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Functional Reassembly of a Split PH Domain

Kenji Sugimoto,[†] Yasuo Mori,[§] Keisuke Makino,[†] Katsutoshi Ohkubo,[†] and Takashi Morii*,[†,‡]

Contribution from the Institute of Advanced Energy, Kyoto University, Uji, Kyoto 611−0011, Japan, Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444−8585, Japan, and PRESTO, JST, Uji, Kyoto 611-0011, Japan

Received November 26, 2002; E-mail: t-morii@iae.kyoto-u.ac.jp

Abstract: The pleckstrin homology (PH) domain forms a structurally conserved protein module of approximately 120 amino acid residues. Several proteins involved in cellular signaling and cytoskeletal organization possess split PH domains while their biological roles and ligand binding activity remain to be clarified. We have designed a split PH domain from a structurally well-characterized PH domain of phospholipase Cδ_1 by dissecting the PH domain and tethering a coiled coil module to each subunit to ask a question of whether the coiled coil could mediate a functional reassembly of the split PH domain. Isothermal titration microcalorimetry measurements indicated a formation of a thermodynamically stable 1:1 complex of the N-terminal and C-terminal halves of the split PH domain by the coiled coil formation. The reassembled split PH domain binds to IP_3, a target molecule of the parent PLCδ_1 PH domain, but not to L-IP_3, indicating that the split PH domain maintains a binding selectivity similar to the native PLCδ_1 PH domain. These results demonstrate that the split PH domain folds into a functional structure when the split halves are brought to close proximity, and suggest that the native split PH domains, such as found in PLCγ_1, have distinctive functions upon the reassembly.

Introduction

The pleckstrin homology (PH) domain forms a structurally conserved protein module of approximately 120 amino acid residues that is associated with many proteins involved in cellular signaling and cytoskeletal organization processes.[1,2] In the three-dimensional structures, PH domains reveal a common core structure with a β-sandwich of two nearly orthogonal β-sheets held by a C-terminal amphipathic α-helix. One sheet consists of four β-strands ($\beta1-\beta4$), and another of three strands ($\beta5-\beta7$). The three loops ($\beta1/\beta2$, $\beta3/\beta4$, and $\beta6/\beta7$) were found to be the most variable in length and sequence and represent a series of different binding sites implemented on the same particularly stable structural scaffold.[3,4,5] Several other domains that share no sequence similarity have been shown to adopt a nearly identical fold as the PH domain, suggesting the existence of a stable PH superfold.[6] The PH domain from Phospholipase Cγ_1 (PLCγ_1) has two Src homology 2 (SH2) domains and one SH3 domain inserted between the strands $\beta3$ and $\beta4$.[7,8] The PH domains from synthrophins also have an entire PDZ domain and a proline-rich linker inserted into this position.[9] These split PH domains are categorized in the PH superfamily from the amino acid sequence homology, but there is no evidence of whether the postulated split PH domain could reconstitute in the stable PH superfold. To ascertain the role of the split PH domain, it is necessary to establish the functional reassembly of the split PH domain when the N-terminal and C-terminal halves are brought into close proximity. We have investigated a reassembly of a split PH domain designed from a PLCδ_1 PH domain by using an antiparallel coiled coil module,[10] which has been successfully applied to reconstitute active proteins from protein fragments.[11,12] The reassembled split PH domain selectively binds inositol-1,4,5-trisphosphate, a target molecule of the parent PLCδ_1 PH domain.

[†] Kyoto University.
[‡] PRESTO, JST.
[§] Okazaki National Research Institute.

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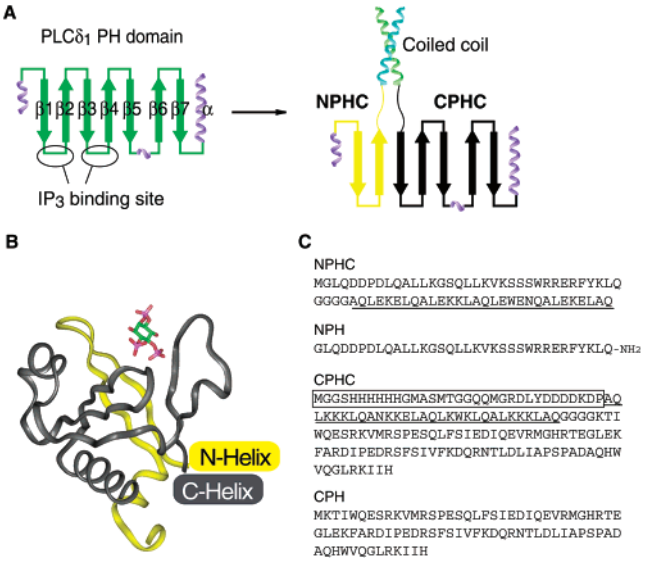

Figure 1. Schematic representation shows a dissection strategy of PLCδ_1 PH domain into a split PH domain. (A) Arrows represent the β-sheets in the PLCδ_1 PH domain (green), the NPHC (yellow), and CPHC (gray). Oligopeptides forming an antiparallel coiled coil segment (coils in green) are attached at the C-terminal and N-terminal of NPHC and CPHC, respectively. (B) A ribbon representation shows a possible structure for NPHC (yellow) and CPHC (gray) reassembled into the PH domain. N-Helix and C-Helix denote the antiparallel coiled coil segment of NPHC and CPHC, respectively. The IP_3 molecule found in the crystal structure[2] of the complex of PLCδ_1 PH domain and IP_3 is shown in the stick representation. (C) Amino acid sequences of the peptides NPHC, NPH, CPHC, and CPH. The coiled coil segments of NPHC and CPHC are underlined. The His-Tag portion introduced to the N-terminal of CPHC is boxed.

Results and Discussion

Design and Synthesis of a Split PH Domain. The PH domain of PLCδ_1 was selected as a parent full domain because its three-dimensional structure and ligand binding characteristics are well characterized.[3,13,14] Inositol-1,4,5-trisphosphate (IP_3) specifically binds in the pocket made from the $\beta1/\beta2$ loop and the $\beta3/\beta4$ loop. The PH domain was divided into two subunits at Gln45 and Lys49 existing in the $\beta2/\beta3$ loop to preserve the functionally important $\beta1/\beta2$ and the $\beta3/\beta4$ loops in separate subdomains. In addition, dissection at the $\beta2/\beta3$ loop seemed to cause the least steric effect when two subdomains were reassembled with an antiparallel coiled coil formation. The leucine zipper peptides[10] were appended to the N-terminal and C-terminal halves through a four-Gly linker to afford an N-terminal subdomain containing the $\beta1/\beta2$ loop (NPHC) and a C-terminal subdomain with the $\beta3/\beta4$ loop (CPHC) subdomains, respectively (Figure 1). The NPHC subdomain contains amino acid residues Lys30, Lys32, Trp36, and Arg40, and the CPHC subdomain contains Glu54, Ser55, Arg56, Lys57, and Thr107 necessary for the direct interaction with IP_3. In addition to NPHC and CPHC, split PH domain derivatives lacking the leucine zipper peptides (NPH and CPH) were synthesized to establish the relationship between structure and function in reassembly.

CD Spectral Analyses of the Split PH Domain. Reassembly of the split PH domain was monitored by comparing CD spectra of NPHC, CPHC, and a 1:1 mixture of NPHC and CPHC (Figure 2A). Both NPHC and CPHC exhibit negative peaks at 208 and 222 nm, suggesting that each fragment contains secondary structures to some extent. In the 1:1 mixture of NPHC and CPHC, intensity of the peak at 208 nm is smaller than that of NPHC alone, and both of the bands at 208 and 222 nm show higher intensity in comparison to those of CPHC. These spectral differences indicated a formation of expected coiled coil structure. The 1:1 mixture of NPHC and CPHC exhibited peaks with stronger intensity than those of parent PH domain both at 208 and at 222 nm. This is expected because the coiled coil formation by NPHC and CPHC would increase the intensity of negative peaks at 208 and 222 nm. Intensity of the negative peaks at 208 and 222 nm are stronger in the mixture containing parent PH domain and coiled coil peptides (1:1 mixture of N-Helix and C-Helix) as compared to that of the 1:1 NPHC/CPHC mixture (Figure 2B). Possible reasons for this observation is that the N−Helix and C−Helix peptides form more stable coiled coil structure than the coiled coil portion of the NPHC/CPHC complex does and/or that only a partial PH fold is formed in the 1:1 NPHC/CPHC complex. In fact, an equilibrium association constant obtained for the antiparallel coiled coil formation by N-Helix and C-Helix was 1.59×10^7 (M^{-1}) under the condition used for the CD analyses (Figure S2). As shown in the following isothermal titration microcalorimetry (ITC) measurements, an equilibrium association constant for N-Helix and C-Helix is 7-fold larger than that for CPHC and NPHC. It is likely that the antiparallel coiled coil in the 1:1 NPHC/CPHC complex forms less helical structure than the nearly 100% helical N-Helix/C-Helix complex does. In addition, CPHC and NPHC contain rather unstructured portions (His-Tag derived 33 residues and four Gly residues of CPHC and four Gly residues of NPHC). It is expected that even a fully reassembled NPHC/CPHC complex reveal smaller molar elipticity than that of the mixture containing parent PH domain and the coiled coil peptides.

When IP_3 was added to the 1:1 NPHC/CPHC complex, a slight CD spectral change at 222 nm was observed (Figure 2B). Such a spectral change was also observed for the parent PH domain upon binding IP_3. The similarity of the spectral changes induced by the addition of IP_3 between the parent PH domain and the NPHC/CPHC complex suggests that association of the split PH domains by the antiparallel coiled coil formation promotes a reassembly of native like PH fold.

Interaction of split PH domains lacking the coiled coil segment (NPH and CPH) was also analyzed by CD spectral analyses (Figure 2C). Compared to the spectrum of parent PH domain (Figure 2B, filled triangles), the spectrum of the 1:1 NPH/CPH mixture indicated little structure formation. The spectrum of the 1:1 NPH/CPH mixture exactly matched with the additive of the NPH and CPH spectra (not shown). The addition of IP_3 to the 1:1 mixture of NPH/CPH did not induce any spectral change, indicating that NPH and CPH did not form the PH domain-like structure. These results demonstrate that IP_3 itself was not inducing functional reassembly of the split PH domains. The observed spectral change for the 1:1 mixture of NPHC/CPHC upon addition of IP_3 also suggests that the antiparallel coiled coil formation is indispensable for the reassembly of NPHC and CPHC.

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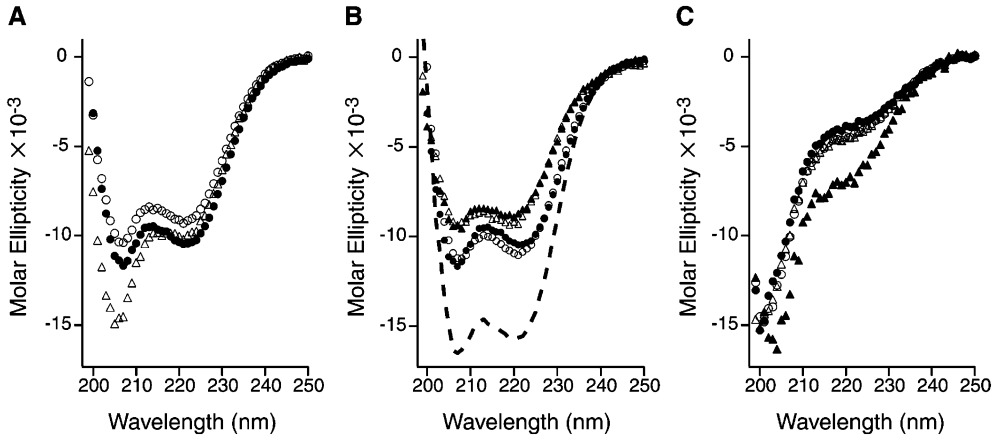

Figure 2. (A) CD spectra of NPHC (4 μM, open triangles), CPHC (4 μM, open circles), and a 1:1 NPHC/CPHC complex (4 μM each, filled circles) show the structural change upon association of NPHC and CPHC. (B) CD spectra of a 1:1 NPHC/CPHC complex (4 μM each, filled circles) and a 1:1 NPHC/CPHC complex with 10 μM IP_3 (4 μM each of NPHC and CPHC, open circles) show the structural change upon addition of IP_3. A similar spectral change was observed between PLCδ_1 derived PH domain (4 μM, filled triangles) and PLCδ_1 derived PH domain (4 μM, open triangles) with 10 μM IP_3. Spectrum of a mixture containing PLCδ_1 derived PH domain (4 μM) and coiled coil peptides (4 μM N-Helix and 4 μM C-Helix) is also shown (dotted line). (C) CD spectra of NPH (4 μM, filled triangles), CPH (4 μM, filled circles) and a 1:1 NPH/CPH mixture (4 μM each of NPH and CPH, open circles) show no structural change upon addition of 10 μM IP_3 to the 1:1 NPH/CPH mixture (4 μM each of NPH and CPH, open triangles).

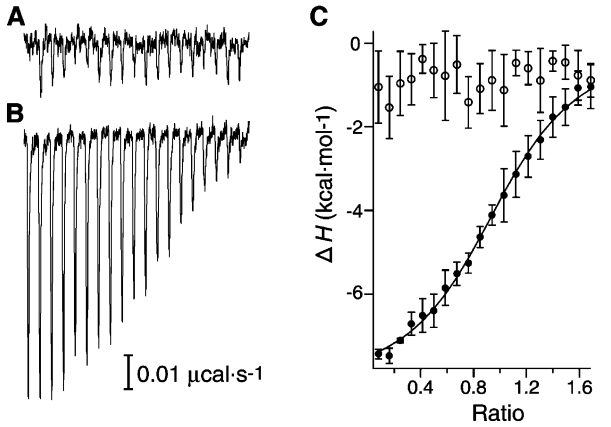

Figure 3. Isothermal titration of CPHC with NPHC. (A) CPH (4.5 μM) was titrated with NPH (35 μM) added in 19 aliquots of 15 μL each in a phosphate buffer (pH 8.0) containing 50 mM NaCl, 0.005% Tween20 at 15 °C. (B) CPHC (4.5 μM) was titrated with NPHC (35 μM) added in 19 aliquots of 15 μL each in a phosphate buffer (pH 8.0) containing 50 mM NaCl, 0.005% Tween20 at 15 °C. (C) Analysis data for isothermal titrations of the NPH/CPH (open circles) and NPHC/CPHC (filled circles) complexes. The heat of dilution was subtracted from the raw data prior to data analysis. All of the data reported represent the mean of three determinations ± SD. The solid line represents the best fit to the data of NPHC/CPHC complex formation.

Isothermal Titration Microcalorimetry Measurements of the Split PH Domain. The coiled coil-assisted reassembly of NPHC and CPHC was analyzed by the isothermal titration microcalorimetry (ITC) measurements (Figure 3), which allow for the simultaneous and direct determination in the same experiment of the binding enthalpy (ΔH), the association constant (K_A), and the stoichiometry (N) of a binding reaction.[15] In the titration of CPH by NPH (Figure 3A), little or no heat formation was observed, indicating that CPH and NPH did not form a reassembled PH domain as indicated by the CD analyses. In contrast, endothermic peaks were observed in the titration of CPHC by NPHC (Figure 3B). The titration data could be fitted best for a stoichiometry of one NPHC per one CPHC (Figure 3C). An average of fitting analyses for three datasets afforded characteristic parameters for the reassembly process, where the stoichiometry (N) was 1.04 ± 0.07 and equilibrium association constant (K_A) was 2.28 ± 1.29 × 10^6 (M^{-1}). The equilibrium binding constant for CPHC and NPHC was 7-fold smaller than that of the antiparallel coiled coil segment alone (C-Helix and N-Helix). It is possible that the -Gly-Gly-Gly-Gly- linker region is not optimized enough to accommodate the antiparallel coiled coil structure. The stability of the NPHC/CPHC complex parallels with that obtained by the surface plasmon analyses (Figure S3). The small enthalpic change (ΔH) for the 1:1 complex formation (−8.26 ± 0.40 kcal·mol^{-1}) indicates that the antiparallel coiled coil segment does not fully fold in the CPHC/NPHC complex as suggested by the CD analyses. It is also possible that the split PH domain fold partially in the reassembled complex or that the hydrophobic packing between CPHC and NPHC is a rather weak one.[16]

IP_3 Binding Activity of the Split PH Domain. The functional reassembly of the split PH domain was examined by an IP_3 binding activity (Figure 4). Judging from the association constant between CPHC and NPHC, about 90% of the molecules were assembled in the 1:1 complex at the concentration (4 μM) used in the CD experiment where structural changes of the 1:1 NPHC/CPHC complex were observed upon addition of IP_3. ITC experiments were carried out in the same condition used for the CD spectral analyses. Titrations of IP_3 to the 1:1 CPHC/NPHC complex resulted endothermic peaks (Figure 4C), and the parameters were obtained as $N = 0.91 \pm 0.07$, $K_A = 8.58 \pm 3.22 \times 10^5$ (M^{-1}), and $\Delta H = -14.17 \pm 1.52$ (kcal·mol^{-1}). Neither CPHC nor NPHC alone showed binding activity to IP_3. As expected from the CD spectral analysis, addition of IP_3 to the 1:1 mixture of NPH and CPH did not cause obvious heat changes (Figure 4A). The association constant of IP_3 and the reassembled split PH domain complex was slightly lower than that reported for the native PLCδ_1 PH domain ($K_A = 5 \times 10^6$ M^{-1}).[13,14] To confirm the functional reassembly of the CPHC/NPHC complex, binding experiment of the 1:1 CPHC/NPHC complex using L-IP_3 was carried out. PLCδ_1 derived PH domain has been shown to

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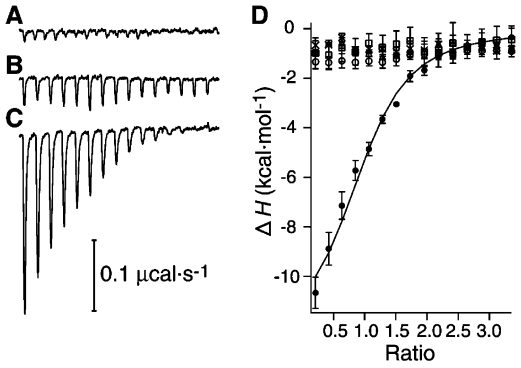

Figure 4. Titration of NPHC/CPHC complex with L-IP_3 and IP_3 by ITC. Titrations of the 1:1 NPH/CPH mixture (4 μM) with 80 μM IP_3 (A), and NPHC/CPHC complex (4 μM) with 80 μM L-IP_3 (B) or IP_3 (C) added in 15 aliquots of 15 μL each. The heat of dilution was subtracted from the raw data prior to data analysis. (D) Analysis data for isothermal titration of the NPHC/CPHC complex with L-IP_3 (open circles) or IP_3 (filled circles) indicate a specific IP_3 binding of the NPHC/CPHC complex. No detectable IP_3 binding was observed for NPHC (open triangles), for CPHC (crosses), and for the 1:1 NPH/CPH complex (open squares). The solid line represents the best fit to the data of NPHC/CPHC complex with IP_3. 4 μM NPHC, CPHC, the 1:1 NPH/CPH complex, or the 1:1 NPHC/CPHC complex was titrated with 80 μM IP_3 added in 15 aliquots of 15 μL each. Binding reactions were carried out in a phosphate buffer (pH 8.0) containing 50 mM NaCl, 0.005% Tween20 at 15 °C. All of the data reported represent the mean of three determinations ± SD.

specifically discriminate IP_3 from L-IP_3.[13,14] As clearly shown in Figure 4B, titration of the 1:1 CPHC/NPHC complex with L-IP_3 resulted little heat change as compared to that with IP_3 (Figure 4C). The split PH domain maintains a binding selectivity of native PLCδ_1 PH domain, which strongly suggests that the reassembled split PH domain forms a nativelike IP_3 binding pocket.

Conclusion

We have shown that association of the split PH domain via an antiparallel coiled coil module promotes a functional reconstitution. The reassembled split PH domain revealed a binding selectivity of native PLCδ_1 PH domain, indicating that the reassembled split PH domain forms a native-like IP_3 binding pocket. The antiparallel coiled coil formation is necessary to reassemble the split PH domain to execute the IP_3 binding activity. Because PH domains form subclass with quite different functions, the dissection positions of the split PH domain would not be limited to the present case. The native split PH domain, such as found in PLCγ_1, would fold in a stable domain when both split subunits are brought in close proximity, and exert distinctive functions upon the reassembly. The SH2 and SH3 domains represent specific binding sites of phosphotyrosine and proline rich motifs, respectively.[17] Insertion of such motifs within the split PH domain would be ideal for building a modular allosteric switch that controls cellular behavior. The reassembly strategy using the coiled coil provides a quite useful tool for delineating the cellular target molecules of native split PH domains.

Experimental Section

Plasmid Constructions. The cDNA corresponding PH domain of PLCδ_1 (11−140) was amplified by the PCR method from a rat brain cDNA library (CLONTECH Labs. Inc.). The amino acid residues Cys48 and Cys96 were mutated to Ser as described previously.[14] The PLCδ_1 PH domain is divided into two subunits by deleting Glu46-Cys48 in β2/β3 loop. The N-terminal half (amino acid number 11−45) was fused at its C-terminal a leucine zipper seuence through a $(Gly)_4$ linker (-GGGGAQLEKELQALEKKLAQLEWENQALEKELAQ) and the C-terminal portion (amino acid number 49−130) was attached at its N-terminal another leucine zipper sequence through a $(Gly)_4$ linker (AQLKKKLQANKKELAQLKWKLQALKKKLAQGGGG-) to give NPHC and CPHC, respectively (Figure 1C). The C-terminal portion (amino acid number 49−130) of the PLCδ_1 PH domain lacking the leucine zipper sequence (CPH) was made as a control split domain. Double-strand oligonucleotides encoding the amino acid sequence of NPHC, CPHC or CPH were synthesized by using PCR-based methods. The expression vectors were constructed by inserting the oligonucleotides corresponding to the amino acid sequence of NPHC or CPH into the NdeI/BamHI sites of pET3b vector. The expression vector for CPHC carrying a His tag at the N-terminal was constructed in a similar manner by using a pTrcHis2 vector. All of the nucleotide sequences were confirmed by dideoxy sequencing.

Protein Purification. The plasmid was transformed into *Escherichia coli* BL21(DE3)-pLysS cells, and the protein was purified by using a HiTrap SP (Amersham Pharmacia) followed by a Mono S cation exchange chromatography (Amersham Pharmacia) with 10 mM phosphate buffer (pH 7.0) containing 50 mM NaCl and 4 M urea. Fractions containing the split PH domain were collected, and purified further by using a reversed phase chromatography (RESOURCE RPC, Amersham Pharmacia). The purity of each split PH domain, NPHC, CPHC, and CPH was confirmed by 15% SDS/polyacrylamide gel (Figure S1).

Synthesis of Oligopeptides. Oligopeptides were synthesized on a Shimadzu PSSM-8 peptide synthesizer according to the Fmoc chemistry protocols by using Fmoc-PAL-PEG resin (0.2 mmol/g, Applied Biosystems), protected Fmoc-amino acids and HBTU, purified by a reversed phase HPLC, and characterized by a Voyager MALDI-TOF spectrometry (Applied Biosystems). The C-termini of all peptides were amidated, and the N-termini of N-Helix (Ac-AQLEKELQALEKKLAQLEWENQALEKELAQ-NH_2) and C-Helix (Ac-AQLKKKLQANKKELAQLKWKLQALKKKLAQ-NH_2) were acetylated. NPH calculated for $[M^+]$ 4004.6, found 4005.5; N-Helix calculated for $[M^+]$ 3565.0, found 3565.6; C-Helix calculated for $[M^+]$ 3559.4, found 3560.0. Peptide concentrations were determined using tryptophan absorbance.

Reassembly of the NPHC/CPHC Complex. The purified NPHC or CPHC solution was replaced for the 10 mM phosphate buffer (pH 8.0) containing 50 mM NaCl and 0.005% Tween20 using a NAP5 gel filtration column (Amersham Pharmacia). The concentration of the protein was calculated from the extinction coefficient at 280 nm under the denatured condition with 8 M urea. The 1:1 NPHC/CPHC complex solution was made by mildly mixing the stock solutions of NPHC and CPHC. Similar procedure was used to prepare the 1:1 NPH/CPH complex.

CD Spectroscopic Measurements of NPHC, CPHC, and the NPHC/CPHC Complex. CD spectra were measured on a J-725 CD spectrometer equipped with a temperature controller using 0.5 cm pathlength cell at 15 °C. Samples contained 10 mM phosphate buffer (pH 8.0), 50 mM NaCl, 0.005% Tween20 and indicated concentrations of peptides. Spectra were the average of 32 scans and were corrected with a spectrum of buffer alone but not smoothed.

Isothermal Titration Calorimetry (ITC) Measurements. ITC was performed on an OMEGA titration calorimeter (MicroCal. Inc.) in 10 mM phosphate buffer (pH 8.0) containing 50 mM NaCl and 0.005% Tween20 at 15 °C. All of the solutions were thoroughly degassed by evacuation. The heat of dilution was subtracted from the raw data prior to data analysis. Analysis of the data was performed using the evaluation software (ORIGIN ver. 5.0) supplied with the instrument.

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Supporting Information Available: SDS−PAGE analyses of CPH, CPHC, and NPHC (Figures S1) and experimental details for the determination of the equilibrium binding constant of antiparallel coiled coil peptides (Figure S2) and the surface plasmon binding studies of NPHC and CPHC (Figures S3) (PDF). This material is available free of charge via the Internet at http://pubs.asc.org.

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