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Analysis of the Non-covalent Interaction Between Metal Ions and the Cysteine-Rich Domain of Protein Kinase C Eta by Electrospray Ionization Mass Spectrometry

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Abstract—Effect of zinc and other metal ions on the folding of the protein kinase C (PKC) surrogate peptide (PKCη-C1B) was analyzed intact under neutral conditions by electrospray ionization mass spectrometry (ESI–MS). ESI–MS spectrum of $^{64}ZnCl_2$-folded PKCη-C1B clearly showed that PKCη-C1B coordinates specifically two atoms of zinc, and that the two thiol protons are lost in each zinc ion coordinate center. $^{113}CdCl_2$-folded PKCη-C1B also showed stoichiometry of two cadmium atoms that was proved by addition of EDTA. The dissociation constants of zinc- and cadmium-folded PKCη-C1B in the phorbol 12,13-dibutyrate binding (PDBu) were similar (0.66 and 0.81 nM) with different B_{max} values (46.4 and 71.4%). The difference would reflect higher coordination potency of cadmium ion that was demonstrated by ESI–MS when PKCη-C1B was folded by 1:1 mixture of zinc and cadmium ions. In contrast, $^{63}CuCl_2$-treated PKCη-C1B did not show any copper-coordinated peak, instead a molecular mass less than 6 mass units smaller than that of apo-PKCη-C1B was observed. The multiple charge mass envelope of copper-treated PKCη-C1B shifted to that of the lower mass charge state like zinc-treated PKCη-C1B. These data suggest that the copper treatment formed three intramolecular S–S bonds to abolish the PDBu binding of PKCη-C1B.

Introduction

Protein kinase C (PKC) isozymes are major targets of tumor-promoting phorbol esters and involved in a wide variety of cell surface signal transduction.[1–3] They consist of a regulatory domain with phorbol ester binding sites and a catalytic domain for protein phosphorylation (Fig. 1). The phorbol ester binding site is within the C1 domains (C1A and C1B),[4] and identified as a cysteine-rich sequence which has six conserved cysteines and two histidines in the pattern $HX_{12}CX_2CX_nCX_2CX_4HX_2CX_7C$ ($n = 13$ or 14).[5,6] Atomic absorption spectroscopy suggested that each cysteine-rich sequence coordinates two atoms of zinc.[7,8] However, determination of exact stoichiometry of zinc coordination is difficult as exemplified by the work of Quest and Bell[9] since precise concentration of the whole PKC isozymes is unknown.

We recently synthesized cysteine-rich sequences with 50–70 residues corresponding to all PKC isozyme C1 domains using an Fmoc solid-phase strategy.[10,11] These C1 peptides were successfully folded by zinc treatment, and bound phorbol 12,13-dibutyrate (PDBu) with dissociation constants (K_d's) in the nano molar range. We also found that treatment of PKC C1 peptides with cadmium ion resulted in potent PDBu binding as the corresponding zinc-treated C1 peptides.[10,12] In contrast, treatment with copper, silver, gold, or mercury completely abolished the PDBu binding. However, it is not known whether these metal effects are attributed to their coordination to the peptides or their irreversible oxidative ability. Elucidation of the mechanism of these metal effects is important since modulation of PKC by neurotoxic heavy metals has been reported by several groups.[13–16]

Electrospray ionization mass spectrometry (ESI–MS) can serve to analyze the non-covalent interactions of proteins with metal ions, ligands, peptides, oligonucleotides, or other proteins.[17] The most important information

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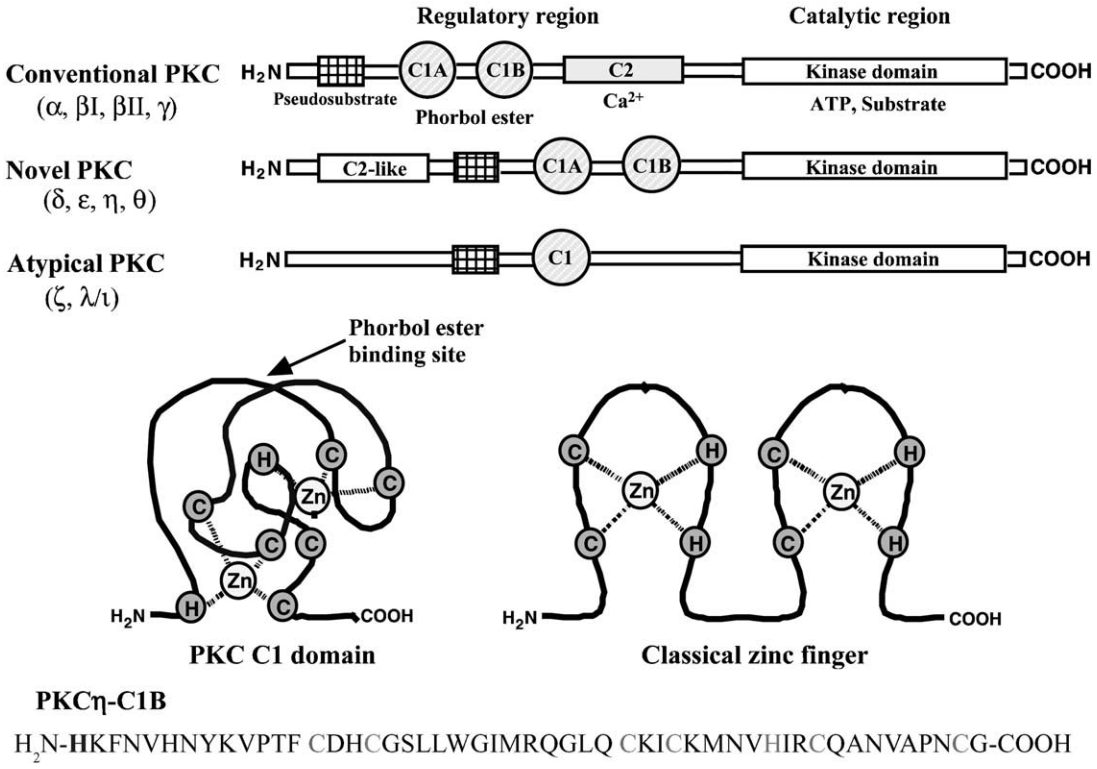

Figure 1. Illustrations of PKC isozymes along with a PKC C1 domain and a classical zinc finger. PKCη-C1B is a 51-mer peptide (mouse PKCη 246–296 + Gly).

obtained by ESI–MS is the stoichiometry of the components which form the complex since ESI–MS provides an accurate molecular mass of the complex being studied. However, there are only a few examples that strictly determine the stoichiometry of the components of the complex.[18,19] Low purity and complexity of the whole proteins due to post-transcriptional modifications are major reasons why accurate determination of the stoichiometry quite difficult. In contrast, our synthetic C1 peptides of PKC isozymes are especially suitable for these analyses since the metal-free C1 peptides can be easily obtained in high purity. It is almost impossible to prepare the metal-free whole PKC isozymes by DNA recombination techniques. Here, we would like to show that effects of metal ions on folding and phorbol ester-binding of the PKC C1 peptides can be analyzed precisely by ESI–MS.

Results and Discussion

Effects of zinc and other metal ions on PDBu binding of PKCη-C1B

There are 12 PKC C1 peptides which show potent PDBu binding affinity.[10,11] Among these peptides, we focused on PKCη-C1B (mouse PKCη 246–296 + Gly) because this peptide was obtained in high yield by an Fmoc solid-phase peptide synthesis, and its solubility in the neutral buffer solution is considerably higher than other PKC C1 peptides. Since potent PDBu binding of PKC C1 peptides occurs in the pH range between 6.0 and 8.0, ESI–MS measurements should be done under the neutral conditions. Use of PKC C1 peptides with poor solubility in the neutral buffer solution such as PKCγ-C1A, PKCγ-C1B, or PKCγ-C1A-C1B[20] often stopped the spray, making detailed analyses impossible.

Effects of zinc and other metal ions on the PDBu binding of PKCη-C1B are summarized in Figure 2. The zinc-treated PKCη-C1B (10 nM) showed potent specific PDBu binding (about 40,000 dpm), which was fixed at 100 as a reference (entry 1). Significant PDBu binding was observed in PKCη-C1B without zinc treatment (entry 2), but disappeared in the presence of 2 mM EDTA in the assay mixture (entry 16). On the other hand, the binding of zinc-treated PKCη-C1B did not change after exposure to 2 mM EDTA (entry 15), indicating that chelatable ions in the assay mixture could account for the background binding, and that the zinc coordination is not readily reversed by EDTA.

Treatment of PKCη-C1B with metal ions other than zinc, Cr^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, and Ca^{2+}, exhibited PDBu binding of the background level (entries 3–9). However, the specific binding completely abolished when treated with ions of group Ib elements such as Cu^{2+}, Ag^{+}, and Au^{+} (entries 10–12). Although the Hg^{2+} treatment eliminated the binding, the Cd^{2+} treatment resulted in significant PDBu binding which exceeded the level observed for zinc-treated PKCη-C1B (entries 13, 14). Moreover, the binding of Cd^{2+}-folded PKCη-C1B, as well as Zn^{2+}-folded, did not change significantly upon exposure to EDTA, indicating that the

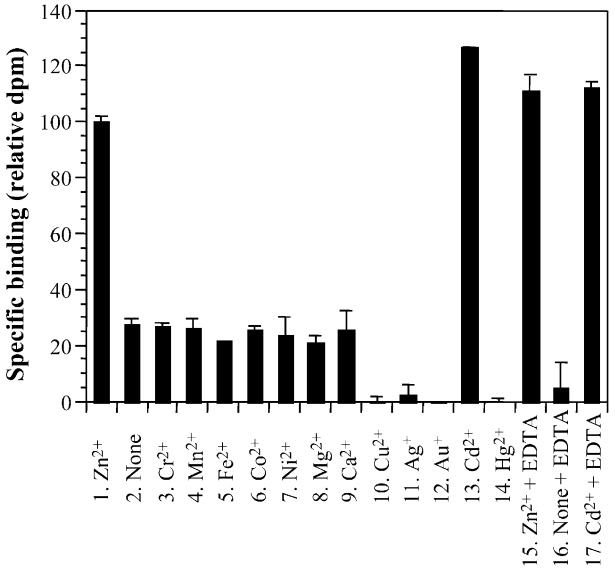

Figure 2. Effects of zinc and other metal ions on the PDBu binding of PKCη-C1B. Metal coordination and PDBu binding assay were carried out by the method described in the Experimental. The specific binding of the $ZnCl_2$-treated PKCη-C1B was fixed at 100 (entry 1). Entry 2: without $ZnCl_2$-treatment; entry 3: $CrCl_2$ treatment; entry 4: $MnCl_2$ treatment; entry 5: $FeSO_4(NH_4)_2SO_4$ treatment; entry 6: $CoCl_2$ treatment; entry 7: $NiCl_2$ treatment; entry 8: $MgCl_2$ treatment; entry 9: $CaCl_2$ treatment; entry 10: $CuCl_2$ treatment; entry 11: $AgNO_3$ treatment; entry 12: AuCN treatment; entry 13: $CdCl_2$ treatment; entry 14: $HgCl_2$ treatment; entry 15: $ZnCl_2$ treatment with 2 mM EDTA in the assay mixture; entry 16: without $ZnCl_2$-treatment with 2 mM EDTA in the assay mixture; entry 17: $CdCl_2$ treatment with 2 mM EDTA in the assay mixture. The bars represent standard deviation of at least three data points.

coordination of cadmium is also potent. We focused on the effects of cadmium and copper as typical examples and analyzed their interaction with PKCη-C1B using ESI–MS.

ESI–MS analysis of zinc-folded PKCη-C1B

ESI–MS analyses of metal–PKCη-C1B complex were performed under the neutral aqueous conditions (10 mM pyridinium acetate, pH 6.8) using Mariner™ (Applied Biosystems) as shown in Figure 3. Mariner™ is a high resolution ESI–MS equipped with time-of-flight (TOF) mass analyzer. In order to obtain more accurate molecular mass, monoisotopic metal ions ($^{64}Zn^{2+}$, $^{113}Cd^{2+}$, and $^{63}Cu^{2+}$) were used for the folding of PKCη-C1B. The ESI–MS spectrum of PKCη-C1B without zinc treatment had the center of mass envelope at +6 and +5 with +7 to +4 charge distribution (Fig. 3a). The molecular mass for apo-PKCη-C1B was 5814.6±0.5 (calculated average mass: 5814.9). Addition of 2 molar equivalents of $^{64}ZnCl_2$ (^{64}Zn: 63.93 Da) shifted the mass envelope to the lower charged state; the 4+ charge species was most abundant (Fig. 3b). This means that the zinc coordination induces significant conformational changes in PKCη-C1B to preserve higher order structure. Additional zinc (4 molar equivalents) did not further change the mass envelope (Fig. 3c), indicating that the 2 molar equivalents of zinc are sufficient for PKCη-C1B to preserve a proper fold. Addition of equimolar $^{64}ZnCl_2$ produced mainly the peak of one zinc atom-coordinated PKCη-C1B (calculated average mass: 5876.9) along with weak peaks of apo-PKCη-C1B and two zinc atoms-coordinated PKCη-C1B (data not shown).

The spectra of Figure 3b and c showed that the molecular mass of zinc-PKCη-C1B complex was 5938.9±0.4 and 5938.8±0.3, respectively, corresponding to two zinc atoms-coordinated PKCη-C1B (calculated average mass: 5938.7 for $M+2^{64}Zn^{2+}-4H^{+}$). This indicates that two zinc atoms coordinate specifically to PKCη-C1B. Although this zinc stoichiometry in PKCη-C1B coincided with that reported previously in whole PKC and some PKC C1 domains using atomic absorption spectroscopy,[7,8] ESI–MS gives a far more accurate number of specifically coordinated zinc. The present result is also in accord with the crystal structure of PKCδ-C1B which has two atoms of zinc.[21]

As shown above, molecular mass of the zinc-coordinated PKCη-C1B coincides with that of $M+2^{64}Zn^{2+}-4H^{+}$, indicating that the charges of the two zinc atoms contribute to the net charge of the zinc–PKCη-C1B complex. Hitherto, the protonation states of the zinc-coordinated cysteine ligands in zinc-finger peptides have not clearly been understood.[22,23] There is a possibility that the thiol protons are retained in the zinc ion coordinate centers or in the neighboring basic amino acid residues. This ambiguity would be partially due to the broadening and shape distortion of the isotopic distribution peak of the zinc–peptide complex[19] since three major isotopic species of zinc (^{64}Zn, ^{66}Zn, and ^{68}Zn) have almost similar relative abundances (48.9, 27.8, and 18.6%, respectively). Recently, Fabris et al.[24] have demonstrated by ESI–MS that two protons are lost for each zinc ion which is chelated in the three types of the classical zinc fingers,[25,26] CCCC, CCHC, and CCHH which is illustrated in Figure 1. The present work shows that two thiol protons are also lost in each zinc ion coordinate center of the RING finger protein family[27] like PKCη-C1B.

ESI–MS analysis of cadmium-folded PKCη-C1B

As for cadmium-folded PKCη-C1B, treatment with 2 or 4 molar equivalents of $^{113}CdCl_2$ (^{113}Cd: 112.90) induced the mass envelop shift to the lower charged state as observed for zinc-treated PKCη-C1B; the 4+ charge species was most abundant in each case (Fig. 4a and b), indicating the formation of a higher order structure. However, in the spectrum of excess cadmium-treated (4 molar equivalents) PKCη-C1B (Fig. 4b), the peak corresponding to the three atoms of cadmium coordination was observed significantly. In order to determine whether the third cadmium coordination is specific or not, ESI–MS was measured in the presence of 4 molar equivalents of EDTA (Fig. 4c). As mentioned in Figure 2, addition of EDTA in the assay mixture did not change the specific PDBu binding of the cadmium-folded PKCη-C1B (entry 17), suggesting that only tightly coordinated cadmiums are detected by ESI–MS in the presence of EDTA. Figure 4c distinctly shows that two cadmiums specifically bind to PKCη-C1B to preserve

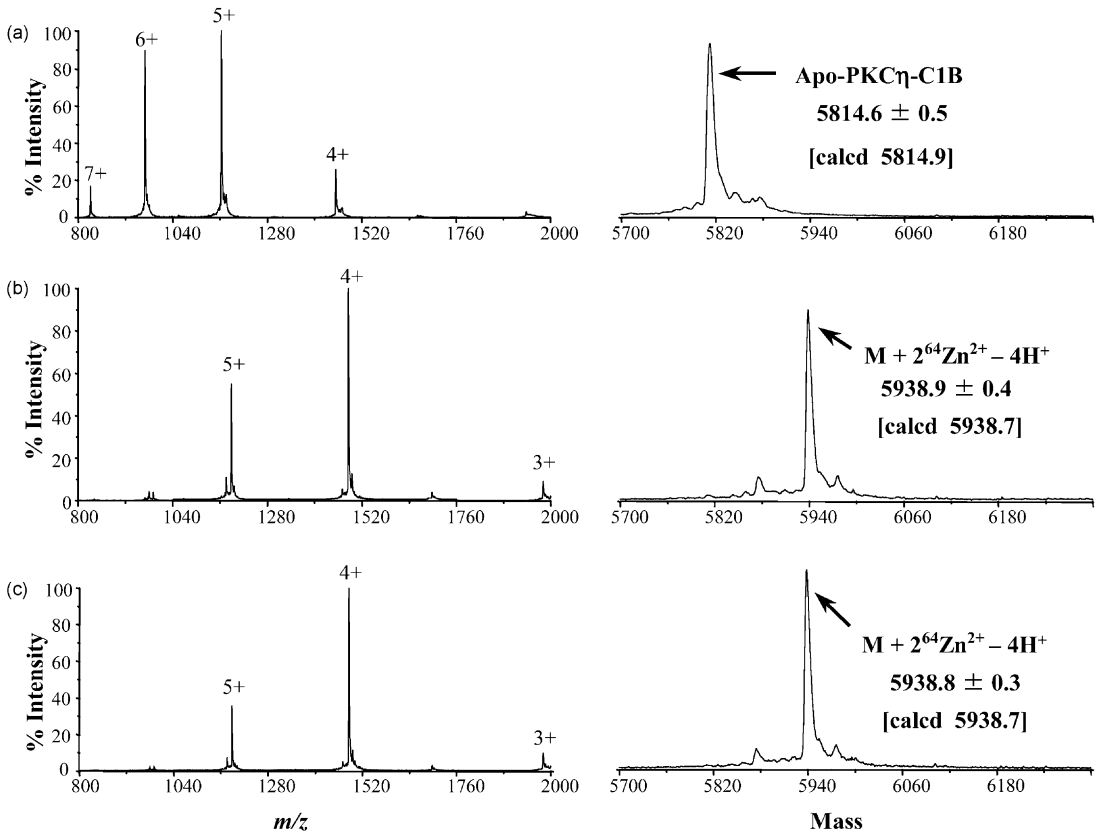

Figure 3. ESI-TOF-MS spectra of zinc-folded PKCη-C1B under the neutral aqueous solution (10 mM pyridinium acetate, pH 6.8): (a) PKCη-C1B without zinc treatment; (b) 2 molar equivalent $^{64}ZnCl_2$-treated PKCη-C1B; (c) 4-molar equivalent $^{64}ZnCl_2$-treated PKCη-C1B. M means calculated average mass of apo-PKCη-C1B (5814.9).

the proper fold for PDBu binding. The spectra of Figure 4a and b show that the molecular mass of cadmium–PKCη-C1B complex is 6036.9±0.1 and 6037.0±0.1, respectively, corresponding to two cadmium atoms-coordinated PKCη-C1B (calculated average mass: 6036.7 for $M+2^{113}Cd^{2+}-4H^{+}$). These results indicate that the stoichiometry of cadmium for PKCη-C1B folding is equal to that of zinc, and that two thiol protons are also lost in each cadmium ion coordinate center of PKCη-C1B.

Comparison of PDBu binding affinity between zinc-folded PKCη-C1B and cadmium-folded PKCη-C1B

Scatchard analysis of cadmium-folded PKCη-C1B for PDBu binding revealed a single binding site with K_d of 0.81±0.05 nM (B_{max}: 71.4±5.4%) as shown in Figure 5b. In a control experiment (Fig. 5a), K_d of zinc-folded PKCη-C1B was 0.66±0.06 nM (B_{max}: 46.4±2.1%). There is no significant difference between these K_d values, which are in good agreement with that reported for whole PKCη (0.58 nM).[28] However, the B_{max} value of cadmium-folded PKCη-C1B was remarkably high, suggesting that cadmium ion effectively folded PKCη-C1B. To interpret these data, PKCη-C1B folded by 1:1 mixture of $^{64}ZnCl_2$ and $^{113}CdCl_2$ was analyzed by ESI–MS. As shown in Figure 4d, the two cadmium atoms-coordinated peak (6037.0±0.1; calculated for $M+2^{113}Cd-4H^{+}$: 6036.7) was predominant while the two-zinc coordinated peak (5938.7) was hardly detected, indicating that cadmium coordinates more strongly than zinc to PKCη-C1B. An extremely high B_{max} value in the Scatchard analysis of cadmium-folded PKCη-C1B would reflect the strong coordinating potency of cadmium ion compared with zinc ion. However, zinc ion can coordinate one of the two binding sites in PKCη-C1B with equal potency to cadmium ion since one zinc- and one cadmium-coordinated peak (5987.9±0.1; calculated for $M+^{113}Cd^{2+}+^{64}Zn^{2+}-4H^{+}$: 5987.7) was significantly detected by ESI–MS.

ESI–MS analysis of copper-treated PKCη-C1B

In contrast to zinc- or cadmium-treated PKCη-C1B, 4 molar equivalent copper-treated PKCη-C1B did not give any copper-coordinated peaks (Fig. 6a). This indicates that coordination of copper to PKCη-C1B is not the main reason for inhibition of the PDBu binding (Fig. 2, entry 10). The spectrum of copper-treated PKCη-C1B gave a molecular mass less than 6 mass units (5809.1±0.1; calculated for M−6H: 5808.9) from that of apo-PKCη-C1B (5814.9) under the neutral conditions (Fig. 6a). This spectrum did not change even under the acidic conditions (molecular mass: 5809.0±0.1), suggesting that the conformation of copper-treated PKCη-C1B under the acidic conditions is quite similar to that under the neutral conditions (Fig. 6b). In a control experiment, PKCη-C1B without copper treatment was directly analyzed under the acidic conditions (Fig. 6c). The spectrum showed that the

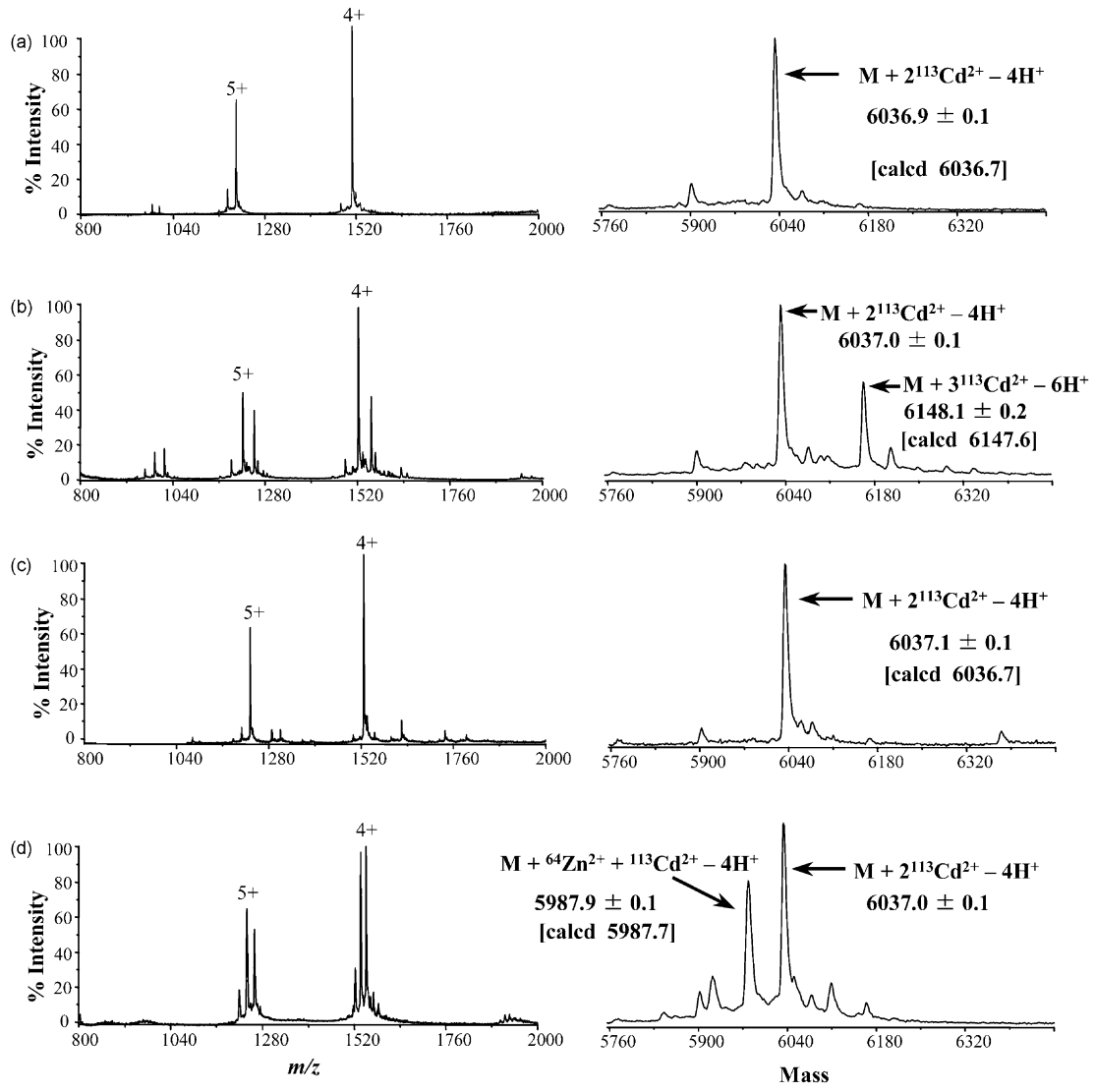

Figure 4. ESI-TOF-MS spectra of cadmium-folded PKCη-C1B under the neutral aqueous solution (10 mM pyridinium acetate, pH 6.8): (a) 2 molar equivalent $^{113}CdCl_2$-treated PKCη-C1B; (b) 4 molar equivalent $^{113}CdCl_2$-treated PKCη-C1B; (c) 4 molar equivalent $^{113}CdCl_2$-treated PKCη-C1B in the presence of 4-molar equivalent of EDTA; (d) PKCη-C1B folded by 1:1 mixture of 2 molar equivalent $^{64}ZnCl_2$ and $^{113}CdCl_2$. M means calculated average mass of apo-PKCη-C1B (5814.9).

molecular mass of PKCη-C1B (5814.6±0.5) coincides with that of the theoretical value (5814.9). However, the multi-charged mass envelop of Fig. 6c (higher charged state), was significantly different from that of Fig. 6b (lower charged state), indicating that copper-treated PKCη-C1B has a higher order structure than control PKCη-C1B without copper treatment. Since PKCη-C1B has six cysteine-residues, the present results strongly suggest that copper-treatment resulted in the formation of three intramolecular S–S bonds to make PKCη-C1B inactive in the PDBu binding assay. It is reported that the copper ion inhibits the enzyme activity of a conventional PKC mixture and its PDBu binding possibly by interacting with the catalytic domain of PKC.[13,14] However, our present data using ESI–MS have unambiguously shown that the copper ion directly oxidizes the cysteine-rich sequences to abolish its PDBu binding affinity. Inactivation by group Ib element ions other than copper (silver and gold ions) could also be understood by a similar oxidation mechanism.

Conclusion

We have shown that ESI–MS coupled with the synthetic functional domains of enzymes is a promising method to analyze precisely the metal ion–functional domain interaction. By using a synthetic surrogate peptide of PKC, such as PKCη-C1B, metal effects on the PDBu binding of PKC C1 domains could be understood at a molecular level. Such an approach can be applicable not only to other PKC C1 homology domains[4] but also to other functional domains related to metal coordination.

Experimental

General procedures

The following spectroscopic and analytical instruments were used: electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), Mariner™ (Applied

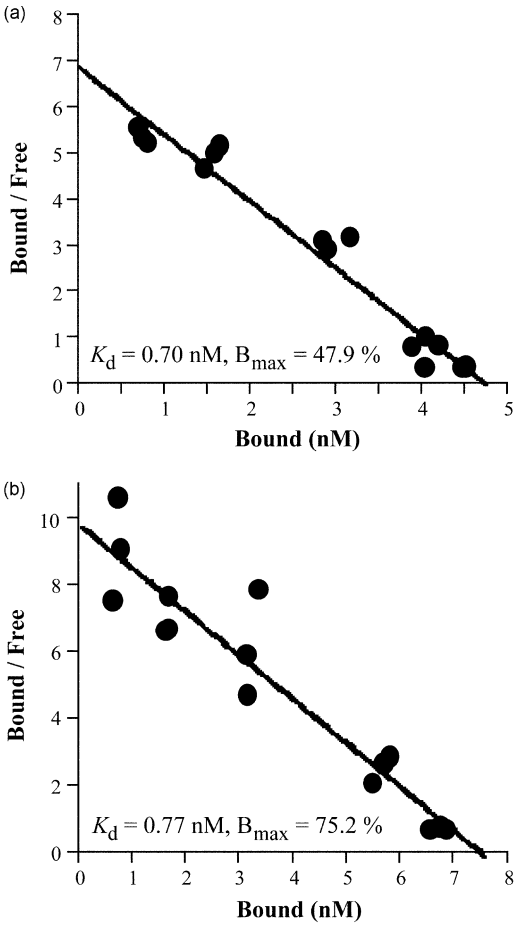

Figure 5. Scatchard analysis of PKCη-C1B folded by: (a) $^{64}ZnCl_2$ or (b) $^{113}CdCl_2$ in PDBu binding. PKCη-C1B folded by $^{64}ZnCl_2$ or $^{113}CdCl_2$ was incubated with increasing concentrations of [^{3}H]PDBu in the presence of 50 μg/mL phosphatidylserine dioleoyl and 3 mg/mL bovine γ-globulin at 4 °C for 10 min as described in the Experimental. One representative experiment is shown. Similar results were obtained in other experiments.

Biosystems, CA, USA); peptide synthesizer, Pioneer™ peptide synthesizer Model 9030 (Applied Biosystems); HPLC, Waters Model 600E with Model 2487 UV detector; HPLC was carried out on a YMC-packed SH-342-5 (ODS, 20 mm i.d.×150 mm) column (Yamamura Chemical Laboratory) for preparative purposes. [^{3}H]PDBu (17.0 Ci/mmol) was purchased from Perkin-Elmer Life Science. $^{64}ZnCl_2$, $^{113}CdCl_2$, and $^{63}CuCl_2$ were obtained from ISOTEC inc. Unless otherwise noted, reagents were purchased from Sigma, Wako Pure Chemical Industries, or Nacalai Tesque. PKCη-C1B was synthesized by the method reported previously.[10,11]

[^{3}H]PDBu binding assay of PKCη-C1B

The PDBu binding assay was carried out using the procedure of Sharkey and Blumberg[29] with slight modification.[10,11] The standard assay mixture (250 μL) in 1.5-mL Eppendorf tube contained 50 mM Tris–maleate (pH 7.4), 50 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine [phosphatidylserine dioleoyl], 3 mg/mL bovine γ-globulin, 20 nM [^{3}H]PDBu (17.0 Ci/mmol), and 10 nM PKCη-C1B. For determination of PDBu saturation curves for Scatchard analysis, concentrations of free [^{3}H]PDBu between 1 and 20 nM were used.

Metal coordination was carried out in a helium-purged distilled water solution (pH 5.5–6.0) of PKCη-C1B. Five mol equivalents of 10 mM $ZnCl_2$, $CdCl_2$, or other metal salts in helium-purged distilled water were added to the PKCη-C1B in a helium-purged distilled water solution, which was allowed to stand at 4 °C for 10 min. After 10 μL of the above peptide solution (172 μM) was diluted with 990 μL of helium-purged distilled water, the resultant solution (1.5 μL) was added to the standard assay mixture described above (248.5 μL), and the solution was incubated at 30 °C for 20 min. For Scatchard analysis, incubation temperature was set at 4 °C for 10 min. To the tubes was added 187 μL of 35% (w/w) poly-(ethyleneglycol) (average molecular weight, 8000), and the mixture was vigorously stirred. The tubes were stood at 4 °C for 10 min, and then centrifuged for 10 min at 12,000 rpm in an Eppendorf microcentrifuge at 4 °C. A 50-μL aliquot of the supernatant of each tube was removed, and its radioactivity was measured to determine the free [^{3}H]PDBu concentration. The remainder of the supernatant of each tube was removed by aspiration. The tips of the tubes were cut off, and the radioactivity in the pellets was measured to determine the bound [^{3}H]PDBu. Specific binding represents the difference between the total and nonspecific binding. The nonspecific binding for each tube was calculated from its measured free [^{3}H]PDBu concentration and its partition coefficient to the pellet (about 3%).

ESI–MS analysis of metal–PKCη-C1B complex

For ESI–MS sample preparation, we used a synthetic PKCη-C1B peptide and monoisotopic metal chlorides ($^{64}ZnCl_2$, $^{113}CdCl_2$, and $^{63}CuCl_2$) for accurate mass measurement. PKCη-C1B in ultra pure water solution (100 μM) was treated with 0–4 molar equivalents of each metal chloride (0.5 mM in ultra pure water). After incubation at 4 °C for 10 min, each solution was neutralized with 50 mM of pyridinium acetate buffer (pH 6.8), and then the PKCη-C1B concentration was adjusted to 25 μM in 10 mM pyridinium acetate. For ESI-MS spectrum in the presence of EDTA (Fig. 4c), 4 molar equivalents of 1.0 mM EDTA (pH 6.8) were added finally to the cadmium-folded PKCη-C1B solution prepared by the method described above. For ESI–MS under acidic conditions (Fig. 6b and c), PKCη-C1B solution with or without copper treatment was added to 50% acetonitrile in water containing 1% acetic acid to adjust the PKCη-C1B concentration to 12.5 μM.

The mass spectra were acquired using Mariner™ (Applied Biosystems). For ESI–MS analysis in the neutral aqueous conditions, 25-μL aliquots of 25 μM PKCη-C1B samples in 10 mM pyridinium acetate buffer (pH 6.8) were injected into the source at a flow rate of 4 μL/min using a syringe pump. In acidic conditions, 25-μL aliquots of 12.5 μM PKCη-C1B samples in 50% acetonitrile in water containing 1% acetic acid were used. The 3.5 kV of the counter-electrode was applied to the spray tip. The interfacing nozzle was heated to

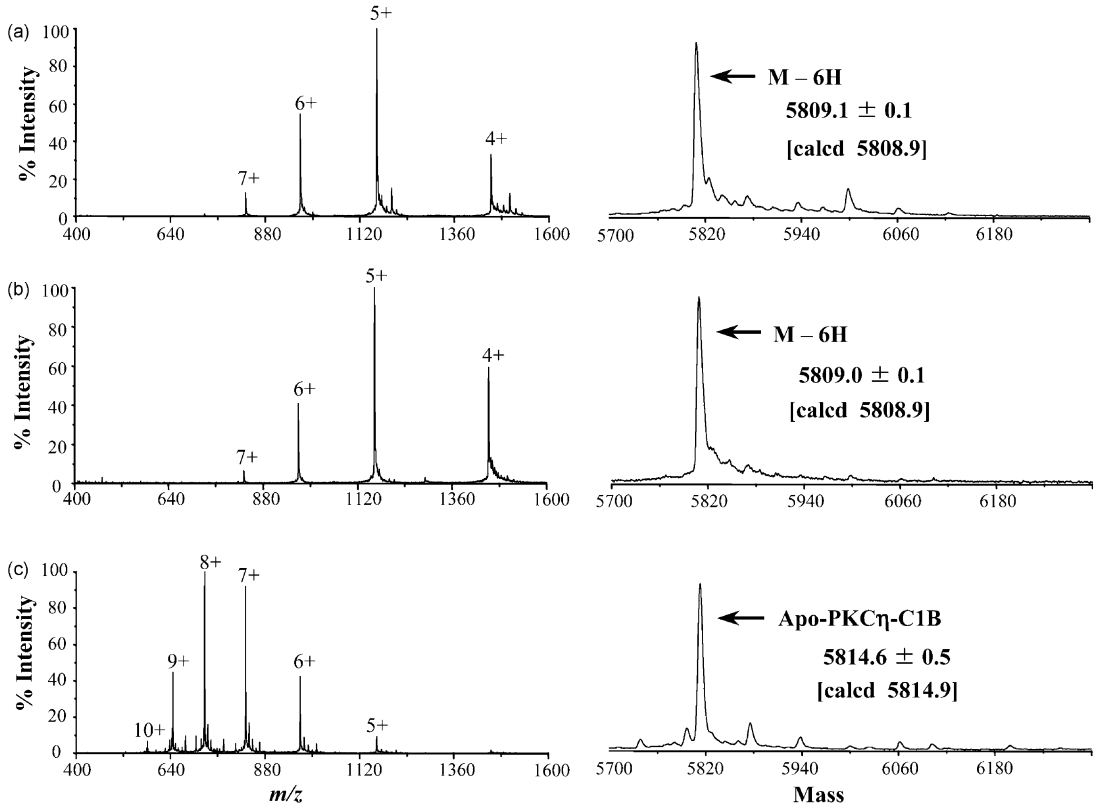

Figure 6. ESI-TOF-MS spectra of copper-treated PKCη-C1B: (a) 4 molar equivalent $^{63}CuCl_2$-treated PKCη-C1B under the neutral aqueous solution (10 mM pyridinium acetate, pH 6.8); (b) remeasurement of the sample (a) in the acidic aqueous solution (50% acetonitrile in water containing 1% acetic acid); (c) apo-PKCη-C1B in the acidic aqueous solution (50% acetonitrile in water containing 1% acetic acid). M means calculated average mass of apo-PKCη-C1B (5814.9).

140 °C and its voltage was set at 150 V. The TOF analyzer was set at 740–200 V of push–pull potential, 4 kV of acceleration potential, 1.5 kV of reflector potential, and 2.2 kV of detector voltage. The mass measurements were carried out at high resolution with neurotensin [monoisotopic protonated molecular ions: 1672.9170 (+1), 836.9621 (+2), and 558.3105 (+3)], using the external calibration method. All measured masses are the average masses and averaged from at least three scans.

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