structurally related to mammalian PC-PLC that plays a role in cell growth and tumor formation [8], it can thus serve as a good model system for the poorly characterized mammalian enzymes [9–11]. Moreover, PC-PLC$_{Bc}$ is also a widely used tool in phospholipid research and thus contributes significantly to the fields of membrane biochemistry, lipid metabolism, and blood coagulation [12–14].

The X-ray crystal structures of PC-PLC$_{Bc}$ and its inhibitor complexes have been solved [15–17]. The active site was revealed to contain three Zn^{2+} ions (Fig. 1), wherein Zn1 and Zn3 in the active site form a dinuclear center (3.3 Å) bridged by Asp122 and a water (or hydroxide) molecule as observed in several other Zn proteins [18,19], including aminopeptidases and phosphotriesterase. Zn2 is not bridged to either Zn1 or Zn3 (with distances 6.0 and 4.7 Å, respectively), and has a coordination similar to carboxypeptidase A [20]. Although the crystal structure showed three bound Zn^{2+} ions in PC-PLC$_{Bc}$ [15], all previous physical studies of the enzyme in

*Corresponding author. Tel.: +1-813-974-2220; fax: +1-813-974-1733.

E-mail address: ming@chuma.cas.usf.edu (L.-J. Ming).

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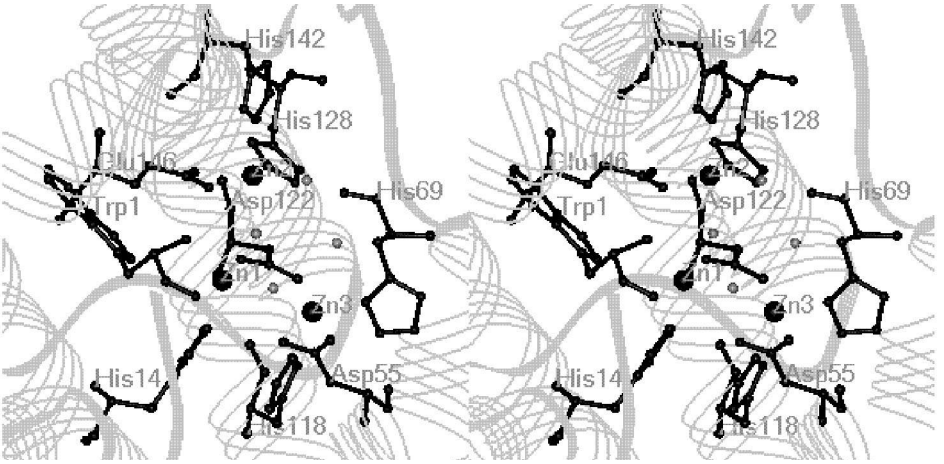

Fig. 1. The active site structure of PC-PLC$_{Bc}$ based on the crystal structure (Protein Data Bank code 1ah7). It is very similar to that of nuclease P1, in which Glu146 at the Zn2 site is replaced by Asp153 in nuclease P1. The coordinated water molecules are represented by small spheres.

solution indicated the presence of only two metal ions per molecule, including atomic absorption [21], EPR [22], EXAFS [23], and ^{113}Cd NMR [24] studies. One explanation for the discrepancy is attributed to the removal of loosely bound metal ions in previous experiments. Since all of the previous studies used an incorrect molecular weight of 23 kDa, a correction changes the metal content from 2 to 2.3 Zn^{2+}/molecule.

Several other metal ions have been shown to bind to PC-PLC$_{Bc}$, including Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, and Ag^{+} [25,26]. Metal substitution was found to decrease the activity of PC-PLC$_{Bc}$ with the effectiveness that follows the trend $Cu^{2+}>Ni^{2+}>Cd^{2+}>Co^{2+}>Hg^{2+}>Ag^{+}>Mn^{2+}$ toward lecithin substrates which may also reflect the trend of metal binding affinity with PLC [26]. A Co^{2+} derivative of PC-PLC$_{Bc}$ was prepared by dialyzing the native enzyme against low concentrations of Co^{2+} (1–5 mM), resulting in exchange of one high-affinity Zn^{2+} with Co^{2+}. The Zn site that did not exchange with Co^{2+} was referred to as the 'structural site' while the exchangeable site was considered the 'catalytic site', possibly Zn2 [25]. However, a recent mutagenesis study indicates that Glu146 in the Zn2 site is not involved in the activation of the nucleophilic water [27]. Furthermore, a molecular dynamic study suggests that Asp55 may act as a general base to deprotonate a water molecule for nucleophilic attack instead of Zn2 since there are no coordinated water molecules directly bound to the three Zn^{2+} when a substrate inhibitor is bound [28]. Co^{2+}-substituted derivatives were also prepared by adding one or two equivalents of Co^{2+} to apo protein [25]. Electronic absorption and EPR studies suggest that the Co^{2+} in PC-PLC$_{Bc}$ has a distorted octahedral coordination sphere in both high-affinity metal binding sites as well as the presence of a magnetic coupling between two paramagnetic Co^{2+} centers in Co_2-PLC$_{Bc}$ [22]; however, X-ray crystal structure shows five-coordinate geometries [15]. In this report, we discuss ^{1}H NMR studies of Co^{2+}- and Cu^{2+}-substituted derivatives of PC-PLC$_{Bc}$. The binding patterns of these two metal ions are suggested based on the spectral features. A stable tri-Cu^{2+}-substituted PC-PLC$_{Bc}$ has been prepared and characterized with NMR and EPR spectroscopies, which represents the first trinuclear Cu^{2+} center in proteins studied by the use of NMR spectroscopy.

2. Experimental section

2.1. Sample preparation

The isolation of PC-PLC$_{Bc}$ followed the literature procedure [29] with some modifications. In brief, *B. cereus* strain ATCC 10987 (AB-1) was grown for 15–17 h and the bacterial supernatant collected. The enzyme in the supernatant was bound to agarose-linked egg-yolk lipoprotein (prepared by cross-linking egg-yolk lipoprotein to BrCN-activated Sepharose 4B), which was packed into a column and washed with 15 mM Hepes buffer at pH 7.4 containing 0.15 M NaCl and 0.1 mM $ZnCl_2$ (Buffer A). The enzyme was then removed from the column with 8 M urea in the same loading buffer, then dialyzed against Buffer A without NaCl. The crude PC-PLC$_{Bc}$ was then loaded on to a DEAE-Sephadex A-50 column (2.5×15 cm) and eluted with a linear NaCl gradient from 0 to 0.35 M [29]. The fractions containing PC-PLC activity were concentrated and applied to a Sephadex G-75 size exclusion column (2.5×90 cm), and eluted with Buffer A. The purity of the enzyme after the steps was determined to have a specific activity of greater than 1200 U/mg, consistent with what is reported in the literature [30]. The concentration of PC-PLC$_{Bc}$ was determined using a spectrophotometer according to ε_{280}=51 000 M^{-1} cm^{-1} [30]. The PC-PLC$_{Bc}$ activity of the supernatant was determined by measuring the release of proton upon hydrolysis of phosphatidylcholine in egg yolk supernatant using a pH-stat automatic titrator (Metrohm model 702 SM Titrino)

with the pH kept at 7.4. One unit of PC-PLC$_{Bc}$ activity is defined as the amount of enzyme that liberates 1 μmol of H^+ per min at 37°C [30].

Since metal binding affinity of one of the metal sites is weak which results in only partial occupation by metal upon extensive dialysis as reported in the literature procedure [25], the Co-substituted derivative was prepared in the presence of an excess amount Co^{2+} (2–25 mM) followed by concentrating the samples with ultrafiltration. On the other hand, Cu^{2+} has a much higher affinity for the enzyme than the native Zn^{2+}, which results in complete exchange of Zn^{2+} for Cu^{2+} in the enzyme as described previously based on atomic absorption measurements [26]. The metal derivatives were concentrated to ~400 μl by means of ultrafiltration to give a concentration of ~1 mM for NMR experiments.

2.2. Nuclear magnetic resonance experiments

All ^{1}H NMR spectra were acquired on a Bruker AMX360 operating at 360.13 MHz. The ^{1}H chemical shifts were referenced to the internal HDO signal at 4.8 ppm. For the typical 1D NMR ^{1}H experiments, 10 000–20 000 transients were accumulated with a total recycle time of ~200 ms and 8 K data points. A line-broadening factor of 10–30 Hz was introduced to the spectra via exponential multiplication prior to Fourier transformation to enhance the signal-to-noise ratio. Water suppression was performed by either continuous irradiation of the solvent peak using the decoupler or the 'super-WEFT' [31] technique. Steady-state 1D NOE difference spectra were obtained with the computer alternately adding and subtracting the detected FIDs that were collected with the decoupler pulse set on a signal of interest and at a reference position, respectively, for 30–80 ms and followed by Fourier transformation.

3. Results and discussion

3.1. ^{1}H NMR of Co(II)-substituted PC-PLC$_{Bc}$

Incubation of the enzyme in Co^{2+} solutions results in the exchange of one Zn^{2+} with Co^{2+} as previously reported [25]. However, how the substituted Co^{2+} ions distributes in the three metal-binding sites cannot be recognized without further spectroscopic identification. The ^{1}H NMR spectrum of the Co^{2+} derivative is presented in Fig. 2. The downfield region greater than 30 ppm shows five broad isotropically shifted ^{1}H NMR signals. Two of

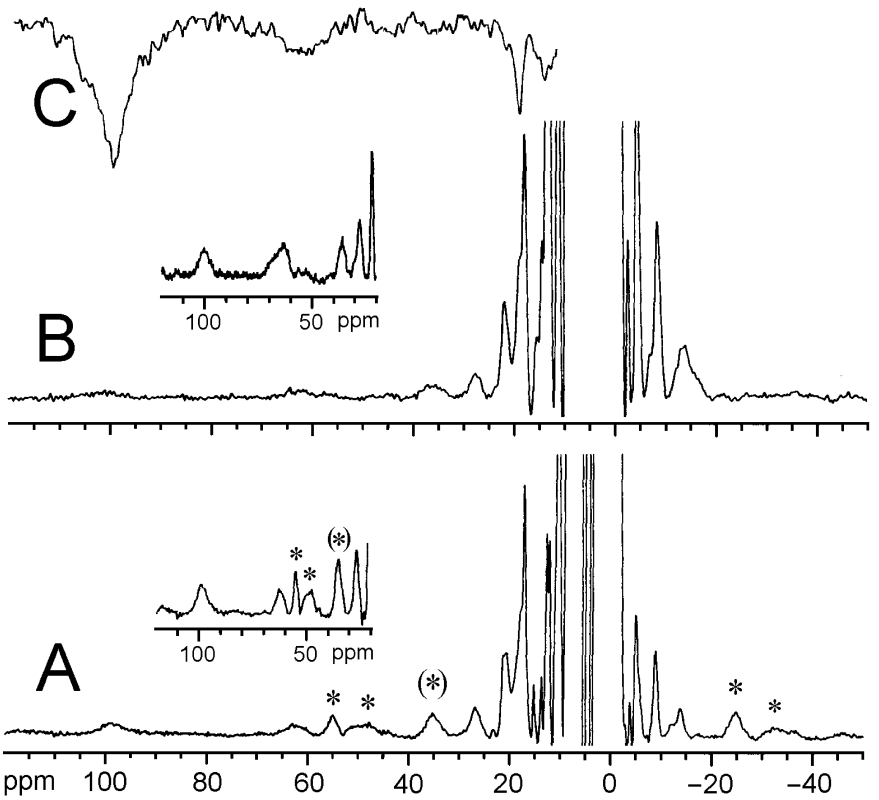

Fig. 2. Proton NMR spectra (360 MHz) of a Co^{2+}-substituted derivative of PC-PLC$_{Bc}$ in (A) H_2O and (B) D_2O buffers formed by dialyzing the native enzyme against 1–5 mM Co^{2+} solution in 15 mM Hepes, 150 mM NaCl, pH 7.4 buffer. The NOE difference spectrum (C) of the derivative in D_2O sample, with the signal at 100 ppm irradiated, is also shown. The insets are the downfield region of the spectra A and B which show the broad features more clearly.

these signals at 49.5 and 55 ppm disappear in D_2O buffer and are thus solvent exchangeable (marked with asterisks). The chemical shifts of these solvent exchangeable signals are in the range typical of *meta*-like His ring NH protons [32–34], thus suggesting the presence of at least two coordinated His in this derivative. Moreover, the other broad signals at 97, 63, and 35 ppm have typical linewidths that are characteristic of non-exchangeable *ortho*-like His ring C–H protons, despite their widely spread chemical shifts. The presence of hyperfine-shifted signals for two His suggests that either Zn2 or Zn3 is replaced by Co^{2+}. Zn1 has only one bound His according to the crystal structure and can thus be eliminated. Furthermore, the Zn3 site can be excluded since a relatively sharp hyperfine-shifted ^{1}H NMR signal of a *meta*-like ring C–H proton with a linewidth like that of the ring N–H signal would be expected for its δ-coordinated His-69. Therefore, the data are more consistent with the replacement of Zn2 by Co^{2+}.

The overall downfield spectral features of the Co^{2+}-derivative of PLC resemble that of the Co^{2+}-substituted alkaline phosphatase (AP_i) [35]. Five downfield shifted signals greater than 30 ppm are observed in E,E,Co-AP_i, including two solvent exchangeable signals at ~53 and 68 ppm. The hyperfine shifted signals in the Co^{2+}-substituted AP_i correspond to two ε-coordinated His and one Asp side chains. Therefore, it is reasonable to suggest that the hyperfine shifted signals of the Co^{2+}-substituted PC-PLC_{Bc} represent similar ligands as in E,E,Co-AP_i. The Zn site in PC-PLC_{Bc} that most closely matches the Co^{2+}-occupied site in E,E,Co-AP_i is the Zn2 site with two ε-coordinated His and one Glu ligand, supporting the view that only the Zn2 has been replaced by Co^{2+}.

It should be noted, however, that the intensity of the NOE (~−10 to −15%) observed between the 97 and 63 ppm signals with $T_1<5$ ms is strong and could be attributed to a geminal pair with an internuclear distance of ~1.9 Å, such as the β-CH_2 and γ-CH_2 protons of aspartate and glutamate ligands, respectively. Therefore, these two shifted signals could represent the γ-CH_2 protons of Glu146 at the Zn2 site. However, smaller downfield shifts are generally expected for the protons next to a coordinated carboxylate, such as the β-CH_2 protons of the coordinated Asp observed in the Co^{2+} derivatives of bovine and human copper–zinc superoxide dismutase (~35–45 ppm) [36]. A slight decrease in signal intensity for the signals at 35 and 27 ppm in a D_2O sample could indicate the presence of additional overlapped solvent exchangeable signals (insets in Fig. 2), supporting the replacement of Zn ions from two different sites by Co^{2+}. Therefore, the possibility that both Zn1 and Zn3 are replaced by Co^{2+} cannot be excluded. In this case, it would be reasonable to suggest that these two NOE-correlated signals may represent the bridging Asp-122 β-CH_2 protons. These protons could be shifted farther downfield than would be expected as a result of coordinating to two paramagnetic ions [37]. There are several hyperfine shifted ^{1}H NMR signals in the region of 30–100 ppm in the spectrum of di-Co^{2+} substituted ribonucleotide reductase [38], of which one or more signals can be attributed to the coordinated Asp/Glu. This spectral similarity between di-Co^{2+} ribonucleotide reductase and the Co^{2+} derivative of PLC here suggests that Co^{2+} might occupy the dinuclear Zn1–Zn3 site in PLC. The broadness of the signals in the Co^{2+}-derivative of PLC hinders further study and thus the assignment is tentative, which is also partially due to the lack of sufficient examples in the literature for different modes of carboxylate coordination. Nevertheless, the replacement of the metal ions in the dinuclear site by Cu^{2+} has been unambiguously determined (discussed in next section).

Two solvent exchangeable signals are found in the upfield region at −20 to −40 ppm in the Co^{2+}-substituted PC-PLC_{Bc}. These signals probably correspond to labile backbone amide NH protons. For example, the Gly45 backbone NH of azurin [39] and the peptidyl NH protons in Co^{2+}-bound peptide antibiotic bacitracin are all found upfield shifted [40]. Moreover, the relatively sharp solvent exchangeable signal at −24.5 ppm might be attributable to the backbone amido-NH proton of Ser2 at 4.2 Å from Zn1, which may gain upfield shift via a spin polarization mechanism [32,34,41] as a result of the coordinated carbonyl group of Trp1 (Fig. 1). This assignment supports the substitution of Co^{2+} for the dinuclear Zn1–Zn3 site. The large number of resonances found between 30 and −10 ppm reflects the presence of sizeable magnetic anisotropy which afford the significant dipolar shifts [32,34], characteristic of distorted five- and six-coordinate Co^{2+} ion as found in the Zn sites of PC-PLC.

Nuclease P1 from the mold *Penicillum citrinum* is a tri-zinc glyco-enzyme (M_r=36 kDa) which hydrolyzes RNA and single-stranded DNA into phosphate and 5′-nucleosides [42]. The active site structures of nuclease P1 and PC-PLC_{Bc} are almost identical (cf. Fig. 1), with a subtle difference found at Zn2 site where the coordinated Glu in PLC is replaced with an Asp in nuclease P1. A mono-Co^{2+}-substituted derivative of nuclease P1 was previously prepared and studied with ^{1}H NMR spectroscopy [43]. Since the active site structure of nuclease P1 is similar to that of PC-PLC_{Bc}, it is instructive to compare the ^{1}H NMR spectra of the Co^{2+}-substituted derivatives of both enzymes. The ^{1}H NMR spectrum of the Co^{2+}-substituted nuclease P1 shows only one solvent exchangeable N–H signal (50.4 ppm) and three broad C–H signals (33, 60 and 78.2 ppm) in the downfield region >30 ppm [43], which suggests that the Zn^{2+} undergoing exchange with the Co^{2+} has only one ε-coordinated histidine, i.e. the N-terminal Zn1 site (cf. Fig. 1). The large number of upfield hyperfine shifted signals also indicates a large magnetic anisotropy that is characteristic of distorted five- or six-coordinate Co^{2+} centers. Despite the structural similarity of these two enzymes, the isotropically shifted ^{1}H NMR spectra of their Co^{2+} derivatives are different in

overall features, which reflects their different Co^{2+} binding properties. In order to gain better understanding about metal binding property of PC-PLC_{Bc}, Cu^{2+} has been used as a probe for further studies discussed below. Since Cu^{2+} is known to bind PL-PLC_{Bc} tightly [26], any weak binding that cannot be revealed for other metal ions can potentially be seen in Cu^{2+} binding.

3.2. ^1H NMR of Cu^{2+}-substituted PC-PLC_{Bc}

Although Cu^{2+} was reported to replace all the Zn^{2+} ions in this enzyme [25], we found that two different Cu^{2+}-substituted derivatives of PC-PLC_{Bc}, a 'transient derivative' and a 'stable derivative', can be detected by means of ^1H NMR spectroscopy. The 'transient derivative' is formed during initial incubation of the native enzyme against an excess amount of Cu^{2+}, which changes completely into the final 'stable derivative' within 2 days. The formation of the transient derivative is demonstrated by the observation of several relatively sharp isotropically shifted ^1H NMR signals in the 110 to −20 ppm range (Spectrum A, Fig. 3). Cu^{2+} ion has not been commonly used as a ^1H NMR probe in the study of proteins owing to its unfavorably slow electronic relaxation time (1–3 ns) which induces excessive line broadening in ^1H NMR signals in almost all the cases [32,34,44]. Recently, a few type-1 Cu^{2+} centers in proteins have been shown to exhibit relatively sharp hyperfine-shifted ^1H NMR features owing to their unique trigonally distorted tetragonal geometry [45–47]. However, such sharp features are not expected to occur in type-2 Cu^{2+} centers. Conversely, magnetically coupled Cu^{2+} systems can show relatively sharp isotropically shifted ^1H NMR signals, as shown in a few recent studies on di-Cu^{2+} complexes [48–52]. Therefore, at least two Zn^{2+} in PC-

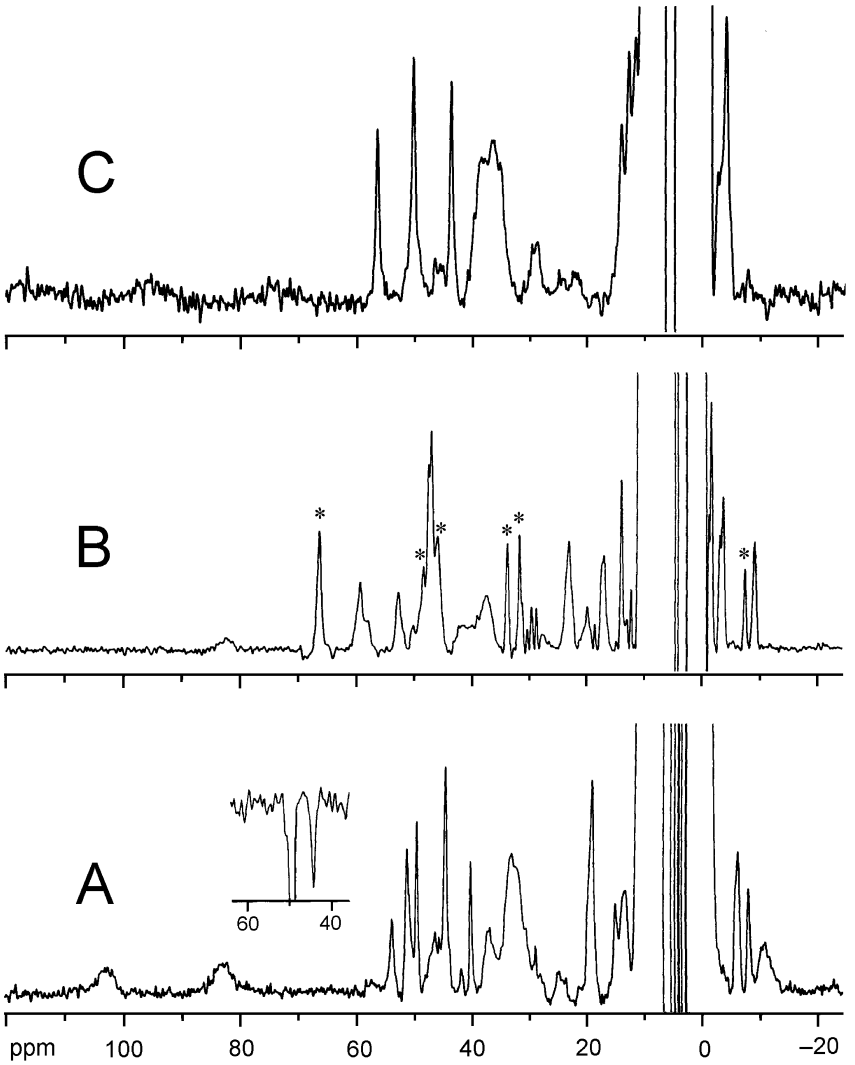

Fig. 3. Proton NMR spectra (360 MHz) of the 'transient' Cu_2Zn-PLC_{Bc} (A) and the final 'stable' Cu_3-PLC_{Bc} (B) in 15 mM Hepes, 150 mM NaCl, pH 7.4 buffer solution in the presence of an excess amount of Cu^{2+}, and of Cu_3-PLC_{Bc} (C) in the presence of an excess amount of the substrate 1,2-dicaproyl-*sn*-glycero-3-phosphocholine. The inset in (A) shows cross relaxation between the two signals at 49.1 and 44.2 ppm. The solvent exchangeable signals in (B) are labeled with asterisks.

PLC_{Bc} *must* have been replaced by Cu^{2+} to produce this 'transient derivative', dubbed Cu_2Zn-PLC_{Bc}, with a magnetically coupled di-Cu^{2+} center in order to show the relatively sharp isotropically shifted ^1H NMR signals.

These isotropically shifted ^1H signals in the transient derivative can be reasonably attributed to replacement of the dinuclear Zn1–Zn3 site bridged by Asp122 and a water or a hydroxide (Fig. 1) to afford a magnetically coupled dinuclear Cu^{2+} center. A similar μ-carboxylate–μ-hydroxyl bridged dinuclear Zn^{2+} center was also recently revealed in the aminopeptidases from *Streptomyces* [53,54] and *Aeromonas* [55], which were shown to exhibit well defined isotropically shifted ^1H NMR features upon replacement of the two Zn^{2+} ions with Cu^{2+} to afford a di-Cu^{2+} center [56,57]. The native di-Cu^{2+} enzyme tyrosinase has also been shown to exhibit sharp ^1H NMR features [58]. The Zn2 and Zn3 sites (separated by 4.7 Å) might also be a potential pair for the formation of a dinuclear metal center since their distance was found to slightly decrease by 0.2 Å upon PO_4^{3-} binding which bridges the two Zn ions as shown in the crystal structure [16]. Nevertheless, the long Zn2–Zn3 distance and the lack of a well-defined bridging ligand (Fig. 1) make these two sites less likely to form the NMR-active di-Cu^{2+} site.

A complete assignment of the isotropically shifted ^1H NMR signals of this transient Cu_2Zn-PLC_{Bc} has not been achieved owing to the complete conversion of this derivative to another derivative within 2 days in the presence of an excess amount of Cu^{2+}, a condition required for complete Cu^{2+}-substitution to occur. Nevertheless, several cross relaxations among some isotropically shifted signals have been observed, particularly a strong NOE interaction between the pair at 49.2 and 44.3 ppm possibly due to a geminal pair (inset, Fig. 3A), such as the $C_\beta H_2$ of Asp55 in the Zn1 site or the bridging Asp122.

The stable terminal derivative (dubbed Cu_3-PLC_{Bc}) with all the three Zn^{2+} ions replaced by Cu^{2+} is obtained with further incubation of Cu_2Zn-PLC_{Bc} in an excess amount of Cu^{2+}, or with extensive dialysis of PC-PLC_{Bc} against Cu^{2+} as demonstrated in a previous study with activity assay and atomic absorption spectroscopy [25]. Again, the presence of magnetic coupling in Cu_3-PLC_{Bc} can be demonstrated by the detection of relatively sharp isotropically shifted ^1H NMR signals in the range of 90 to −20 ppm (Fig. 3B). The spectral features are completely different from those of Cu_2Zn-PLC_{Bc} (Fig. 3A), indicating that the di-Cu^{2+} center in the transient Cu_2Zn-PLC_{Bc} is affected by the third Cu^{2+} site structurally or/and magnetically. The well defined sharp ^1H NMR features reflect the presence of magnetic coupling in this derivative. The EPR spectrum of this derivative after the removal of the excess Cu^{2+} shows $g_\parallel=2.22$, $g_\perp=2.06$ and $A_\parallel=185$ G (19.2×10^{-3} cm^{-1}), indicative of an overall $S'=1/2$ spin system (Fig. 4). The $g_\parallel$ and $A_\parallel$ values of the EPR spectrum are consistent with a nitrogen-rich coordination sphere [59], as expected in PC-PLC_{Bc} (Fig. 1). The overall features of this

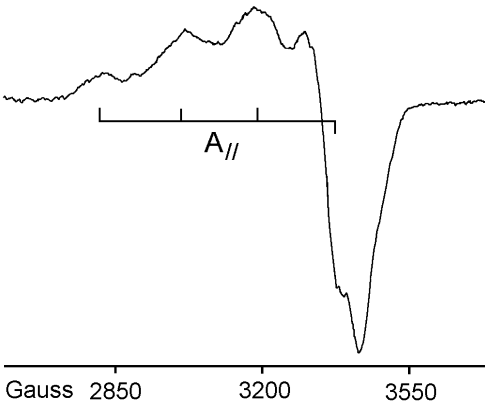

Fig. 4. The X-band EPR spectrum of the stable Cu_3-PLC_{Bc} at 9.5486 GHz and 77 K (Bruker ER200D spectrometer at UCLA). The parameters $g_\parallel=2.22$, $g_\perp=2.06$ and $A_\parallel=185$ G (19.2×10^{-3} cm^{-1}) indicate the presence of an overall $S'=1/2$ spin system.

EPR spectrum are different from those of tetragonally distorted mononuclear Cu^{2+} centers in complexes and proteins; however, the g and $A_\parallel$ values are consistent with the presence of a 'type-2' Cu^{2+} center [60]. This is in agreement with the formation of a trinuclear Cu^{2+} center in which the antiferromagnetically coupled di-Cu^{2+} of $S'=0$ is further coupled with the third 'type 2' Cu^{2+} center of $S=1/2$ to yield a spin system with a ground state $S'=1/2$ [61,62].

Several solvent exchangeable signals are detected in the ^1H NMR spectrum of Cu_3-PLC_{Bc} at 66.4, 48.2, 46.1, 33.9, and 31.8 ppm (Fig. 3B), which can be assigned to the ring NH protons of five-coordinated His residues in the active site (Fig. 1). The detection of five-coordinated His residues is consistent with that observed in the crystal structure, which also indicates that all the three Zn^{2+} sites are occupied by Cu^{2+} to afford the final stable derivative Cu_3-PLC_{Bc} with a magnetically coupled trinuclear Cu^{2+} center. The detection of an upfield-shifted solvent-exchangeable signal is also consistent with a coordinated Trp-1 through the backbone carbonyl group, which gives rise to the upfield-shifted peptidyl NH signals via a spin polarization mechanism [32,34]. Attempts to acquire through-bond COSY and TOCSY and through-space NOESY spectra of this derivative have not been successful, which prevents a full assignment of this unique ^1H NMR spectrum. Nevertheless, this primary ^1H NMR study of a trinuclear Cu^{2+} center in protein represents the first of its kind, and worth further investigation.

3.3. ^1H NMR of Cu_3-PLC_{Bc} with a bound substrate

The catalytic inertness of Cu_3-PLC_{Bc} allowed the study of its interaction with substrate by means of NMR spectroscopy. This idea was previously applied to the studies of several other inert enzyme–substrate ES systems, such as Cu^{2+}-carbonic anhydrase [63] and Co^{2+}- and Cu^{2+}-substi-

tuted isopenicillin N synthase [64,65], by the use of different spectroscopic methods. The isotropically shifted ^{1}H NMR spectrum of Cu_3-PLC_{Bc} is dramatically changed upon the addition of an excess amount of the substrate 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (Fig. 3C). Since the substrate is not hydrolyzed by the Cu_3-PLC_{Bc} derivative, the change of the spectrum is thus indicative of the formation of a stable ES complex. This observation has provided significant insight into substrate binding and the mechanism of PC-PLC_{Bc} that otherwise cannot be observed conveniently on the native enzyme. For example: (a) The dramatic change of the spectral features indicates direct substrate binding to the metal centers likely through the phospho-group of the substrate, suggested in previous crystallographic study of the enzyme–inhibitor complexes [16,17] and molecular modeling study [66]. However, a recent molecular dynamic calculation [28] study suggests that the substrate is not directly coordinated to the metal, which is attacked by a nucleophilic water activated by the coordinated Asp-55. (b) All the signals are dramatically changed which suggests that all the three metal ions are involved in substrate binding, as observed in the crystal structure of the enzyme–inhibitor complexes [16,17]. (c) The activity of PC-PLC is significantly enhanced toward phospholipid micelles. Nevertheless, the observation of an ES complex indicates that the interaction of the enzyme with a single substrate must also be a key step in the interfacial catalysis by PLC. The formation of the ES complexes in enzymes has been indicated to have significant energetic contribution toward enzyme catalysis [67,68]. The observation of the well resolved isotropically shifted ^{1}H NMR spectrum of this inert ES complex suggests that it can serve as a useful model for the understanding of the transient Michaelis ES complex of the native enzyme, which cannot be studied conveniently.

4. Conclusion and perspectives

The detection of a magnetically coupled dinuclear Cu^{2+} center in Cu_2Zn-PLC_{Bc} indicates that the Zn^{2+} ions in the dinuclear (Zn1 and Zn3) metal-binding sites are more easily replaced by Cu^{2+} than the one in the mononuclear (Zn2) site, which might be also the case for Co^{2+} binding. We have also reported for the first time a rare case of a magnetically coupled tri-Cu^{2+} center of $S'=1/2$ in proteins which affords relatively sharp isotropically shifted ^{1}H NMR signals. There are a number of multinuclear proteins discovered in recent years which are involved in normal and disease-related biological processes [18,19]. The study presented here and a few studies reported previously [56–58], suggest that ^{1}H NMR spectroscopy can be a valuable tool for the characterization of proteins with magnetically coupled di- and multi-nuclear centers by the use of Cu^{2+} as an 'NMR friendly' probe.

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