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Interdomain interaction of cyclic AMP receptor protein in the absence of cyclic AMP

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Interdomain interaction of apo-cyclic AMP receptor protein (apo-CRP) was qualified using its isolated domains. The cAMP-binding domain was prepared by a limited proteolysis, while the DNA-binding domain was constructed as a recombinant protein. Three different regions making interdomain contacts in apo-CRP were identified by a sequence-specific comparison of the HSQC spectra. The results indicated that apo-CRP possesses characteristic modules of interdomain interaction that are properly organized to suppress activity and to sense and transfer the cAMP binding signals. Particularly, the inertness of the DNA-binding motif in apo-CRP was attributable to the participation of F-helices in the interdomain contacts.

Key words: allostery, cyclic AMP receptor protein, heteronuclear single quantum coherence, interdomain interaction, nuclear magnetic resonance.

Abbreviations: CRP, cyclic AMP receptor protein; NTD, N-terminal domain; CTD, C-terminal domain; CHαCRP, dimeric NTD of CRP (residues 1–136) generated by chymotrypsin digestion; βCRP^{C}, dimeric CTD of CRP (residues 110–209) containing the C-helices; HSQC, heteronuclear single quantum coherence.

Cyclic AMP receptor protein (CRP) is well known as a global transcription regulator in enteric bacteria and nearly 200 promoters have been suggested to be under the regulation of CRP (*1*). The protein is inactive in its apo form, *i.e*. in the absence of cAMP, but it is activated by cAMP binding and functions by binding to specific DNA sites, as well as by interacting with RNA polymerase (*2–5*). The structure of CRP has been well characterized by X-ray crystallography, at the three different states of complexes: CRP–cAMP (*6*, *7*), CRP–cAMP–DNA (*8–10*) and CRP–cAMP–DNA–RNA polymerase (*11*). Unfortunately, however, the apo-CRP structure, which is essential to understand the activation mechanism, has not been solved yet. CRP is a 47 kDa dimer composed of two identical subunits, each 209 amino acids long. In the crystal structures, each subunit is folded into two structurally distinct domains, which are covalently connected by a short polypeptide stretch (named the hinge region, residues 135–138; Fig. 1). The larger N-terminal domain (NTD, residues 1–134) is responsible for CRP dimerization and cAMP binding, while the smaller C-terminal domain (CTD, residues 139–209) is involved in specific recognition of DNA *via* a helix-turn-helix motif (E-helix and F-helix). In the crystal structure of the cAMP-bound CRP, the relative orientation of the two domains differs between the two subunits (*6*, *7*). In the 'open' subunit, there is a large cleft between the domains which is not present in the 'closed' subunit. However, when bound to DNA and RNA polymerase, both CRP subunits are in the closed conformation (*8–11*).

The activation process of CRP is typically demonstrated to be allosteric, where the cAMP-binding signals are transmitted from the NTD to the CTD (*4*, *12*, *13*). This allosteric conformational change of CRP is known to result in subunit realignment and domain rearrangement. However, majority of the researches has been focused on the intersubunit communications and the detailed features of interdomain interaction in CRP have been elucidated only in its cAMP-bound state, since the apo-state structure was not available. Thus, the structural information of apo-CRP, particularly concerning its interdomain communications, would be a fundamental requisite to understand the mechanism of CRP allostery. At this point, domain contacts in apo-CRP would be simply monitored by a comparative analysis between intact form and its isolated domains. In this work, individual domains of CRP were independently prepared, as well as the intact CRP. Then a simple spectroscopic approach was performed to characterize the interdomain interaction of CRP in apo state and to figure out its difference from the known properties in the cAMP-bound state.

In the presence of cAMP, CRP dimer can be cleaved with various proteases, producing a dimer of the NTD (αCRP), which retains cAMP-binding capability (*14*). Particularly, the fragment produced by chymotrypsin digestion (designated as CHαCRP, residues 1–136) corresponds to the whole region of NTD. Thus, we used CHαCRP as the NTD construct of CRP. Detailed protocol for the CHαCRP preparation by a limited proteolysis is described in Supplementary Data. The ^{1}H–^{15}N HSQC spectrum of the apo-CHαCRP in Fig. 2 was well

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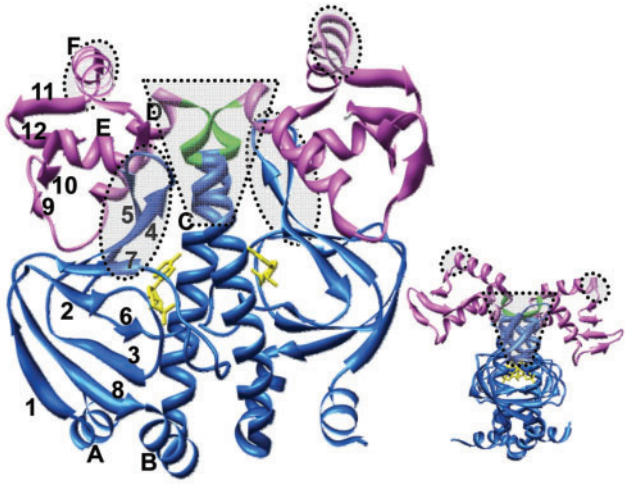

αA: D8 ~ S16	αB: Y99 ~ N109	αC: P110 ~ L137
αD: D138 ~ A151	αE: T168 ~ G177	αF: S179 ~ Q193
β1: H19 ~ Y23	β2: T28 ~ I30	β3: T38 ~ K44
β4: S46 ~ K52	β5: E58 ~ N65	β6: F69 ~ I70
β7: W85 ~ A88	β8: C92 ~ S98	β9: M157 ~ H159
β10: G162 ~ K166	β11: I196 ~ H199	β12: T202 ~ Y206

Fig. 1. **Ribbon presentation of the CRP-cAMP$_2$ structure (PDB entry 1G6N).** Both the front-view (larger figure) and the side-view (smaller figure) drawings are coloured blue, magenta, green and yellow for the NTDs, CTDs, hinge regions and cAMP molecules, respectively. The secondary structure is labelled on the left subunit of the font-view drawing, with letters for α-helices and numbers for β-strands. The residues constituting each secondary structure element are denoted under the figures. The regions responsible for the interdomain interaction in apo-CRP, suggested by the present results, are broadly indicated by dotted circles and boxes.

consistent with that of the recombinant NTD dimer, which was recently reported by Popovych *et al*. (*15*). Although the authors completed the NMR assignments of the protein, the data are not open to access yet.

It has been noted that the recombinant CTD (βCRP), which can be recognized by anti-CRP antibodies (*16*), could not be expressed to any significant amount, since it is very unstable and highly susceptible to proteolytic degradation (*16*, *17*). Thus, for the CTD construct, we cloned and expressed the domain including the C-helix, as used by Li *et al*. (*17*). This protein, designated as βCRP^C (residues 110–209), is known to be a cooperatively folded structural entity that behaves as a dimer (*17*). Detailed protocol for the βCRP^C preparation is also described in Supplementary Data. The ^{1}H–^{15}N HSQC spectrum of the βCRP^C in Fig. 2 also supported a well-folded conformation of the protein, by showing a goodness of spectral dispersion. Unfortunately, however, the expression level, solubility, stability and the dynamic properties of the protein were so poor that a series of three-dimensional spectra for NMR assignments could not be obtained with enough quality.

Figure 2 depicts that both the two isolated domains retained the structures comparable to those in the intact apo-CRP. The resonances from the apo-CHαCRP (blue in Fig. 2) and βCRP^C (red in Fig. 2) were broadly well matched with those from the intact apo-CRP (black in Fig. 2), with some exceptional peaks indicated in Fig. 3. Figure 3 shows a detailed comparison of the ^{1}H–^{15}N HSQC spectra between the intact apo-CRP and its isolated domains. Since the resonance assignments of the CHαCRP and βCRP^C are not available, as mentioned above, the residue-specific inspection of the spectra was just qualitatively approached using the previous backbone NMR assignments of apo-CRP (*18*, *19*), mainly in the well-resolved regions. Generally, independent domains not interacting with each other in an intact protein retain the resonance chemical shifts without any significant change, when isolated individually (*20*). In that case, only a few resonances from the termini where the covalent linkage was broken would be expected to show a reasonably altered chemical shift. However, we could identify at least 18 peaks that markedly shifted between the spectra, although other resonances in the apo-CRP spectrum are broadly well matched with those in either the apo-CHαCRP or the βCRP^C spectrum. Thus, the corresponding residues of the resonances identified can reflect the sites of interdomain interaction in apo-CRP. They could be divided into three groups according to their spatial locations in the cAMP-bound CRP structure (Fig. 1).

The first group of resonances involves the residues R123, V126, T127, S128, V131, G132, N133, L134, T140 and G141. They are mapped onto the around-hinge region from the C-terminus of the C-helix to the N-terminus of the D-helix (Fig. 1). This region in the cAMP-bound CRP is responsible for the abundant interdomain networks that constitute the interdomain and intersubunit interface of the protein (*6*, *7*). Particularly, the residues R123, T127 and S128 belong to the members that critically stabilize the cAMP binding. Thus, cAMP binding could easily modulate the interdomain networks in this region, to achieve allosteric transition. The second group, containing E54, I60, L61 and W85, is localized in the NTD, especially on or near the β-strands 4 and 5 (Fig. 1). This region also contributes to the interdomain contacts in the cAMP-bound CRP (*6*, *7*). The region around E54 (the hairpin turn between residues 53 and 56), which has a different conformation between the two subunits in the CRP-cAMP structure, lies close to the hinge region of the opposite subunit. The residues 58–63 (N-terminus of β-strand 5) are involved in non-covalent interdomain interaction with the residues 171 to 175 (C-terminus of E-helix). In particular, the residue I60 forms a direct hydrogen bond to Q174 and a water-mediated hydrogen bond to I175. However, in the present data, the residues E171 to C178 (indicated in Figs 2 and 3) showed no significant change of resonances. Thus, in apo-CRP, the N-terminus of the β-strand 5 seems to contribute to the interdomain interaction by a different way from that in the cAMP-bound CRP. This result supports the following model of CRP allostery established *via* a comprehensive insight into the CRP-cAMP structure. Passner *et al*. (*7*) have hypothesized that cAMP binding cause a rearrangement of domains *via* a dynamic movement of a flap consisting of β-strands 4 and 5, which switch between the cAMP-binding pocket and the interdomain networks. In this model, the loop connecting β-strands 6 and 7 is

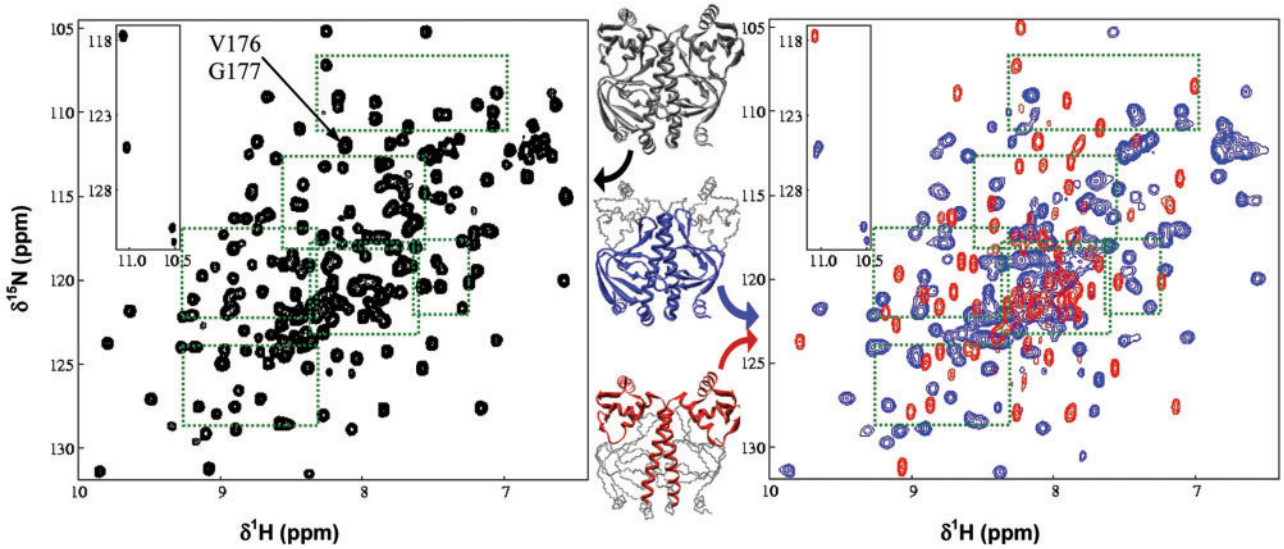

Fig. 2. **^{1}H–^{15}N HSQC spectra of apo-CRP (black), apo-CHαCRP (blue) and βCRP^{C} (red).** The spectra of 0.5–1.0 mM protein dissolved in the 50 mM potassium phosphate buffer (pH 6.7) containing 500 mM KCl and 7% D_2O were obtained at 313 K on a Bruker DRX 600 spectrometer. The constructed regions were indicated by the corresponding colours in the crystal structure of the cAMP-bound CRP (PDB entry 1G6N). The green-boxed regions were enlarged in Fig. 3.

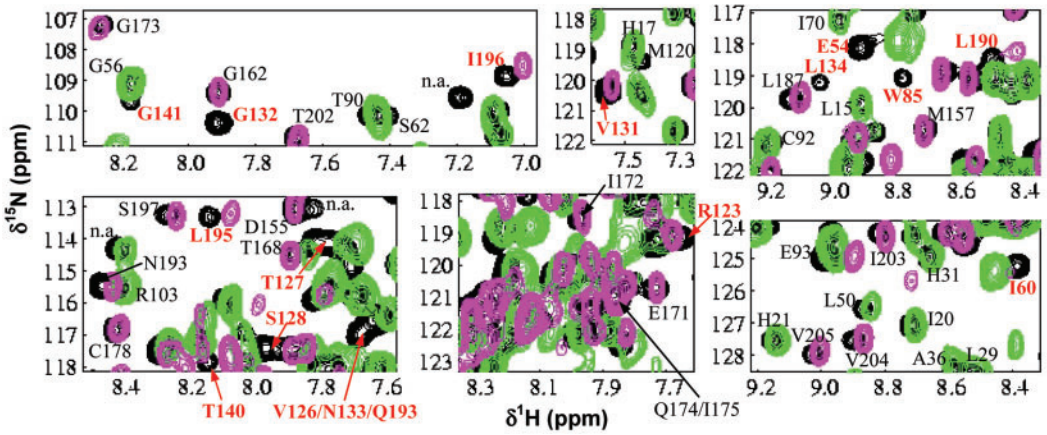

Fig. 3. **Close-up views into the selected regions taken from the superimposed ^{1}H-^{15}N HSQC spectra of apo-CRP (black), apo-CHαCRP (green) and βCRP^{C} (magenta).** The backbone NMR assignments of apo-CRP (*18*) are partly labelled. The apo-CRP resonances that totally shifted in the other spectra have red assignment labels. The resonances labelled 'n.a.', of which assignments are not available, mostly originate from side-chains.

also important to sense the cAMP-binding signal. The residue W85 of the present identifications, which does not contribute to interdomain interaction in the cAMP-bound CRP, is located at the N-terminus of the β-strand 7. Thus, if W85 is indeed involved in the interdomain interaction in apo-CRP, the residue could also play a role to transmit the cAMP-binding signal to the CTD.

The most interesting observations are taken from the third group of residues L190, Q193, L195 and I196, which is localized in the C-terminus of the F-helix and the turn between F-helix and β-strand 11 (Fig. 1). Other resonances from this region, such as L187, N193 and S197 in Fig. 3, also showed a moderate change of chemical shifts. This region is apart from the NTD and thus never involved in the interdomain communication in the cAMP-bound CRP. However, it has been suggested that the F-helix is protruded as a consequence of cAMP binding, to bind to DNA (*4*, *12*, *21*). Thus, the present identification reveals that the DNA-binding motif of CRP is inert in apo state, as it is constrained to face toward NTD to make interdomain contacts.

Some mutants of CRP, namely *CRP**, can activate CRP-dependent promoters in the absence of cAMP (*4*). Interestingly, almost all of the known positions of the *CRP** mutation, such as 53, 62, 127, 128, 138, 140, 141, 142, 144, 148 and particularly 195, are involved in the

three regions identified in the present results. Thus, it can be concluded that the interdomain networks in apo-CRP are cooperatively suppressing the activity of the protein. At this point, the *CRP** mutation or the cAMP binding could confer activity to the protein, by perturbing or modifying the interdomain networks.

In summary, structural information of CRP in the absence of cAMP is a key requisite to fully understand its allostery. In this study, the sites of interdomain contacts in apo-CRP could be identified by a simple comparison of the 2D ^{1}H–^{15}N HSQC NMR spectra between the intact apo-CRP and its isolated domains. In this comparison, for the following reasons, we could rule out the possibility that the identified inconsistency between spectra might be induced from local structural changes such as partial unfolding, regardless of interdomain networks. First, we could not observe any indication of unfolding, such as narrowing of spectral dispersion. Second, the two domain constructs, apo-CHαCRP and βCRP^{C}, have been previously characterized to adopt their structure highly comparable to that of the corresponding parts in the intact apo-CRP (*15*, *17*, *22*). The present data also supported the conservation of structural folds, by showing an overall consistency of spectra. In addition, even if the spectral changes alternatively originate from the local structural perturbation, the loss of interdomain contacts would be inevitably responsible for those structural changes. Consequently, the identified regions in the present study prove the existence of the noncovalent interdomain interaction and reflect the interdomain contact sites in apo-CRP. The results also verified that apo-CRP possesses different modules of interdomain interaction from those in the cAMP-bound state. The interdomain networks in apo-CRP showed favourable characteristics to efficiently suppress its activity. They were also properly organized to sense the cAMP-binding signal at the NTD and to transmit the signal to the CTD. In particular, the inertness of the DNA-binding motif in apo-CRP was attributable to the participation of F-helices in the interdomain contacts.

The present results constitute the first structural data of apo-CRP that provides new information about the interdomain contacts occurring in the protein. We expect the results will contribute to more profound understanding of CRP allostery and will encourage further biophysical investigations to detail the structure and interactions of the apo-CRP domains.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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