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Revisiting the mechanism of activation of cyclic AMP receptor protein (CRP) by cAMP in *Escherichia coli*: Lessons from a subunit-crosslinked form of CRP

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ABSTRACT

Cyclic AMP receptor protein (CRP), the global transcription regulator in prokaryotes, is active only as a cAMP–CRP complex. Binding of cAMP changes the conformation of CRP, transforming it from a transcriptionally 'inactive' to an 'active' molecule. These conformers are also characterized by distinct biochemical properties including the ability to form an S–S crosslink between the C178 residues of its two monomeric subunits. We studied a CRP variant (CRP^{cl}), in which the subunits are crosslinked. We demonstrate that CRP^{cl} can activate transcription even in the absence of cAMP. Implications of these results for the crystallographically-determined structure of cAMP–CRP are discussed.

1. Introduction

The cyclic AMP receptor protein (CRP, also known as CAP) of *Escherichia coli* is a transcription factor that regulates the expression of more than 100 genes in the bacterium [1–5]. It is a homodimer of 209-residue subunits, comprising the C-terminal DNA binding domain (DBD, residues 139–209) and the N-terminal dimerisation domain that binds cAMP (CBD, residues 1–134), with a short hinge region (residues 135–138) connecting the domains. CRP is functionally active only when complexed with cAMP, the binding of which brings about an allosteric change in the protein, causing it to bind to specific sites on DNA and activate (or repress) a number of genes. Changes in several biochemical and physicochemical properties distinguish cAMP bound CRP from the unliganded protein. Binding of cAMP (a) makes CRP sensitive to proteases, (b) reduces the accessibility of its sole accessible cysteine residue (C178) and (c) enables the crosslinking of the two subunits of the protein by an S–S bond at the C178 residues, apart from other changes in conformation probed by ANS fluorescence, small angle X-ray scattering or Raman spectroscopy [1].

The first three dimensional structures of *E. coli* cAMP–CRP appeared in 1981 [6]. Since then, several structures have appeared for the ligand-bound protein in various forms: alone, as a complex with DNA or with DNA and a fragment of RNA polymerase [7–11]. However, the molecular details of the conformational changes that occur in CRP upon cAMP binding became clear only when the structure of apo-CRP (CRP without cAMP) was obtained by X-ray crystallography [12] and NMR [13] in 2009. In both the structures it was observed that binding of cAMP to CBD induces an allosteric conformational change in DBD mainly through structural changes in the C-helix of CBD. However, there were certain differences in the structural change between apo-CRP and cAMP–CRP, as probed by X-ray crystallography or NMR. In the NMR structure that represents the conformation of apo-CRP in the solution state, the C-helices in apo-CRP are 11 residues shorter than in the ligand-bound structure, while in the X-ray structure, the C-helices are shorter by 6 residues in apo-CRP. In addition, the F-helices (the recognition helices of the HTH motif of CRP) are buried in the apo-CRP crystal structure, while the D-helices are shorter by 4 residues. This structure also suggests that upon cAMP binding, the DBD domain reorients itself as one rigid body and rotates over the hinge for proper repositioning of the F helices, making them fit in the DNA major grooves at the cognate binding site. However, these changes in the DBD were not clearly observed in the NMR structure [13]. In fact, the differences in the positioning and conformations of the different structural elements within the DBD in apo-CRP and cAMP–CRP suggests a conformational flexibility of the DBD. This is further reflected in the distance between two Cys-178 residues that lie at the loop connecting the D and E helices in the DBD.

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The distance of Cys-178 residues of apo-CRP in the NMR structure is almost 8 Å shorter compared to that in the crystallographic structure. These results are in general agreement with earlier speculations on the cAMP-induced conformational changes in CRP, obtained from biochemical experiments using mutant proteins [14–16], as well as from molecular dynamics studies [17,18].

It has also been proposed that the level of cAMP rather than its mere presence or absence plays an important role in different biophysical and biochemical properties of CRP [19,20]. Two different conformers of CRP exist at lower and at higher concentrations of cAMP, with only the low-cAMP conformer being able to activate transcription [20]. Among other cAMP-dependent structural properties, CRP undergoes DTNB-induced intersubunit crosslinking of C178 residues, only at low cAMP [19,20]. It is possible that upon such crosslinking, the CRP conformation gets locked in the 'active state', formed at low cAMP. If this is true, the crosslinked form of CRP would represent a conformation in which the hinge movement is restricted and the molecule is frozen in the active state. Therefore, this conformer of CRP should be able to exhibit properties of the molecule that are characteristic of the cAMP–CRP complex, even in the absence of cAMP. In this paper, we have examined this interesting possibility.

2. Materials and methods

2.1. Materials

The expression vector pET28a (Novagen), *E. coli* strains XL1Blue and BL21 (DE3) were used for cloning and purification of CRP. The plasmid pSA509 containing *E. coli galactose* promoter was obtained from Dr. Sankar Adhya, NCI, NIH, USA. *E. coli* RNA polymerase was purchased from Epicenter, USA, as a 100% sigma-saturated holoenzyme. Chymotrypsin, 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB), cAMP, IPTG, dithiothreitol (DTT) and NTPs (Sigma), [α-^{32}P] UTP (BRIT, India), and Ni–NTA agarose (Qiagen), were purchased from the indicated sources. All other enzymes were from Promega, USA.

2.2. Cloning, expression, and purification of CRP from E. coli

Chromosomal DNA was isolated from *E. coli* (XL1 Blue) by the bacterial DNA isolation protocol [21]. From the purified chromosomal DNA, the *crp* gene was PCR-amplified using the primers PFCRP (5′-CGAAACATATGGTGCTTGGCAAACCGCA-3′) and PRCRP (5′-GCGTTGGATCCAATTTAACGAGTGCCGT-3′). The PCR product was digested with *Nde*I and *Bam*HI and ligated into the corresponding sites of the expression vector pET28a. The resulting plasmid pABS01 was used to transform the BL21 (DE3) strain. Following induction at 37 °C for 3 h with 0.3 mM IPTG, the expressed protein was purified through a Ni–NTA agarose column (pre-equilibrated with buffer A: 20 mM sodium phosphate (pH 7), 500 mM NaCl, 10% glycerol and 0.5 mM DTT). Elution was done by the addition of buffer A plus 250 mM imidazole. The protein obtained was 98% pure as estimated from a 13.5% SDS–PAGE followed by Coomassie blue staining.

2.3. Intersubunit crosslinking of Cys-178 residues by DTNB and chymotrypsin digestion

10 μM CRP was treated with 100 μM DTNB in presence of 500 μM of cAMP for 20 min at 22 °C, to effect S–S crosslinking between the Cys-178 residues of the two subunits. Cyclic AMP and DTNB were removed from the mixture by extensive dialysis against 50 mM sodium phosphate buffer (pH 7) with three to four changes. The absorbance spectra of the crosslinked protein samples were recorded before and after dialysis to ensure that cAMP was completely removed. Proteolysis of CRP or crosslinked CRP (CRP^{cl}) was carried out in 40 mM Tris–Cl (pH 8.0) with 0.1 M KCl, 10 mM $MgCl_2$ by incubating 20 μl of protein (6–8 μg) with chymotrypsin (0.4 μg) at 28 °C for 20 min, in the absence or presence of varying concentrations of cAMP as specified (20 μM–10 mM). In each case, the reaction was started with the addition of 2 μl of 0.2 mg/ml chymotrypsin solution and incubated at 28 °C for 20 min. Reactions were stopped by adding the gel loading dye followed by boiling for 5 min. The digested products were run on a 13.5% SDS–PAGE followed by Coomassie blue staining.

2.4. Preparation of DNA fragments containing different promoter/operator regions of E.coli

The −145 to +155 region of the *E. coli lactose* promoter was PCR-amplified from *E. coli* (XL1 Blue) using the primers LacPF (5′-CGCCCATATGGTTGGCCGATTCATTAATGC-3′) and LacPR (5′-TTAGGGATCCATTACGCCAGCTGGCGAAAG-3′), resulting in a 300-bp product which was cloned at the *Nde*I and *Bam*HI sites of pET28a vector. The cloned plasmid (pABS02) was then transformed into an *E. coli* XL1 Blue strain. The *lac* promoter operator region used in this work was PCR-amplified with suitable primers (LacPF and LacPR). The other promoter region i.e. −197 to +91 of *galactose* promoter of *E. coli* region used in this work was also PCR-amplified from plasmid pSA509 with appropriate primers PP287 (5′-TGCATGAATTCTTGGCCAACG-3′) and PP288 (5′-ATTTGCTGCAGTAATTGCACA-3′).

2.5. In vitro transcription

Reactions were performed in a 20 μl volume, in transcription buffer (40 mM Tris–Cl, pH 8.0, 0.1 M K-glutamate, 1 mM DTT, 20 mM $MgCl_2$), using 5 nM of DNA template and 50 nM of RNA polymerase. After incubation with 100 nM of CRP at 37 °C for 20 min, transcription was initiated by the addition of the nucleotide mix (0.1 mM each of ATP, GTP, CTP; 0.01 mM UTP, 5 μCi [^{32}P] UTP at 3000 Ci/mmol and 1 μg heparin) and terminated after 20 min by adding 10 μl of formamide loading buffer (90% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). However in case of CRP^{cl}, 200 nM of protein (including ~50% of the protein that remained in the uncrosslinked form, as detected from SDS–PAGE) was used and the reaction mixture did not contain any DTT. The protein was freed from cAMP as described earlier. As a negative control, 200 nM of CRP^{cl} was treated with 2 mM DTT and added to the same reaction mixtures. Transcription experiments were also done with free CRP in the absence and presence of 20 μM cAMP as additional control. Transcribed RNA was resolved by electrophoresis on a 10% polyacrylamide-7 M urea gel. The amounts of transcripts were quantitated with a typhoon scanner system. To test the effect of varying concentrations of cAMP on the transcriptional activity of CRP and CRP^{cl}, specified amounts of cAMP (16 μM–10 mM) were included in the reaction mixtures.

2.6. In vitro transcription of CRP and CRP^{cl} for determination of half-maximal concentrations for transcription activation

The experiments were done as for the in vitro *transcription* experiments described above, where all the components of the reaction mixture were mixed and incubated simultaneously. Increasing concentrations of proteins were used. In the case of CRP, a specified amount of protein (0, 20, 40, 60, 80 or 160 nM) was incubated with 100 μM of cAMP for 5 min on ice. However for CRP^{cl}, 0, 40, 80, 120, 160 and 320 nM of total protein (including ~50% of the protein that remained in the uncrosslinked form, as detected from SDS–PAGE) were used, and the reaction mixture did not contain any DTT or cAMP. Transcribed RNA was resolved