Cyclic AMP receptor protein (CRP) is a homodimeric protein, which is activated by cAMP binding to function as a transcriptional regulator of many genes in prokaryotes. Until now, the actual number of cAMP molecules that can be bound by CRP in solution has been ambiguous. In this work, we performed a nuclear magnetic resonance study on CRP to investigate the stoichiometry of cyclic nucleotide binding to CRP. A series of 1H-15N heteronuclear single quantum coherence (HSQC) spectra of the protein in the absence and in the presence of cAMP or cGMP were analyzed. The addition of cAMP to CRP induced a biphasic spectral change up to 4 equivalents, whereas the cGMP addition made a monophasic change up to 2 equivalents. Altogether, the results not only established for the first time that CRP possesses two cyclic AMP-binding sites in each monomer, even in a solution without DNA, but also suggest that the syn-cAMP binding sites of the CRP dimer can be formed by an allosteric conformational change of the protein upon the binding of two anti-cAMPs at the N-terminal domain. In addition, a residue-specific inspection of the spectral changes provides some new structural information about the cAMP-induced allosteric activation of CRP.

Cyclic AMP receptor protein (abbreviated as CRP); also referred to as catabolite gene activator protein, CAP) plays a key role in the regulation of more than 150 genes in prokaryotes (1–4). CRP 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. The protein is a dimer composed of two identical subunits, each 209 amino acids long. Several three-dimensional structures of the CRP-cAMP complex, with or without DNA, have been solved by x-ray crystallography (4–7). In these structures, each subunit of CRP is folded into two structurally distinct domains, which are covalently connected by a short polypeptide stretch named the hinge region (residues 135–138). The larger N-terminal domain, which is predominantly β-stranded, is responsible for CRP dimerization, while the smaller C-terminal domain, which is predominantly α-helical, is involved in specific recognition of DNA. Although many biochemical and biophysical studies have implied that cAMP binding allosterically induces CRP to assume an active conformation (8–17), this allostery of CRP activation is not clearly understood at the structural level, since the three-dimensional structure of apo-CRP has not been solved yet.

At present, another matter of primary concern and interest regarding CRP is the number of conformational states the protein can adopt with cAMP and which one of the states is the active conformation. The many biochemical and biophysical properties of CRP that exhibit a bimodal dependence on cAMP concentration have been interpreted as evidence for the existence of three conformational states of the protein (18–20). During the 3 decades since its discovery, the protein has been thought to be able to bind two cAMP molecules, and thus three conformational states of CRP have been considered, namely apo-CRP, CRP-cAMP1, and CRP-cAMP2 (18, 19). This assumption has been supported by the crystal structures of the CRP-cAMP2 and CRP-cAMP2-DNA complexes (4–6), where two cAMP molecules are bound with an anti-conformation in the N-terminal domains of the protein. In addition, the second state, CRP-cAMP1, has been considered to be the only active conformation of CRP (18, 19). Recently, Passner and Steitz (4) solved the crystal structure of the CRP-cAMP2-DNA complex, where two additional cAMP molecules with a syn-conformation are bound to the region between the interdomain hinge and the C-terminal domain of the protein. Thus, they reinterpreted the three conformers as represented by apo-CRP, CRP-cAMP2, and CRP-cAMP4. Afterward, several reports adopting this new model have appeared in the literature (20, 21). However, this new paradigm for CRP is doubtful for the following reasons, as pointed out by Harman (2). First, the new model could not explain the fact that CRP is functionally activated with one equivalent of cAMP (22–25). Second, aside from the existence of a CRP-cAMP2-DNA crystal structure, there has been no compelling experimental evidence for CRP-cAMP4 in solution. In addition, the protein footprinting results of CRP showed that the DNA binding of CRP probably alters the conformation of the protein (26), and the DNA molecule also contributes to the syn-cAMP binding in the crystal structure of CRP-cAMP4-DNA. Thus, it is not clear, in solution, whether a CRP dimer in nature can bind four cAMP molecules or CRP-cAMP2 can bind the additional two syn-cAMP molecules only after binding DNA.

In the present work, we concisely analyzed the stoichiometry of the cAMP binding to CRP in solution and in the absence of DNA, by NMR spectroscopy. The results clearly revealed that CRP binds four cAMP molecules as its maximum, even in the solution without DNA. In addition, the comparative study of cGMP, an antagonist of cAMP (13–15), provides new information about the allosteric conformational change of CRP by cAMP binding.
**EXPERIMENTAL PROCEDURES**

99.9% D$_2$O, cAMP, and cGMP were purchased from Sigma, and all other materials were either analytical or biotechnological grade. Uniformly 15N-labeled CRP was prepared as reported previously (9). The absence of cAMP in the purified CRP solution was checked dually by the absorbance ratio at 278 and 260 nm and by the near-UV circular dichroism spectrum shape of the protein (9, 27–30). The concentrations of the protein and cyclic nucleotides were determined spectrophoto metrically using the following extinction coefficients: 41,000 M$^{-1}$ cm$^{-1}$ at 278 nm for the CRP dimer (8, 9); 14,650 M$^{-1}$ cm$^{-1}$ at 258 nm for cAMP (30); 12,950 M$^{-1}$ cm$^{-1}$ at 254 nm for cGMP (30). The conventional two-dimensional 1H-15N HSQC spectra of 50 $\mu$M [U-15N]CRP dissolved in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 M potassium chloride, 7% D$_2$O, and diverse concentrations of cAMP or cGMP were obtained at 313 K on a Bruker DRX 600 spectrometer. All of the spectra were processed and analyzed using the NMRPipe/NMRDraw software (31) and the NMRView program (32). Residue-specific assignments were based on the previous report for apo-CRP (8). Peak intensities were calibrated between spectra by making the Gln6 resonance intensity of each spectrum equal to each other, since the resonance retained the most constant chemical shift and resonance intensity independently of the addition of cyclic nucleotides.

**RESULTS**

As shown in Fig. 1, most of the resonances in the HSQC spectra of CRP were perturbed upon the addition of cAMP. This spectral change was completed with 4 molar equivalents of cAMP for CRP (Fig. 1C) and showed a biphasic pattern with a phase from 0 to 2 equivalents and the other from 2 to 4 equivalents of cAMP. For example, as shown in Fig. 2, the resonances Glu$^{72}$ and Gly$^{74}$ were broadened by the addition of the first equivalent of cAMP and subsequently disappeared with 2 equivalents of cAMP binding. Concomitantly, the peaks a and d, which seem to correspond to the resonances of Glu$^{72}$ and Gly$^{74}$, respectively, in the presence of cAMP, appeared through the binding of the first two cAMPs, and no significant chemical shift change in these resonances occurred with the further addition of cAMP. In contrast, the chemical shift changes from the resonances Asp$^{161}$, Gly$^{173}$, and Gly$^{184}$, probably to the respectively corresponding peaks b, e, and f, began when the molar ratio of CRP to cAMP was 1:3 and were completed at the molar ratio of 1:4. Fig. 3 also clearly depicts the biphasic spectral change of CRP upon cAMP binding. The resonance Ile$^{203}$ shifted, probably to peak a, with a remarkable broadening through the binding of the first two equivalents of cAMP, and then shifted again through the binding of the second two equivalents of cAMP, probably to peak b. Fig. 4 also semiquantitatively shows the spectral change of CRP by the binding of cAMP. Since the sequence-specific NMR assignments of the cAMP-bound CRP were impossible, we could not plot the chemical shift differences between the apo- and cAMP-bound CRP forms. Instead, we analyzed the remaining intensities of individual resonances. For several representative resonances, the square root values of the relative intensity, $I/I_0$, are plotted in Fig. 4 as a function of the molar equivalents of cAMP to CRP, where $I$ and $I_0$ are the resonance intensities in the presence or absence of cAMP, respectively. The $(I/I_0)^{1/2}$ values are representative of the unchanged or remaining fraction, based on the fact that the resonance intensity is approximately proportional in general to the square of the concentration, and its zero value indicates that the resonance was completely broadened or shifted elsewhere. Some cAMP-dependent intensity decreases were completed (i.e. the peaks disappeared) when the molar
ratio of CRP to cAMP was 1–2, while the others continued up to the ratio of 1–4. Only a few resonances from the N-terminal residues retained both their chemical shift and intensity, independently of the added cAMP concentration (Fig. 4). Although almost all of the resonances, except for those from −10 N-terminal residues, were perturbed upon cAMP addition, the individual ending points of change could be identified qualitatively or semiquantitatively for only 76 resonances; the changing group A, consisting of 46 resonances that were completely broadened or shifted elsewhere by the binding of 2 cAMP equivalents to CRP, and the changing group B, consisting of 30 resonances whose spectral changes were completed with 4 equivalents of cAMP. Their corresponding residues are respectively summarized in Fig. 5. It is very likely that some of the resonances of the former changing group were affected subsequently by the second two equivalents of cAMP binding, as depicted for resonance Ile^{203} (Fig. 3). Unfortunately, however, this could rarely be identified for the changing group A resonances, due to the lack of sequence-specific assignments in the [CRP]/2[cAMP] spectrum (Fig. 1B). It was also likely that some resonances in group B were changed by the first two equivalents of cAMP. For example, the resonances Glu^{54} and Gly^{56},

![Image of two-dimensional 1H-15N HSQC spectra](image-url)
whose intensity reductions were completed by 4 equivalents of cAMP, showed more severe intensity decreases through the first 2 equivalents of cAMP than the other resonances in group B (Fig. 4). Their chemical shifts seemed to be slightly different in the [CRP]/2[cAMP] spectrum from those in the apo-CRP spectrum (data not shown), but these chemical shift changes were so small, with the concomitant broadening and overlap, that they could not be confirmed.

The spectral changes induced by the addition of cGMP were quite different from those caused by cAMP. For instance, in Fig. 2, although the resonances Glu72 and Gly74 disappeared, as observed with cAMP, through the 2 equivalents of cGMP binding, the corresponding shift of the Glu72 resonance was gradually positioned probably to peak c instead of peak a and that for Gly74 could not be observed. Moreover, the further addition of cGMP above 2 equivalents caused no significant change within the entire region in the HSQC spectra of CRP (Fig. 1D), indicating saturation at the molar ratio of 1:2 (CRP dimer versus cGMP). All of the resonances changed by cGMP binding were included in the changing group A, indicating that cGMP seems to bind at a site in CRP similar to that occupied by the first 2 equivalents of cAMP. However, the identified chemical shift changes by cGMP were not only different in their pattern, but were also smaller in their degree than those caused by cAMP. This means that the cGMP-induced conformational change of CRP, in contrast to that caused by cAMP, is neither dramatic nor correct for activation.

**DISCUSSION**

Despite the high molecular mass of the apo-CRP dimer (47 kDa), which has been considered rather large to be assigned by NMR, we previously obtained a nearly complete set of backbone NMR assignments of the protein, and determined the secondary structure of apo-CRP, by a series of multidimensional NMR spectra on the triply (13C, 15N, and 2H) labeled apo-CRP (8, 9). However, the low solubility, the low stability at high temperature (313 K), and the rapid relaxation time of the cyclic nucleotide-bound CRP prevented us from obtaining diverse three-dimensional NMR spectra, such as triple resonance spectra, which are essential for its sequence-specific NMR assignments (9). Instead, in this work, we obtained only the two-dimensional 1H–15N HSQC spectra of CRP in the presence of cyclic nucleotides, cAMP or cGMP, with rather good quality for the low concentration; higher concentrations (than 50 μM) reduced the spectral quality, due to precipitation during the measurements at 313 K. Both the impossibility of NMR assignments for the cyclic nucleotide-bound CRP and the severe spectral overlap in many regions of the HSQC spectra (Fig. 1) prevented us from achieving a complete and quantitative analysis of the individual resonances. Thus, the spectra were qualitatively or semi-quantitatively analyzed, mainly in the well resolved region, on the basis of the previous backbone NMR assignments (8) of apo-CRP.

Despite some ambiguities, due to the insufficiency of the assignments for the cyclic nucleotide-bound CRP, the present results clearly revealed for the first time that a CRP dimer could bind four cAMP molecules in the absence of DNA in solution, forming two distinct cAMP-binding sites in each monomer. At least three stable conformational states of CRP with cAMP exist, namely apo-CRP, CRP-cAMP2, and CRP-cAMP4, as revealed by the present HSQC spectra in line with their crystal structures (3–6). In the presence of 1 or 3 equivalents of cAMP, the HSQC spectra of CRP became heterogeneous, with the doubling of many resonances. Unfortunately, it could not be determined whether the spectrum of CRP with 1 equivalent of cAMP reflects the intramolecular heterogeneity from a heterodimer with only one subunit that bound cAMP, or the intermolecular heterogeneity from the mixing of two distinct homodimeric proteins, apo-CRP and CRP-cAMP2. Likewise, the spectrum of CRP with 3 equivalents of cAMP could not prove by itself the conformational state of CRP-cAMP3. However, many
functional aspects of CRP have been observed with only one equivalent of cAMP, and the crystal structure of a CRP mutant that bound three cAMP molecules has been solved (2, 22–25, 33). More experiments designed to distinguish the diverse conformational and functional states of CRP are necessary to understand the action mechanism of the protein, and we expect that the present results will provide important fundamental information for them.

The present study revealed that the maximum binding of cGMP, which is structurally quite similar to cAMP, is 2 equivalents for CRP. Cyclic GMP, compared with cAMP, made different and relatively small perturbations in the cAMP spectrum; nevertheless, its binding site and affinity to CRP are relevant to those of the first 2 cAMP equivalents (9, 13–15). The different pattern and stoichiometry between cAMP and cGMP binding to CRP suggest the following hypothesis: the second two cAMP-binding sites in the CRP dimer are not innate and are formed by a conformational change of the protein upon the binding of the first two cAMP molecules at the two innate cyclic nucleotide-binding sites. The biphasic ordering of the cAMP-induced spectral change of CRP supports this assumption. This structural model can be depicted as a positive cooperativity between anti- and syn-cAMP binding. The severe broadening in the [CRP]/2cAMP spectrum (Fig. 1B), which was greater than that in any other CRP spectrum measured in this work, seems to support the positive cooperativity between the two distinct cAMP binding sites in CRP, under the conditions employed in this study. As a conclusion, the allosteric activation of CRP involves the conformational change by the cAMP binding in the N-terminal domain, and it probably leads to the formation of additional camp-binding sites near the C-terminal domain. In addition, it is apparent that the inactivity of CRP with cGMP is attributable to the absence of the correct allosteric conformational change of the protein.

A detailed inspection of the results provides additional information about the cAMP-mediated conformational change of CRP. In the crystal structure of CRP-cAMP2-DNA (3), two anti-cAMP molecules are bound in the N-terminal domain, and the other two syn-cAMP molecules are near the C-terminal domain (Fig. 5). Consistently, in the present results, the residues that were affected by the first 2 equivalents of cAMP binding to CRP were mapped mainly in the N-terminal domain, including the interdomain hinge and the helix D, while those affected by the second 2 equivalents were mainly in the C-terminal domain. There are two exceptional regions in these maps. First, the region around residues Glu184 and Gly356, despite its location in the N-terminal domain, seems to be affected by the second 2 equivalents of camp binding. This is since the region involves some residues that directly interact with the syn-cAMP molecules in the crystal structure of CRP-cAMP2-DNA (4). The second exception is the C-terminal β-sheet, composed of β-strands 9, 10, 11, and 12, where at least 5 residues (Ile165, Leu185, Ile203, Val205, and Gly207), which were reasonably affected by the first 2 equivalents of cAMP binding. This was unexpected, since this β-sheet in the cAMP-bound CRP crystal is located apart from the anti-cAMP binding sites, spatially as well as in the sequence. Thus, it can be concluded that the C terminus is closer to the N-terminal domain in apo-CRP than in cAMP-bound CRP. A previous NMR study (9) showed that the C-terminal β-sheet is conserved both in the apo- and cAMP-bound CRP, but its interstrand hydrogen-bonding pattern in apo-CRP in solution slightly differed from that of CRP-cAMP2 in the crystal (Fig. 4). For example, apo-CRP in solution probably lacks the hydrogen-bonding between Leu190 and Val1505, which was observed in the cAMP-bound CRP crystal. However, it is not clear whether these small differences are really related to the conformational change or due to the different conditions (solution versus crystal). The present results clearly support the former proposal, in that the resonances Ile165, Leu185, Ile203, Val205, and Gly207 were obviously affected by the binding of the first 2 equivalents of cAMP. In this β-sheet, β-strands 11 and 12 are important for DNA binding (34), and β-strands 9 and 10 are crucial for RNAP binding of CRP (35, 36). Thus, the results indicate that the allosteric conformational change of CRP, which is the functional activation process induced upon cAMP binding, involves the spatial repositioning and the reconstitution of the hydrogen-bonding pattern of the C-terminal β-sheet.

Although the structure of CRP bound to DNA has been solved, its structure complexed with RNAP is not available. As a consequence, a lot is known about the CRP-DNA contact, while in contrast, less is known about the structural mechanism of the CRP-RNAP interaction. Cyclic AMP mediates the protein-protein interaction between CRP and RNAP as well as the CRP-DNA interaction, and the cAMP-bound CRP interacts with RNAP in solution even in the absence of DNA (36, 37). As shown in the present results, the cAMP-induced spectral perturbation of CRP was so enormous that it necessarily involved the resonances from the residues at or adjacent to the three RNAP-interacting regions (38) of the protein. In addition, the binding of both the first (anti-) and the second (syn-) cAMP affected those resonances (at least His124, Glu154, Glu196, Arg103, Asp155, Met157, Thr158, His159, Asp161, Gly162, Gin164, and Ile165, for example). The C-terminal β-sheet of CRP, which seems to be readjusted by the cAMP binding, also involves the major RNAP-binding sites (residues Ala156-Gln164, Refs. 35 and 36). Until now, no clear evidence that supports the cAMP-mediated conformational change in the RNAP-interacting region of CRP has been reported. Thus, the present results provide rather detailed structural data for that conformational change and suggest that the cAMP-dependent biphase feature of the transcriptional regulation by CRP should be further studied at the structural level, focusing on the CRP-RNAP interaction as well as the CRP-DNA interaction.

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