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Study on the association between CTD peptides and zinc(ii)-dipicolylamine appended beta-cyclodextrin

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Abstract: Association between zinc(ii)-dipicolylamine appended beta-cyclodextrin and CTD (carboxy-terminal domain of RNA polymerase II) peptides with different phosphorylation patterns was studied by isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR). The receptor displayed association constant of 6.52×10^3 to 1.49×10^5 towards the phosphorylated peptides, which are 16 to 72 times higher than that of zinc(ii)-dipicolylamine (ZnDpa), indicating the involvement of molecular recognition of beta-cyclodextrin (beta-CD). NMR results confirm the involvement of beta-CD in the association, i.e. aromatic ring on tyrosine of the peptide was included in the cavity of CD, while the inclusion manners are different among the peptides.

Keywords: zinc(ii)-dipicolylamine; phosphorylated peptide; beta-cyclodextrin; isothermal titration calorimetry; nuclear magnetic resonance

Introduction

Recognition of oligopeptides using synthetic receptor is a preliminary step for protein-targeted biotechnology including bioimaging and drug development[1-2]. Considerable research effort has been devoted to this subject during the past two decades. Part of the effort has been focused on phosphorylated peptides, because phosphorylation is the most important post-transcriptional modification of protein, and one-third of proteins are phosphorylated[3].

A typical characteristic of phosphorylated peptides is the $-PO_4^{2-}$ group, which could be efficiently associated by coordination-based receptor. The well-developed bi-nuclear Zn^{2+}-based receptors[4-6] have been reported to effectively associate phosphorylated peptides with association constants in the range of 10^5 to 10^8. Notably, Hamachi and coworkers demonstrate the high selectivity of ZnDpa towards phosphate[4]. Simth and coworkers employ ZnDpa in combination with a near-infrared fluorophore for in vivo optical imaging[7]. Mohr and coworkers explored a fluorescent naphthalimide chemosensor for ATP bearing a dipicolylamine group complexed with

a Zn(II) metal as a receptor moiety[8]. Ngo et.al. reviewed studies employing ZnDpa as phosphate recognition moiety[9]. Though ZnDpa associate phosphate with high selectivity, a single coordination force cannot afford the receptors with selectivity for different phosphorylated species.

Bearing hydrophobic cavity and hydrophilic shell, beta-CD is widely employed as host for complexation with different guest molecules via hydrophobic inclusion. Breslow[10], Inoue[11] and Liu[12] et.al. make great contribution to molecular recognition properties of beta-CD and its derivatives, furthermore, these compounds were employed as enzyme mimics for hydrolysis of ester[13] and amide[14] as well as construction of supramolecular structures[15].

Combining coordination of zinc(ii)-dipicolylamine (ZnDpa) and hydrophobic inclusion of beta-CD, in this study, zinc(ii)-dipicolylamine appended beta-CD (ZnDpaCD) was synthesized (Figure 1a), and its association with CTD (carboxy-terminal domain of RNA polymerase II) peptides with different phosphorylation patterns (Figure 1b) was studied.

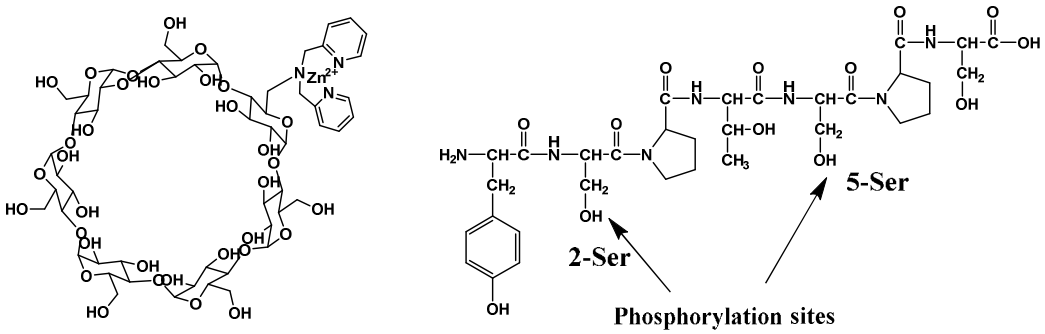

Figure 1. Molecular structures of (a) ZnDpaCD and (b) CTD peptides.

Experimental section

Materials: Beta-CD was recrystallized from distilled water twice, and dried in vaccum. 4-toluene sulfonyl chloride was recrystallized from petroleum ether. DMF of analytical purity was distilled under reduced pressure. Peptides with purity >98% were purchased from GL Biochem (Shanghai) Ltd. All other reagents were commercially available analytical grade and used as received.

Instrumentation: ^{31}P NMR and 2D ^{1}H NMR spectra were recorded on a Varian Mercury/VX 300 and Bruker AV600, 1D ^{1}H NMR spectra was performed on VARIAN UNITY-plus 400. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was conducted with an AUTOFLEX-III LRF200-CID MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). ITC

measurement was performed using VP-ITC.

NMR samples preparation: For ^{31}P NMR and ^{1}H NMR experiments, samples were dissolved in D_2O, and the pH was adjusted to 7 by NaOH/D_2O solution.

ITC measurements: ITC measurements were conducted at 298K. The receptor and the peptides were dissolved in 50 mM HEPES pH = 7.2. Peptides were loaded in the cell, while receptor was loaded in the syringe. To get more data points and facilitate data fitting, the initial titrations contain smaller injection volume. To eliminate the influence of titrant diffusion at the tip of the syringe, a much smaller volume of first injection was employed and the data was discarded in curve fitting. The titration contains an initial injection of 2 μL followed by 5 injections of 6 μL followed by 5 injections of 8 μL followed by 22 injections of 10 μL. Heat of dilution was subtracted from titration data before curve-fitting in Origin. For titration of ZnDpaCD to bis-phosphorylated CTD peptide, the concentrations were 5 mM for ZnDpaCD and 100 μM for the peptide. For titration of ZnDpaCD to 5-Ser-phosphorylated peptide, the concentrations were 5 mM for ZnDpaCD and 500 μM for the peptide. For titration of ZnDpaCD to 2-Ser-phosphorylated peptide, the concentrations were 10 mM for ZnDpaCD and 500 μM for the peptide. For titration of ZnDpaCD to non-phosphorylated peptide, the concentrations were 10 mM for ZnDpaCD and 100 μM for the peptide.

Synthesis of 6-tosylated cyclodextrin (6-Tos-CD)[16]: At 6 ^{o}C, 15 g of dry β-cyclodextrin was added to 150 mL distilled water, then 1.7 g NaOH in 15 mL distilled water was added under stirring. When the solution was homogeneous, 2.52 g 4-toluene sulfonyl chloride in 10 mL acetonitrile was quickly added, and white precipitate appeared immediately. The reaction was kept at 25 ^{o}C for another 2 hour, then the mixture was filtered, and the filtrate was neutralized to pH 7.6 with 1M HCl and kept at 4 ^{o}C overnight. The crude product was obtained by filtration, and the product was obtained by recrystallizing the crude from water. After dried in vacuum for 24 hours, 1.488 g 6-tosylated cyclodextrin was obtained, yield 8.6%. ^{1}H NMR (400 MHz, DMSO): δ 7.75 (d, 2H), 7.43 (d, 2H), 5.95 – 5.49 (m, 14H), 4.80 (d, 7H), 4.55 – 4.26 (m, 6H), 3.80 – 3.42 (m, 102H including water peak), 2.43 (s, 3H). MALDI-TOF: m/z $C_{49}H_{76}O_{37}S$ calculated for [M+Na^{+}] 1311.37, found 1311.41.

Synthesis of 6-dipicolylamine-appended cyclodextrin (DpaCD): Under N_2 atmosphere, 100 mg 6-Tos-CD and 500 mg dipicolylamine were added to 1 mL DMF, the reaction was kept for 24 hours at 90 ^{o}C. After cooled to room temperature, the solution was precipitated with 100 mL acetone, then centrifuged at 5000 rpm for 10 min. After filtration, the precipitate was dissolved in 2 mL distilled water, and

precipitated with 60 mL acetone, then centrifuged at 5000 rpm for 20 min, these steps were repeated once, then the product was obtained as a pale yellow solid. ^{1}H NMR (400 MHz, DMSO) δ 8.44 (d, 2H), 7.73 (t, 2H), 7.53 (d, 2H), 7.38–7.04 (m, 2H), 6.12–5.35 (m, 85 H including water peak), 4.83 (d, 7H), 4.74–4.35 (m, 6H), 3.90 (d, 3H), 3.77-3.49 (m, 27H), MALDI-TOF: m/z calculated for $C_{54}H_{79}N_3O_{34}$ [M+H$^+$] 1314.46, found 1314.76.

Synthesis of 6-Zn^{2+}-dipicolylamine-appended cyclodextrin (ZnDpaCD): When DpaCD was dried, it difficult to redissolve in H_2O, thus, DpaCD without drying was used. DpaCD and $Zn(NO_3)_2 \cdot 6H_2O$ with molar ratio of 1:2 was dissolved in 1.2 mL H_2O, after 2 hour, the solution was precipitated with 30 mL acetone, then centrifuged at 8000 rpm for 30 min, after vacuum dried, the product was obtain as a pale yellow crystalline solid, yield 66.5%. ^{1}H NMR (400 MHz, DMSO) δ 8.60 (d, 2H), 8.04 (dd, J = 33.0, 2H), 7.58 (d, 3H), 5.89 (dd, 14H), 4.84 (dd, 7H), 4.53 (dd, 7H), 4.13 (s, 1H), 3.92–3.41 (m, 113H including water peak); ESI: calculated for $C_{54}H_{79}N_3O_{34}Zn^{2+}$, [M/2] 689.79, found 689.69.

Results and discussion

mRNA transcription is generally mediated by CTD of RNA polymerase II[17]. The binding of specific processing factors depends on the phosphorylation pattern of the CTD, which changes during the transcription cycle[18]. The CTD consists of heptapeptide repeats of the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7, with phosphorylation mainly occurs at Ser2 and Ser5[19]. Receptors that associate CTD with selectivity may function as regulator of the transcription process.

Fujita and coworkers synthesized DpaCD by reaction between amine substituted beta-CD and 2-pyridinecarboxaldehyde[14], in comparison with this strategy, substituting 6-Tos-CD with dipicolylamine should afford better yield, because amine substituted beta-CD suffers from low yield.

When ZnDpaCD was added to peptide solutions, coordination between ZnDpa and phosphates of the peptides occurs as revealed by the chemical shift in ^{31}P NMR spectra (Figure 2).

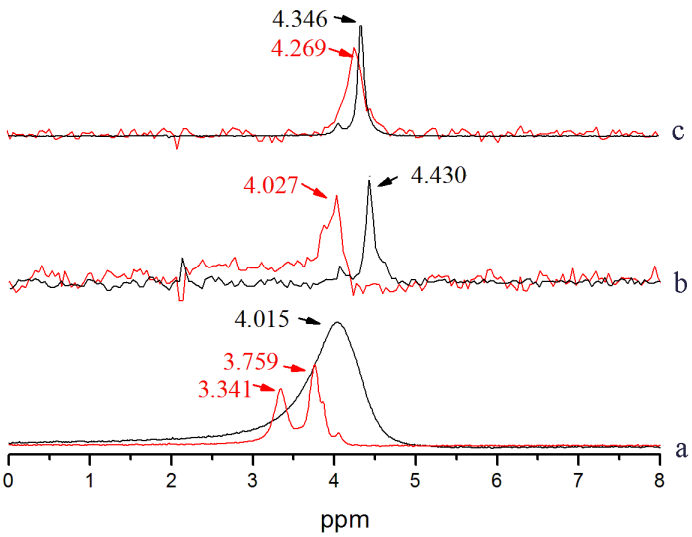

Figure 2. ^{31}P NMR spectra of phosphorylated CTD peptides (a) $Y_{2p5p}S$, (b) $Y_{5p}S$, (c)$Y_{2p}S$ before (in black line) and after (in red line) the addition of ZnDpaCD. $Y_{2p}S$, $Y_{5p}S$ and $Y_{2p5p}S$ denote Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 phosphorylated at Ser2, Ser5 and at both 2, 5 position, respectively.

ITC results indicate the association constants follow the order of $Y_{2p5p}S$ > $Y_{5p}S$ > $Y_{2p}S$ (Table1). Notably, the association constants from ZnDpaCD are significantly larger than that from ZnDpa, indicating the beta-CD moiety of ZnDpaCD cooperatively involved in the association. Binuclear coordination-type receptors for phosphorylated peptides show association constants in the range of 10^5 to 10^8, though ZnDpaCD contains a single coordination site, it shows comparable affinity in comparison with some binuclear coordination-type receptor.

Table 1. Thermodynamic parameters of the association between ZnDpaCD, ZnDpa[20] and the CTD peptides.

Receptor	Peptide	K/ M^{-1}	ΔH/ cal·mol^{-1}	ΔS /cal·mol^{-1}·K^{-1}
ZnDpaCD	$Y_{2p}S$	6.52×10^3	390.7	18.8
ZnDpaCD	$Y_{5p}S$	3.16×10^4	752.1	23.1
ZnDpaCD	$Y_{2p5p}S$	1.49×10^5	1522	28.8
ZnDpaCD	YS	—	—	—
ZnDpa	$Y_{2p}S$	392	4.62×10^3	27.2
ZnDpa	$Y_{5p}S$	435	4.74×10^3	28.1
ZnDpa	$Y_{2p5p}S$	4.93×10^3	2.89×10^3	26.4
ZnDpa	YS	—	—	—

'—' denote that the enthalpy change is too small to be accurately fitted in Origin. YS denote non-phosphorylated CTD peptide Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. The titrations were

conducted at 298K.

^{1}H NMR was used to study the association in detail (Figure 3). Upon association, signal from H-10 of dipicolylamine broaden and shifts downfield, indicating the involvement of ZnDpa in the coordination. On the other hand, signal from aromatic group of tyrosine on the peptide broaden and shifts upfield, indicating the aromatic ring of tyrosine is included in the cavity of beta-CD. Notably, for Ser5 phosphorylated peptide, the aromatic signal splits into two groups, i.e. 7.2 ppm, 6.8 ppm and 7.0 ppm, 6.7 ppm, indicating two different inclusion modes. This is attributed to the asymmetric structure of ZnDpaCD, and aromatic ring of tyrosine could enter the cavity from ether the narrow rim or the wider rim. The overall association modes is illustrated in Figure 4.

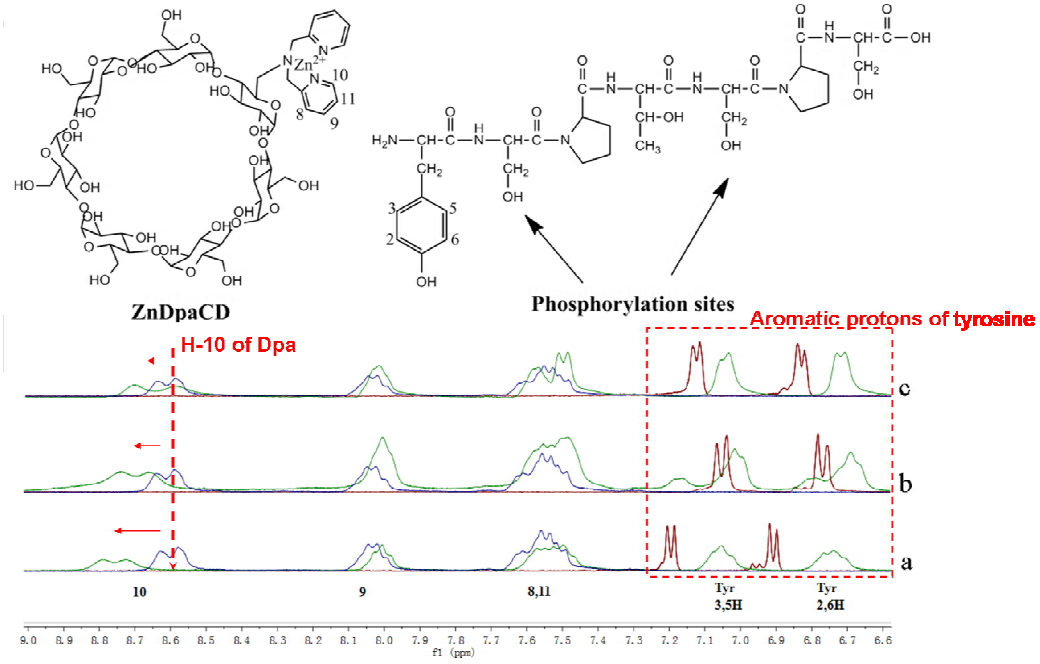

Figure 3. ^{1}H NMR spectra of ZnDpaCD (in blue line) and phosphorylated CTD peptides (in red line) (a) $Y_{2p5p}S$, (b) $Y_{5p}S$, (c)$Y_{2p}S$ and the mixture (in green line) of CTD peptides and ZnDpaCD with 1:1 molar ratio. (400 MHz, D_2O, pH = 7.0)

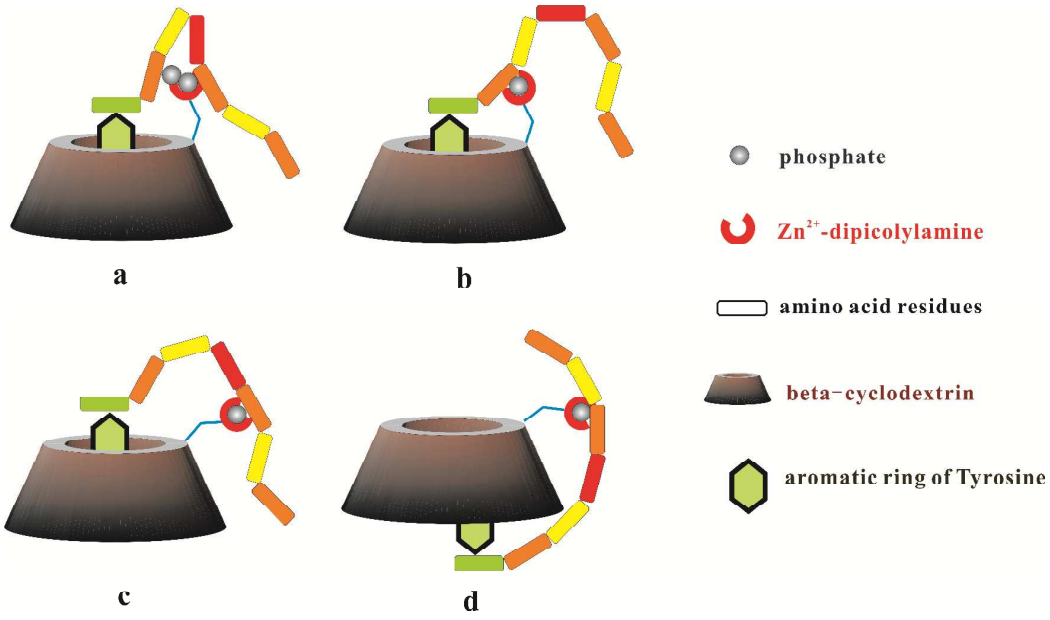

Figure 4. Schematic illustration of the association between ZnDpaCD and CTD peptides with different phosphorylation patters. (a) $Y_{2p5p}S$, (b) $Y_{2p}S$, (c)$Y_{5p}S$ with tyrosine enter the cavity from the narrower rim, (d)$Y_{5p}S$ with tyrosine enter the cavity from the wider rim.

The inclusion modes are further proved by 2D ROESY spectrum. Figure 5 is ROESY spectrum of ZnDpaCD. Signal in **A** region is the cross peaks between aromatic protons of Dpa and protons of CD. Considering ZnDpa moiety is highly hydrated, it cannot be self-included in the cavity of CD. Thus, the cross peaks are inferred to be from H-6 of CD rather than H-3 and H-5.

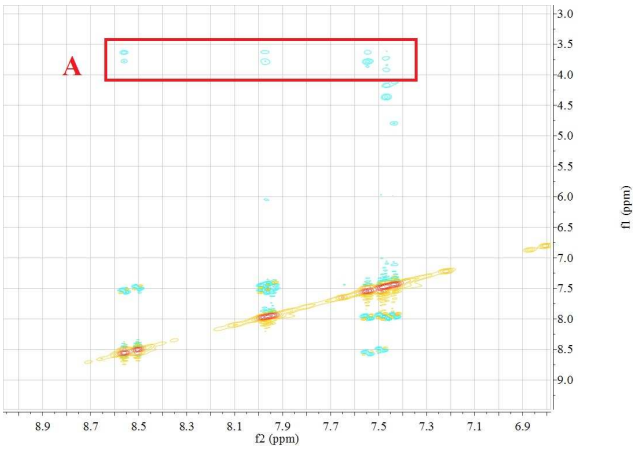

Figure 5 2D ROESY spectrum of ZnDpaCD (600 MHz, D_2O pH = 7.0).

Figure 6 is ROESY spectrum of $Y_{2p5p}S$ and ZnDpaCD in 1:1 molar ratio. Signals in region **A** indicate the spatial proximity of 6-H of beta-CD and dipicolylamine as well

as tyrosine. Considering the aromatic ring of tyrosine is included in the cavity and only H-3,5 of tyrosine display cross peaks with 6-H of CD, the inclusion mode is inferred to be insertion of phenol-OH side of tyrosine from the narrow rim of CD. Signals in region B indicate the spatial proximity of dipicolylamine and tyrosine. Combining the spatial proximity information, the association mode is illustrated in Figure 4a.

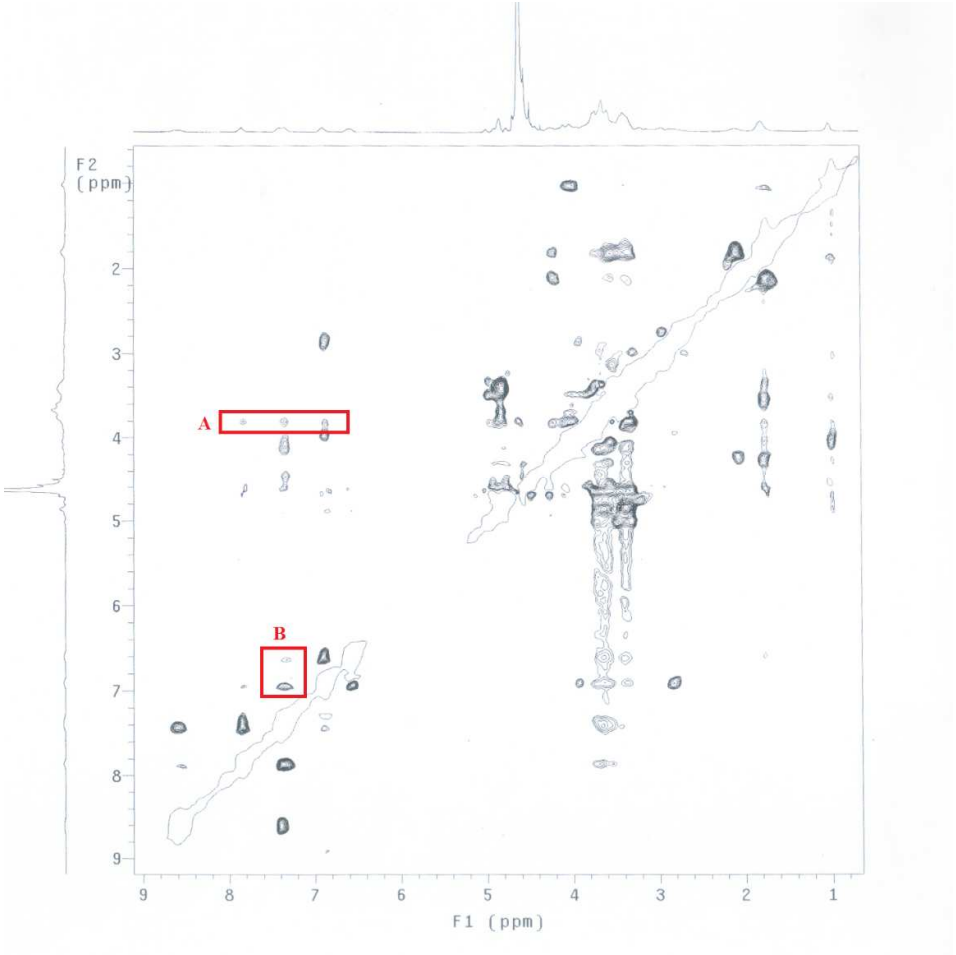

Figure 6 2D ROESY spectrum of $Y_{\mathbf{2p5p}}S$ and ZnDpaCD in 1:1 molar ratio (300 MHz, D_2O pH = 7.0).

Figure 7 is ROESY spectrum of $Y_{\mathbf{2p}}S$ and ZnDpaCD in 1:1 molar ratio. The signals are similar to that of $Y_{\mathbf{2p5p}}S$/ZnDpaCD, except for more intense cross peaks from aromatic protons of Dpa and tyrosine, indicating the similar association mode (Figure 4b).

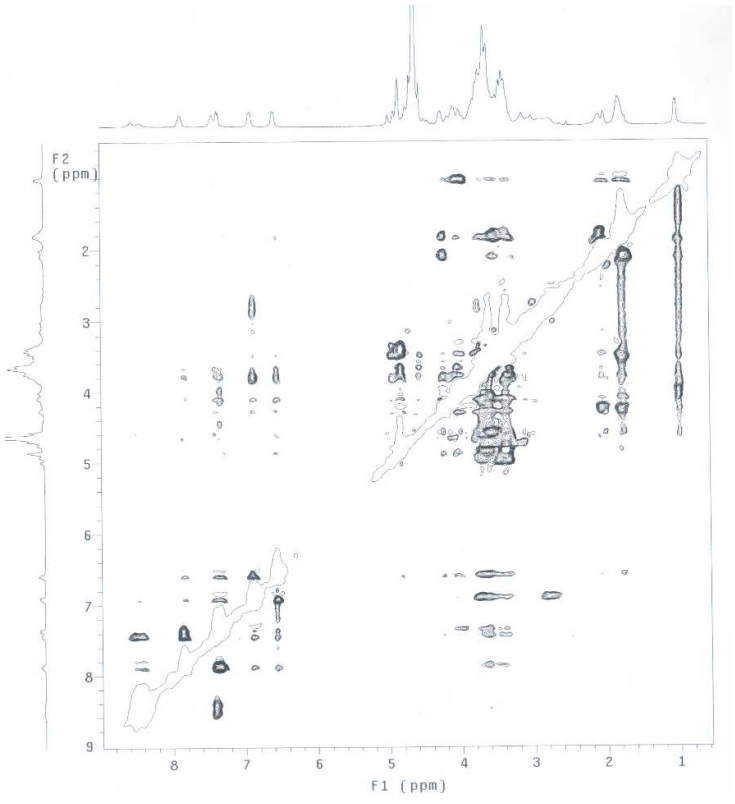

Figure 7 2D ROESY spectrum of $Y_{2p}S$ and ZnDpaCD in 1:1 molar ratio (300 MHz, D_2O pH = 7.0).

Figure 8 is ROESY spectrum of $Y_{5p}S$ and ZnDpaCD in 1:1 molar ratio. In comparison with the above two spectra, new signals in region A1 and A2 appear, indicating tyrosine is included in CD pocket in another different manner. Additionally, these signals do not show cross peaks with Dpa, indicating tyrosine is far away from Dpa. Combining the spatial proximity information, the association modes is illustrated in Figure 4c and 4d.

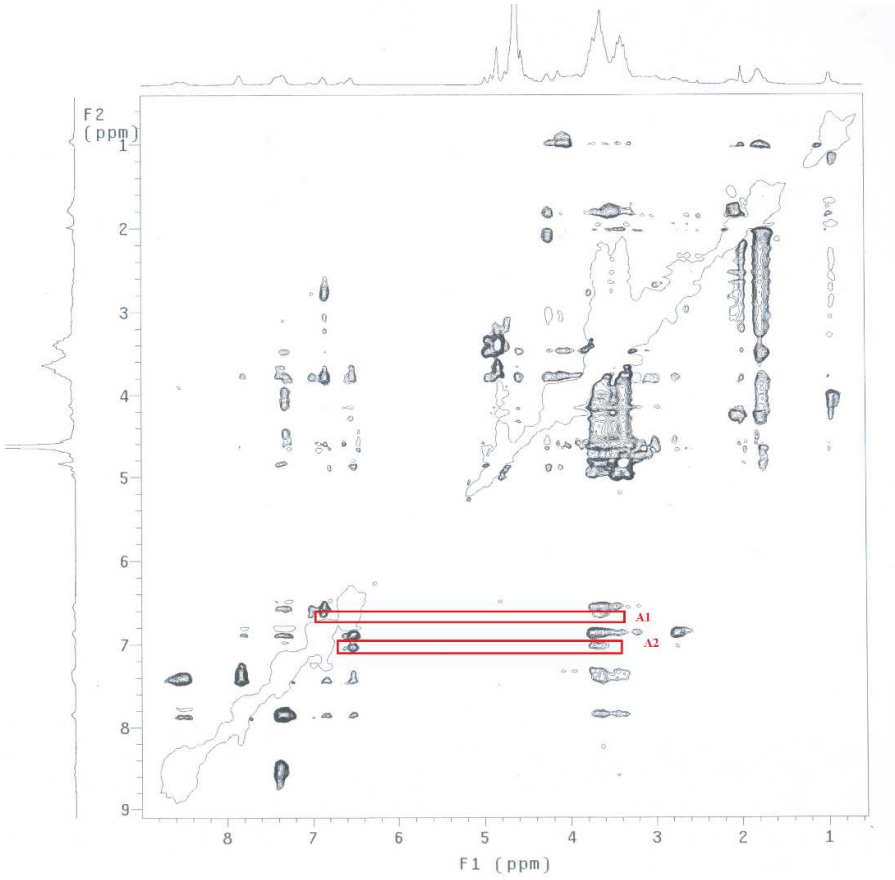

Figure 8 2D ROESY spectrum of $Y_{5p}S$ and ZnDpaCD in 1:1 molar ratio (300 MHz, D_2O pH = 7.0).

Conclusions

In summary, ZnDpaCD associate phosphorylated CTD peptides through coordination in combination with hydrophobic inclusion. The inclusion manner of tyrosine into hydrophobic cavity of CD are different depending on the phosphorylation pattern of the peptides. ZnDpaCD shows selectivity towards the peptides, i.e. the association constants ranging from 6.52×10^3 to 1.49×10^5, and follows the order of $Y_{2p5p}S$ > $Y_{5p}S$ > $Y_{2p}S$. Though the receptor discriminates bis-phosphorylated peptides and mono-phosphorylated peptides, but the selectivity for different mono-phosphorylated peptides is not high.

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Notes

The authors declare no competing financial interest.

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