J. Biochem. 130, 57–61 (2001)

# Detection of the Protein–Protein Interaction between Cyclic AMP Receptor Protein and RNA Polymerase, by $^{13}$C-Carbonyl NMR[1]

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Received February 26, 2001; accepted April 11, 2001

**Cyclic AMP receptor protein (CRP) plays a key role in the transcription regulation of many prokaryotic genes. Upon the binding of cyclic AMP, CRP is allosterically activated, binds to target DNA sites, and interacts with RNA polymerase. Although the protein–protein interaction between CRP and RNA polymerase is known to be important for the transcription initiation of the target genes, its structural understanding is still lacking, particularly due to the high molecular mass (~120 kDa) of the protein complex. We assigned all of the $^{13}$C-carbonyl resonances of methionine residues in CRP by using the double labeling and the enzyme digestion techniques. The result of $^{13}$C-carbonyl NMR experiment on [$^{13}$C′-Met]-CRP in the presence of both cyclic AMP and RNA polymerase α subunit showed that the two proteins interact with each other in solution in the absence of DNA *via* the region around the residues from Met 157 to Met 163 in CRP. The results also showed the effectiveness of the selective labeling and $^{13}$C-carbonyl NMR spectroscopy in the specific detection of the protein–protein interaction between large molecules.**

**Key words: $^{13}$C-carbonyl NMR, CRP, protein–protein interaction, RNA polymerase, transcription regulation.**

Protein–protein interactions are known to be crucial for the biological functions of many proteins including transcription factors. However, protein–protein interactions are often not understood at the structural level, since the protein complex generally has a rather high molecular weight, which hinders study by structure-determining tools such as X-ray crystallography or NMR spectroscopy. Thus, in many cases, the structural features of the protein–protein interaction between large proteins remain obscure. The transcription regulation by cyclic AMP receptor protein (abbreviated as CRP; also referred to as catabolite gene activator protein, CAP) and RNA polymerase is a typical example of such a protein–protein interaction.

CRP is well known as a DNA-binding protein that regulates the expression of more than 150 genes in prokaryotes (*1*). CRP is a homo-dimeric protein consisting of 209 amino acids in each monomer (Fig. 1), and functions by binding, in the presence of the allosteric effector cAMP, to specific DNA sites, and interacting with RNA polymerase (*2–4*). RNA polymerase is a holoenzyme consisting of α, β, β′, and one of several species of σ subunits, and the RNA polymerase α subunit is also a homo-dimeric protein (*5, 6*). Several three-dimensional structures of CRP and RNA polymerase are now available. The structure of the cAMP-bound CRP was determined by X-ray crystallography in both the presence and absence of the bound DNA (*1, 7–9*). In the case of RNA polymerase, the three-dimensional structures of the C-terminal and the N-terminal domain of α subunit were solved by NMR spectroscopy and X-ray crystallography, respectively (*10, 11*). The crystal structure of a fragment of RNA polymerase σ subunit has also been reported (*12*). However, the structures of CRP without a bound molecule (apo-CRP), β, β′, and intact σ subunits of RNA polymerase, and the protein complex of CRP with RNA polymerase have not been solved yet.

Although it has been proposed for several decades that the direct interaction of CRP with RNA polymerase is important for the transcription regulation (*2, 13*), it is only recently that the site of interaction of CRP with RNA polymerase was tentatively identified (*6*). Isolated mutants of CRP defective in transcription activation but not defective in DNA binding were all found to contain amino-acid substitutions within a surface loop consisting of residues 156–164 (*14*). Thus it has been proposed that transcription regulation by CRP involves direct protein–protein contact between the surface loop of CRP and RNA polymerase. Study of truncated or mutated RNA polymerase has further suggested that the region of RNA polymerase contacting with the surface loop (residues 156–164) of CRP is located in the C-terminal domain of its α subunit (*5, 6, 15*). However, no detailed data from direct observation of the interaction between the two proteins has been reported. This paper reports the first observation of the specific protein–protein interaction between the region of wild-type CRP containing Met 157 and Met 163 residues and RNA polymerase α subunit.

[1] This study was supported by the Genetic Engineering Research Program, Ministry of Education, Korea. This work was also supported in part by year 2000 BK21 project for Medicine, Dentistry and Pharmacy, Korea.

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Abbreviations: αCRP, N-terminal fragment of CRP; cAMP, adenosine 3′,5′-cyclic monophosphate; CRP, cAMP receptor protein; NMR, nuclear magnetic resonance; RNAPα, RNA polymerase α subunit.

This paper also shows that the $^{13}C$-carbonyl NMR method can be broadly generalized to the study of protein–protein interactions. If the carbonyl carbons of a specific amino acid in a protein are selectively labeled with $^{13}C$, the one-dimensional $^{13}C$ NMR spectrum can be highly simplified with the NMR signals of only the labeled amino acids. Use of one-dimensional $^{13}C$-carbonyl NMR in the structural study of large proteins was first proposed by Kainosho and Tsuji (*16*), who developed an assignment strategy of the selectively labeled $^{13}C$-carbonyl NMR spectrum by specific double labeling. Subsequently, the double labeling method combined with the use of subdomains was applied to the spectral assignment of the $^{13}C$-carbonyl resonances of human annexin I (*17*). After complete assignment of the $^{13}C$-carbonyl resonances, the ligand-binding sites of a protein could be studied by $^{13}C$-carbonyl NMR spectroscopy (*17–20*). In this research, we assigned $^{13}C$-carbonyl-Met resonances of CRP by double labeling and enzyme digestion techniques, and extended the use of $^{13}C$-carbonyl NMR technique to the structural study of the protein–protein interaction as well as ligand-binding of CRP.

## MATERIALS AND METHODS

*Materials*—Isotope $^{13}C'$- or $^{15}N^{\alpha}$-labeled amino acids and 99.9% $D_2O$ were purchased from Isotec (Miamisburg, OH) and Cambridge Isotope (Andover, MA). The isotope enrichment was 95% or higher for each of the amino acids. Subtilisin and cAMP were purchased from Sigma (St. Louis, MO), and all other materials were either analytical or biotechnological grade. Column resins, Bio-Rex 70 and hydroxyapatite were purchased from Bio-Rad Laboratories (Hercules, CA).

*Sample Preparation*—Selectively $^{13}C'$-labeled CRP and $^{13}C'/^{15}N^{\alpha}$-double-labeled CRP were obtained from the overproducing *E. coli* strain BL21 containing the plasmid pT7-CRP, with the isotope-labeled amino acids, and purified by sequential chromatography on Bio-Rex 70 and hydroxyapatite, as described previously (*20, 21*). Five kinds of selectively labeled CRP were prepared: [$^{13}C'$-Met], [$^{13}C'$-Met/$^{15}N^{\alpha}$-Arg], [$^{13}C'$-Met/$^{15}N^{\alpha}$-Ala], [$^{13}C'$-Met/$^{15}N^{\alpha}$-Thr], and [$^{13}C'$-Met/$^{15}N^{\alpha}$-Gln]CRP. The intact $\alpha$ subunit of RNA polymerase was expressed in *E. coli* BL21($\lambda$DE3) cells transformed with the expression plasmid pGEMAX185 (*24*), and purified by the method of Negishi *et al.* (*25*). Half-size deleted CRP ($\alpha$CRP) was obtained by limited digestion of CRP by subtilisin, as reported previously (*20*).

*$^{13}C$-Carbonyl NMR Spectroscopy*—$^{13}C$ NMR spectra were recorded on a Bruker AMX 500 FT NMR spectrometer at 303 K with the protein dissolved in 50 mM potassium phosphate buffer (pH 6.7) containing 0.3 M KCl. Before measurement, the solvent water was changed to $D_2O$ after lyophilization. The NMR spectra were recorded at 125 MHz using a Waltz-16 composite pulse decoupling sequence. The free induction decay was recorded with 32 K data points and a spectral width of 12 ppm centered at 176 ppm. The $^{13}C$ chemical shifts are given in ppm from the methyl group resonance of 2,2-dimethyl-2-silapentane-5-sulfonate.

## RESULTS

*Assignments of the $^{13}C$-Carbonyl Resonances of Met Residues in apo-CRP*—CRP monomer has six methionine residues, three (Met 59, 114, and 120) in the N-terminal domain and the others (Met 157, 163, and 189) in the C-terminal domain, and the methionine residues are well dispersed in the spatial structure of CRP (Fig. 1). In the $^{13}C$-carbonyl NMR spectrum of apo-CRP labeled with [1-$^{13}C$]Met, the carbonyl resonances of the six methionine residues in a subunit of apo-CRP were well resolved (Fig. 2A), indicating the symmetric structure of CRP dimer. In the $^{13}C$-carbonyl NMR spectrum of apo-CRP, in which the Met and Arg residues were labeled simultaneously with [1-$^{13}C$]Met and [$\alpha$-$^{15}N$]Arg, resonance "b" was severely decreased in intensity (Fig. 2B). In a $^{13}C'/^{15}N^{\alpha}$-double-labeled protein, the peak of a $^{13}C$-carbonyl carbon is split and/or decreases in intensity due to spin coupling with the amide $^{15}N^{\alpha}$ of the next residue in the sequence (*16–20*). Accordingly, the resonance "b" could be assigned to Met 114, since only this of the six methionine residues is directly followed by arginine (Fig. 1A). Resonance "c", whose intensity was specifically decreased in the spectrum of [$^{13}C'$-Met/$^{15}N^{\alpha}$-Ala]CRP, was assigned to Met 120 (Fig. 2C), which is followed by Ala 121 (Fig. 1A). Resonances "d" and "e" significantly broadened in the spectra of [$^{13}C'$-Met/$^{15}N^{\alpha}$-Thr]CRP and [$^{13}C'$-Met/$^{15}N^{\alpha}$-Gln]CRP, respectively (Fig. 2, D and E). Therefore the resonances "d" and "e" were assigned to Met 157 and Met 163, which are followed by Thr 158 and Gln

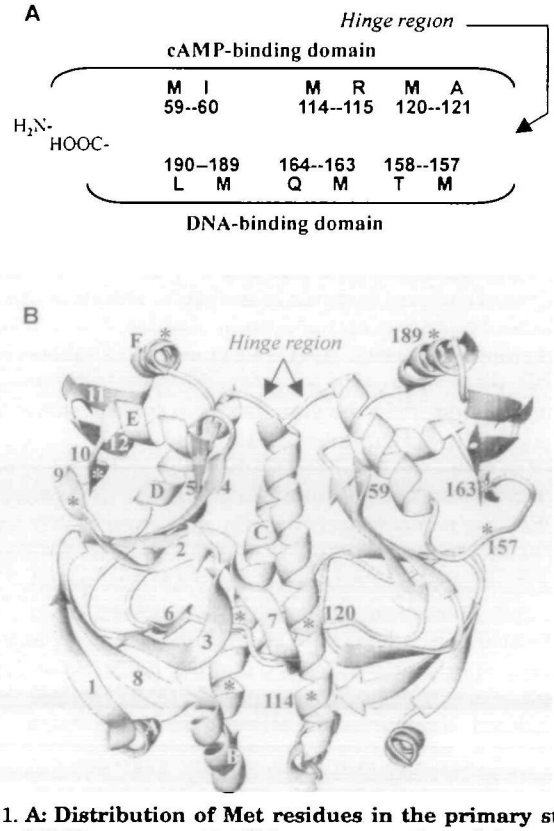


Fig. 1. **A: Distribution of Met residues in the primary structure of CRP monomer. B: Ribbon presentation of the crystal structure of CRP dimer.** The secondary structure is labeled with letters for $\alpha$-helices and numbers for $\beta$-strands in the left subunit. The positions of methionine residues are indicated by asterisks in both subunits, and labeled with the sequence number in the right subunit. This drawing was produced with the UCSF MidasPlus program using the coordinates for CRP complexed with cAMP and DNA (*8*).

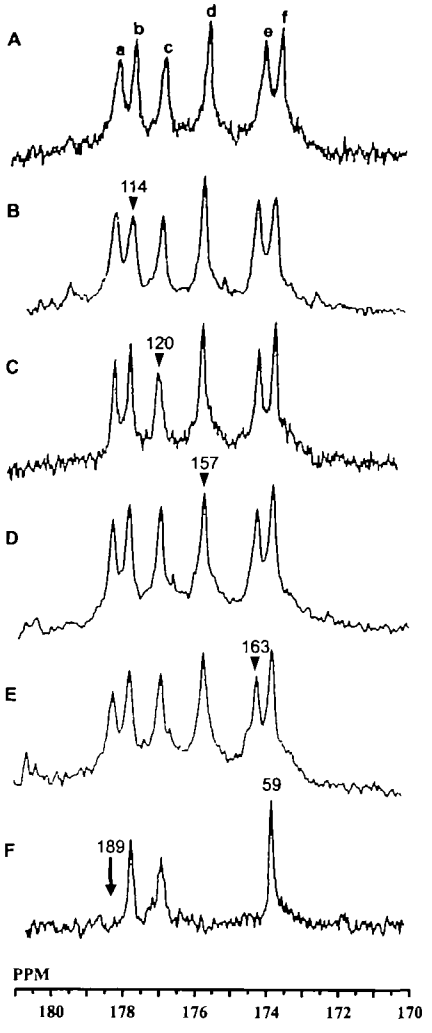


Fig. 2. **125 MHz $^{13}$C-carbonyl NMR spectra of [$^{13}$C′-Met] (A), [$^{13}$C′-Met/$^{15}$N$^{\alpha}$-Arg] (B), [$^{13}$C′-Met/$^{15}$N$^{\alpha}$-Ala] (C), [$^{13}$C′-Met/$^{15}$N$^{\alpha}$-Thr] (D), and [$^{13}$C′-Met/$^{15}$N$^{\alpha}$-Gln]CRP (E), and [$^{13}$C′-Met]αCRP (F).** The concentration of CRP was in 0.1–0.2 mM for dimer.

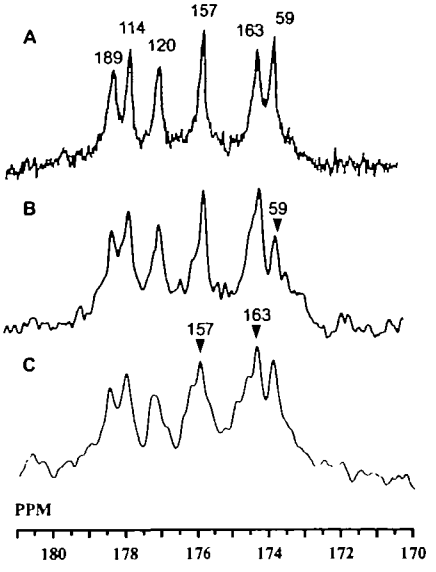


Fig. 3. **125 MHz $^{13}$C-carbonyl NMR spectra of [$^{13}$C′-Met]CRP in the absence of cAMP and RNAPα (A), in the presence of cAMP (B), in the presence of both cAMP and RNAPα (C).** Concentrations of cAMP and proteins (CRP dimer and RNAPα dimer) were 0.4 and 0.2 mM, respectively.

164, respectively. When the [$^{13}$C′-Met]CRP was cleaved by subtilisin, three resonances ("a", "d", and "e") disappeared, and the others (resonances "b", "c", and "f") remained with the unchanged chemical shifts (Fig. 2F). The limited subtilisin digestion of the CRP dimer produces αCRP, a dimer of the N-terminal domain of CRP, which retains the same structure as found in the intact protein (*24, 25*). Thus, the disappearing resonance "a" and the remaining resonance "f" could be assigned to Met 189 and Met 59, respectively. In addition, the $^{13}$C-carbonyl NMR spectrum of the [$^{13}$C′-Met]αCRP (Fig. 2F) confirmed that the structure of αCRP dimer is symmetric and nearly identical to that of the N-terminal domain in the intact CRP dimer. The completely assigned chemical shifts of these resonances (Fig. 3A) were in good agreement with those determined previously by the 3D HNCO experiment (*21*).

*Protein–Protein Interaction between CRP and RNA Polymerase α Subunit*—To interact with DNA and RNA polymerase, CRP requires allosteric activation by the binding of cAMP (*13, 26–28*). When cAMP was added to apo-CRP, overall broadening occurred in the $^{13}$C-carbonyl NMR spectrum of the [$^{13}$C′-Met]CRP (Fig. 3B), implying a motional suppression of the protein by the ligand binding (*20*). Apparently, several resonances (Met 114, Met 120, Met 157, and Met 163) seem to have shoulders at the left sides of their main peaks. The peak shoulders of Met 157 and Met 163 remain in the spectrum of CRP-cAMP-RNAPα complex (Fig. 3C). But it is not clear whether these asymmetric peak shapes indicate a specific conformational change of the region or are due to a technical problem such as line broadening. Nevertheless, in the presence of cAMP, the Met 59 resonance showed a specific and sharp decrease in intensity, showing that the residue contributes to the formation of the cAMP-binding pocket in CRP (*20*). In the presence of both cAMP and RNAPα, the overall $^{13}$C-carbonyl NMR spectrum of the [$^{13}$C′-Met]CRP broadened (Fig. 3C), which indicates that the rotational correlation time of CRP was increased by its interaction with RNAPα. In particular, the Met 157 and Met 163 resonances were remarkably broadened by the contact with RNAP. Thus it can be concluded that at least the region around the residues from Met 157 to Met 163 in CRP makes direct contact with the α subunit of RNA polymerase. This region is consistent with the region, residues 156–164, suggested previously by point mutation experiments (*14*). The apparently higher peak of the Met 59 resonance in the presence of RNAPα (Fig. 3C) than that in its absence (Fig. 3B) is probably due to the broadening effect of the adjacent Met 163 resonance. Similarly, the apparent change in the chemical shift of the Met 120 resonance in the spectrum of CRP-cAMP-RNAPα complex from that of CRP-cAMP complex may be due to the asymmetric shape of the peak, which has a left shoulder in the spectrum of CRP-cAMP (Fig. 3B) but a right shoulder in that of CRP-cAMP-RNAPα complex.

## DISCUSSION

The RNAPα-binding region of CRP detected in this work is involved in the C-terminal DNA-binding domain of the protein. Previously we have studied the structural mechanism of the cAMP-induced allosteric activation of CRP by heteronuclear multidimensional NMR spectroscopy and a series of $^{13}$C-carbonyl NMR experiments (*20*). From those results, we concluded that the cAMP binding to CRP changes the secondary structure around only the cAMP-binding pocket, whereas the C-terminal DNA-binding domain is reoriented without any significant change of secondary structure. The present results support this conclusion, although they are not sufficient to allow a clear interpretation of the allosteric mechanism of CRP. On addition of cAMP, the $^{13}$C-carbonyl resonance of Met 59, which is located in the cAMP-binding pocket, was specifically changed; but those of Met 157 and Met 163, which are involved in the DNA-binding domain, remained without any reasonable alteration. Nevertheless, CRP acquired the RNAPα-binding ability after cAMP binding *via* at least the residues Met 157 and Met 163. This indicates that the microenvironments around the RNAPα-binding region detected in this work are not perturbed by the cAMP binding. Thus, as concluded previously, we can infer that the cAMP-induced allosteric mechanism of CRP involves the global reorientation of the DNA-binding domain with no dramatic change of secondary structure.

Heyduk *et al.* (*13*) suggested first that CRP interacts with RNAP in solution in the absence of promoter DNA. They observed a large increase in the fluorescence anisotropy of the complex of CRP with the fluorochrome-labeled DNA containing a CRP-binding site and no known RNA polymerase-binding site on addition of RNAP holoenzyme to the complex. However, their fluorescence result did not show whether CRP that was not bound to DNA could interact with RNAP. Thus, it can be said that our result is the first direct observation of the interaction between the two proteins in solution in the absence of DNA. In addition, our result confirmed that the sites of interaction of the two proteins are located in the region around the residues Met 157 and Met 163 of CRP, and in the α subunit of RNAP. Our result also raises a new question about the mechanism of transcription initiation *in vivo*: does the RNA polymerase recognize the CRP-DNA complex, in which the DNA is bent to about 90° (*8, 9*), or does the CRP-RNAP complex recognize the target DNA? Both mechanisms could operate in cells, as could the synchronized formation of a complex of the three molecules. It is worth clarifying which mechanism or mechanisms actually operate, since it would provide a key to the question of whether the CRP-induced bent geometry of DNA is related to the recognition by RNA polymerase.

Despite the high molecular mass of apo-CRP dimer (47 kDa), which has been considered rather large to be assigned by NMR, we previously obtained the nearly complete NMR backbone assignments of the protein by a triple ($^{13}$C, $^{15}$N, and $^{2}$H) labeling and a series of multidimensional NMR spectroscopy (*21*). However, it is very difficult to study the cAMP-bound CRP by multi-dimensional NMR spectroscopy, due to the low solubility, the low stability at high temperature (313 K), and the rapid relaxation time of the cAMP-bound CRP (*20*). Moreover, when CRP is complexed with RNAPα, which is also a dimer with molecular mass of about 70 kDa, the total molecular mass is about 120 kDa, which is beyond the limit of general NMR study. We simplified the NMR spectrum of the complex to its maximum by selective labeling and one-dimensional detection with enough data points to ensure spectral resolution. Thus we could investigate the protein–protein interaction in the high molecular weight complex of CRP with RNAPα. The results confirmed that this method can be effectively used not only to detect the protein–protein interactions between large proteins but also to define the interacting sites.

We are grateful to Prof. Hiroji Aiba (Nagoya University, Nagoya) for his generous gift of the CRP gene. We also thank Prof. Akira Ishihama (National Institute of Genetics, Mishima) for his generous gift of RNA polymerase α subunit gene.

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