Functional roles of the two cyclic AMP-dependent forms of cyclic AMP receptor protein from *Escherichia coli*

Jayanta Mukhopadhyay, Runa Sur, Pradeep Parrack*

Department of Biochemistry, Bose Institute, P-1/12, C.I.T. Scheme VIIM, Calcutta 700 054, India

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Abstract The cyclic AMP receptor protein activates transcription in *Escherichia coli*, only when complexed with cyclic AMP. The cyclic AMP receptor protein-cyclic AMP complex formed at low concentrations of cyclic AMP has a different conformation from either cyclic AMP receptor protein alone or its complex with cyclic AMP formed at high cyclic AMP concentrations. Various biophysical data suggest that the latter complex resembles free cyclic AMP receptor protein. We have examined the conformational and biological properties of cyclic AMP receptor protein as a function of cyclic AMP concentrations, using the *gal* operon of *E. coli*. A biphasic behavior is observed. It is shown that only the complex formed at lower concentrations of cyclic AMP is the transcriptionally active form. This difference between the complexes at different levels of cyclic AMP arises from a decreased ability of the cyclic AMP receptor protein-cyclic AMP complex at high cyclic AMP concentrations to bind to DNA at specific sites.

Key words: Cyclic AMP receptor protein; Cyclic AMP; Transcription activation; Gene regulation; *Escherichia coli*

1. Introduction

The cyclic AMP (cAMP) receptor protein (CRP) plays a key role in the regulation of expression of many genes [1–3] in bacteria. CRP is a homodimer of two 209 residue subunits [3]. Activation or repression of transcription by CRP requires that it binds cAMP and undergoes an allosteric conformational change which is essential for its binding to DNA at specific sites [4–7]. However, cAMP binds to each CRP monomer at two different sites with different affinities [8]. Higher affinity sites where the nucleotide binds in the anti conformation are located in the N-terminal domains, whereas lower affinity binding sites (where the bound cAMP has a syn conformation) are located at the interface formed by the two C-terminal domains of the CRP subunits. Therefore, it has been suggested that CRP exists in three conformational states: free CRP, CRP with two cAMP molecules bound to N-terminal domains, CRP-$(cAMP)_2$, and CRP with four cAMP molecules bound to both N-terminal and C-terminal domains, CRP-$(cAMP)_4$. Earlier, it was proposed [7] that the three conformational states of CRP consisted of the following species: free CRP, CRP-$(cAMP)_1$ and CRP-$(cAMP)_2$, which have been re-interpreted by Passner and Steitz [8] in the light of the recent crystal structure data. For our purpose, it is sufficient to note that the behavior of CRP at different concentrations of cAMP is essentially biphasic, so that two different conformers exist at lower and higher concentrations of cAMP. We shall refer to these conformers as the 'anti complex' and the 'syn complex'.

It is known that the level of cAMP regulates the expression of many genes in *Escherichia coli*, this regulation being mediated by CRP. It is, however, not yet known whether or not both forms of cAMP-CRP complexes are competent in transcription regulation. Predominantly from biophysical experiments, it has been suggested that the syn complex resembles unliganded CRP, while the conformation of the anti complex is different [7]. This suggestion has led to the hypothesis that perhaps the anti complex is the active form that plays a role in regulation of transcription, while the syn complex is inactive. We have examined the effect of increasing concentrations of cAMP on (a) the conformation of CRP, (b) regulation of transcription in the *E. coli* galactose operon and (c) DNA binding properties of CRP, to evaluate the above hypothesis.

2. Materials and methods

2.1. Materials

CRP was purified from the strain pp47 harboring plasmid pHA7 according to the method used by Ryu et al. [9] with the following modification. A Superdex-75 column (Pharmacia) was used in the final step, instead of the mono-S column. The protein obtained was at least 98% pure, as judged by Coomassie blue staining following SDS-PAGE. RNA polymerase and nucleotide triphosphates (Pharmacia), cAMP and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma) and [^{32}P-α]UTP (BRIT, India) were purchased from the indicated sources. Concentrations were determined spectrophotometrically, using $\varepsilon = 14\,650$ M^{-1} cm^{-1} at 258 nm for cAMP [10] and 20 400 M^{-1} cm^{-1} at 278 nm for CRP [11].

2.2. Chymotrypsin digestion

Proteolysis of CRP was carried out in 40 mM Tris-HCl (pH 8.0) with 0.1 M KCl, 0.1 mM DTT, 10 mM $MgCl_2$, 0.2 mM EDTA by incubating CRP (8.5 μg) with chymotrypsin (0.4 μg) at 28°C for 20 min at various cAMP concentrations. Digested products were run on a 13.5% SDS-PAGE followed by Coomassie blue staining and then analyzed by densitometric scanning with a GS-700 densitometer (Bio-Rad).

2.3. Determination of the accessibility of SH groups

The accessibility of cysteine residues in CRP at different cAMP concentrations was determined by treating 900 μl protein solution (in 100 mM K-phosphate buffer, pH 8.0) with 100 μl DTNB, at room temperature (25°C). Final concentrations of protein and DTNB were 1 μM and 30 μM, respectively. The number of free cysteine residues was determined from the changes in absorbance at 412 nm as 2-nitro-5-thiobenzoate (TNB) molecules released in the reaction, using ε of TNB = 13 600 M^{-1} cm^{-1} at 412 nm [12].

2.4. Intersubunit crosslinking of Cys-178 residues by DTNB

10 μM CRP was treated with 100 μM DTNB for 20 min at 22°C, to

*Corresponding author. Fax: (91) (33) 334 3886.
E-mail: pradeep@boseinst.ernet.in

Abbreviations: CRP, cyclic AMP receptor protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate

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effect S-S crosslinking between the Cys-178 residues of the two subunits. The mixture was then run on a 13.5% SDS-PAGE to separate the crosslinked species from the uncrosslinked one.

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 2 nM of a 346 bp DNA fragment (*Eco*RI-*Bam*HI fragment of pSA 509 [13]) containing *gal* promoters as template and 100 nM CRP, cAMP (0–400 μM) and 50 nM RNA polymerase. Experiments were done in two ways. In the first procedure, 100 nM CRP and a specific amount of cAMP were incubated on ice for 5 min to form the cAMP-CRP complex and then added to the reaction mixture. In the second method, cAMP was added to the reaction mixture containing 100 nM CRP and the rest of the components. After incubation 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). Transcribed RNA was resolved by electrophoresis on a 10% polyacrylamide-7 M urea gel. The amounts of transcripts were quantitated with a Bio-Rad Phosphorimager.

2.6. DNA binding studies

DNA fragments containing gal promoters as well as the CRP binding site were amplified by PCR from the plasmid pSA509 [13], obtaining 298 bp double-stranded linear DNA. 1.0 μM CRP (in 40 mM Tris-Cl (pH 8.0) with 0.2 M KCl, 0.1 mM DTT, 20 mM $MgCl_2$) was titrated with the above DNA in the presence of cAMP at different concentrations. The DNA binding of CRP was monitored by the change in the fluorescence intensity at 345 nm, measured in a Hitachi F-3000 spectrofluorometer. Excitation was at 295 nm. Bandwidths of 5 nm were used on both sides.

3. Results

3.1. Proteolysis by chymotrypsin

It has been shown that digestion by various proteases including chymotrypsin is an indicator of the conformational state of CRP [6,7]. Free CRP is resistant to proteolysis and is digested only in the presence of cAMP, producing a 13 kDa fragment in SDS-PAGE (indicating that the cleavage occurs at F136). We studied the effect of increasing amounts of cAMP on the cleavage of CRP. Our results (Fig. 1) show that the sensitivity to chymotrypsin exhibits a biphasic behavior, with the maximum sensitivity (~80%) occurring near 200 μM cAMP (lane d). A further increase in the concentration of the ligand reduces this sensitivity and at 10 mM cAMP, this becomes 38% (lane h). Thus, the conformational change induced by the binding of cAMP is reversed at higher levels of the ligand.

3.2. Accessibility of C-178 residues

CRP has three Cys residues per monomer, of which only C-178, located near the DNA binding region of the protein, is accessible to modification by DTNB [12]. Thus, two Cys residues are accessible to react with DTNB, per dimer of CRP. When the number of accessible Cys residues was estimated as a function of cAMP concentrations, this number also showed a biphasic dependence on cAMP, decreasing to a minimum value of 1.5 at 0.1 mM cAMP and increasing again when the cAMP concentration was increased (Fig. 2). It has been reported that DTNB induces disulfide bond formation between the C-178 residues of the two subunits of CRP [12]. Thus, the observed decrease in the sensitivity of Cys -SH groups at intermediate levels of cAMP may result from the crosslinking of the -SH groups. Such a crosslinking may not be possible

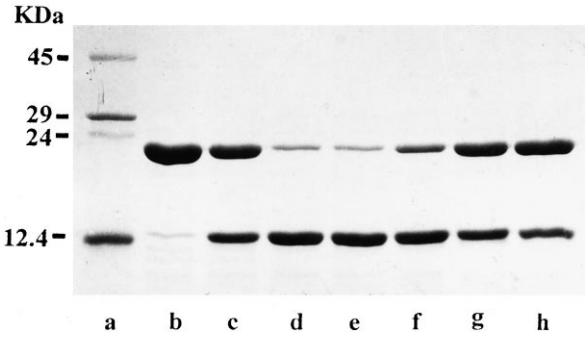

Fig. 1. Proteolysis of CRP by chymotrypsin, in the presence of varying concentrations of cAMP. Lane a, molecular weight marker. Lanes b–h contained cAMP as shown (figures in brackets represent the percentage of CRP digested, as measured by densitometry): (b) 0 (5%), (c) 20 μM (41%), (d) 200 μM (79%), (e) 1 mM (84%), (f) 2 mM (67%), (g) 5 mM (44%) and (h) 10 mM (38%).

for the syn complex, which has an altered conformation. We verified this possibility in the intersubunit crosslinking experiment carried out at different cAMP concentrations described below.

Crosslinking between the -SH groups of C-178 residues in CRP subunits by DTNB results in a 45 kDa band in SDS-PAGE, as shown in Fig. 3 (lanes a–e). CRP was subjected to crosslinking by DTNB at cAMP concentrations ranging from 0 to 10 mM. It is observed that crosslinking occurs at low cAMP concentrations but the extent of crosslinking goes down at high cAMP concentrations. The differential behavior of the C-178 groups at low and high cAMP suggests that the conformations of the two cAMP-CRP complexes are different. It was further examined whether the crosslinked protein (as in lane c) is sensitive to chymotrypsin cleavage, after removal of cAMP (Fig. 3, lane g). As a control, a CRP-cAMP complex was formed at 1 mM cAMP and was also subjected to a similar treatment by chymotrypsin before (lane f) and after (lane h) removing cAMP. Indeed, cAMP could be removed from these complexes, as judged from the absence of

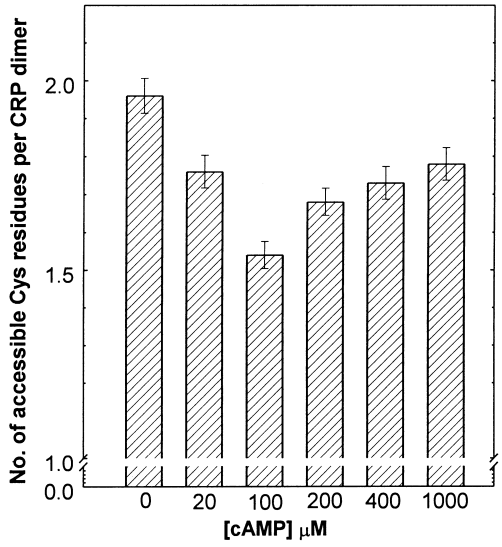

Fig. 2. The number of accessible cysteine residues per CRP dimer, at various cAMP concentrations. S.D.s are indicated.

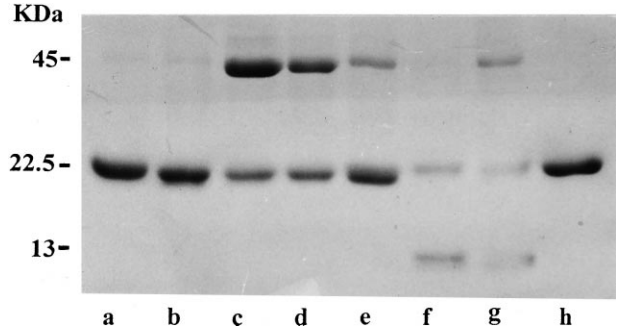

Fig. 3. Intersubunit crosslinking of CRP monomers by DTNB, at various cAMP concentrations. Lane a: no cAMP, no DTNB; lanes b–e: treated with DTNB, at [cAMP] = 0, 1, 5 and 10 mM. Sensitivity to proteolysis by chymotrypsin was also checked for the crosslinked protein, after removal of cAMP (lane g, which is similar to CRP as in lane c, having 1 mM cAMP). For comparison, proteolysis of CRP at 1 mM cAMP before (lane f) and after (lane h) removal of cAMP by passing through a Sephadex G-25 spun column is also shown.

digested product in lane h. The crosslinked protein shows sensitivity to chymotrypsin (as evidenced by the 13 kDa band) even in the absence of cAMP (lane g). Thus, the crosslinking of the subunits probably fixes the conformation of the protein in the form that is similar to the anti complex.

3.3. *In vitro transcription of gal promoters*

All the results described above clearly point to different conformations of the cAMP-CRP complexes at low and high cAMP. Are these conformations involved in the regulation of transcription? A 346 bp linear DNA containing promoter region of the galactose operon of *E. coli* was used as a template to carry out in vitro transcription experiments at various cAMP concentrations, to answer the above question. This operon has two overlapping promoters, P1 and P2, separated by 5 bp, which are differentially regulated by cAMP-CRP. In the absence of cAMP, transcripts are produced both from P1 and P2 while in its presence, P1 is activated and P2 is repressed [14,15]. Thus, the galactose operon is a suitable system to test the effects of the two types of cAMP-CRP complexes described above. Run-off transcripts of 149 and 154 bp lengths are expected to be produced from P1 and P2, respectively, from the template used. CRP was pre-incubated with increasing concentrations of cAMP, before addition of the other components and initiating transcription. As seen from Fig. 4A, P1 is activated and P2 repressed, up to [cAMP] = 100 μM (lane e). Further addition of cAMP tends to reverse this effect and at 400 μM cAMP (lane g), about 30% of the transcripts are from P2. This biphasic nature of the activation/repression by CRP as a function of cAMP concentrations correlates with a similar biphasic change in the conformations of the complexes. The same experiment was also performed by changing the order of addition. CRP was first incubated with DNA and RNA polymerase, followed by the addition of cAMP and NTP mix to initiate transcription (Fig. 4B). In this case, there is a monotonic increase in the activation of P1 coupled with a repression of P2, all the way up to 400 μM cAMP. A result similar to that shown in Fig. 4B was also reported by Choy and Adhya [13], for cAMP up to 200 μM. The difference between the results of Fig. 4A and B is probably due to the fact that when anti complex is bound to DNA at specific sites, further binding of cAMP is precluded.

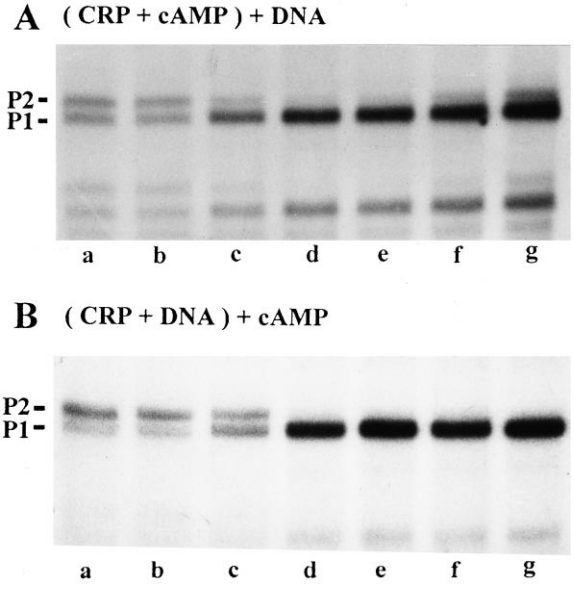

Fig. 4. The effect of cAMP on in vitro transcription from *gal* promoters. Run-off transcripts from P1 and P2 promoters are indicated. cAMP concentrations used were 0, 0.2, 2, 20, 100, 200 and 400 μM (lanes a–g). (A) CRP was pre-incubated with the indicated amount of cAMP, allowing cAMP-CRP interaction to precede interaction with DNA. (B) CRP was added to DNA and RNA polymerase, prior to addition of the specified amount of cAMP, so that CRP-DNA interactions preceded interaction with cAMP. The radioactive counts in each lane were not always equal. The minor transcripts migrating faster than *gal* P1 and P2 originate from premature termination at a partial rho-independent terminator sequence near the 5′ end of the DNA template.

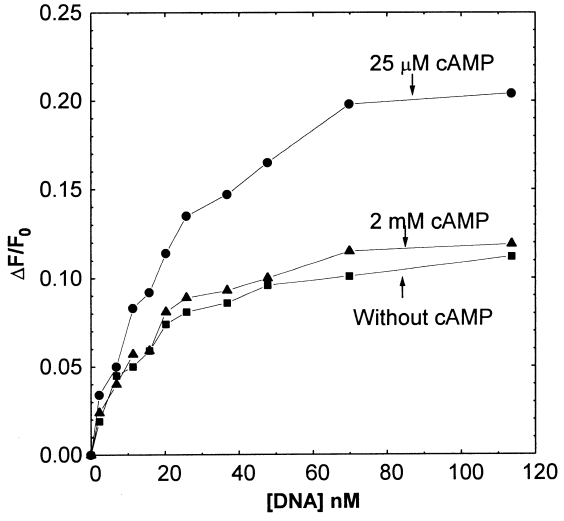

Fig. 5. Binding of CRP to DNA, at different cAMP conditions. Relative quenching of the fluorescence intensity ($\Delta F/F_0$, where $\Delta F = F_0 - F$, F_0 and F indicate the fluorescence in the absence and presence of DNA) is plotted as a function of the added DNA concentration. CRP was kept fixed at 1 μM. The quenching curves arise from binding of the protein to DNA and the initial slopes indicate the strength of binding. Note the increase of this slope when cAMP is changed from 0 to 25 μM and its return to the starting level at 2 mM cAMP.

3.4. Interaction of the different cAMP-CRP conformers with DNA

In the light of the recent three dimensional structure of the cAMP-CRP complex [8], it is known that at high cAMP concentrations, the lower affinity (syn) cAMP binding sites are likely to be occupied, which might interfere with DNA binding at specific sites. This loss of DNA binding may account for the reversal of activation of P1 (and repression of P2) at high cAMP, as in Fig. 4A. We tested this possibility by measuring the DNA binding capability of the two CRP complexes (formed at low and high cAMP) described below.

When DNA is added to CRP, the tryptophan fluorescence of the protein is quenched. In a control experiment, we checked that when DNA was added to bovine serum albumin, which does not bind to DNA, no quenching occurred (data not shown). This quenching was therefore used to assess the DNA binding ability of CRP. The tryptophan fluorescence emission was measured at DNA concentrations ranging from 0 to 120 nM, for each of the following three cases, viz. (i) CRP alone, (ii) low (25 μM) cAMP, and (iii) high (2 mM) cAMP (see Fig. 5). The quenching curves (i) and (iii) almost coincide, whereas that for the low cAMP conformer (ii) is distinct, with an enhanced quenching effect. Since it is known that free CRP does not exhibit strong and specific binding to DNA [16], we interpret these quenching data (at zero and high cAMP) as an indication of non-specific binding of CRP to DNA. On the other hand, the anti complex binds to DNA at specific sites, with a strong affinity. Thus, the reduced activation of transcription by CRP at high cAMP levels is a consequence of the inability of the complex to bind to DNA at specific sites. It may be pointed out that Heyduk and Lee [7] also showed by gel mobility shift experiments that CRP loses its capacity to bind to DNA as the cAMP concentration is increased.

4. Discussion

The allosteric transformation of CRP from a transcriptionally inert complex to an active one in the presence of cAMP appears to be crucially dependent on the cAMP:CRP ratio. While activation/repression is apparent at low cAMP concentrations, at high cAMP concentrations, a second inactive conformer of cAMP-CRP is formed that is unable to specifically bind to DNA with a significant affinity. Extra cAMP molecules bound at the low affinity sites in the C-terminal domain of CRP would interfere with its DNA binding property. Thus, when the syn complex formed at high concentrations of cAMP is allowed to interact with DNA, there is little binding (Fig. 5) and also the activation of *gal* P1 is reduced (Fig. 4A). When the anti complex interacts with DNA, further cAMP binding cannot occur and there is activation of *gal* P1 even at high concentrations of cAMP (Fig. 4B). This observation has two consequences. First, the regulation of transcription by cAMP-CRP may have a much finer control than believed so far, a change in the level of cAMP can cause a significant perturbation to the activation. Thus, the level of cAMP would dictate whether or not the cAMP-CRP complex would be involved in regulation, rather than the mere presence or absence of the ligand. Moreover, the order of cAMP binding, i.e. whether cAMP binds to free CRP or to DNA-bound CRP, should also dictate the regulatory events. Secondly, the reported crystal structure of the ternary cAMP-CRP-DNA complex [8] contains high cAMP concentrations in which the syn complex (i.e. the complex that does not inactivate) predominates. It is thus possible that the interactions between the ligand, the protein and DNA, as predicted from the crystal structure data, only indicate certain possible interactions and do not represent the interactions occurring in the activation complex.

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