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Cyclic AMP-dependent functional forms of cyclic AMP receptor protein from *Vibrio cholerae*

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Abstract

The cyclic AMP receptor protein (CRP) from *Escherichia coli*, involved in the transcriptional regulation of a number of genes and operons, works by binding to specific sites upstream of promoters. CRP also binds cyclic AMP (cAMP), and this binding, which causes conformational changes in CRP, is mandatory for its activity. A cAMP-dependent variation in the conformation as well as biological activity of *E. coli* CRP has been reported, with the cAMP–CRP complex formed at high cAMP concentrations resembling the uncomplexed apoprotein CRP. CRP from *Vibrio cholerae*, which plays an important role in the regulation of virulence gene expression, has a 95% sequence identity with the *E. coli* protein. We have purified and characterized *V. cholerae* CRP and studied its transcription activation properties as a function of increasing cAMP concentrations. A biphasic dependence on cAMP levels was observed, similar to that found for *E.coli* CRP. The implications of these results on regulation of cAMP–CRP dependent promoters in *V. cholerae* has been discussed.

Keywords: Cyclic AMP receptor protein; Cyclic AMP; Transcription activation; *Vibrio cholerae*; Allosteric change; Gene regulation

The cyclic AMP (cAMP)[1] receptor protein (CRP) is a well-known transcription factor that regulates transcription in many promoters in *Escherichia coli* [1–3]. CRP alone does not possess the regulatory activity, nor is it able to bind to DNA with significant affinity or specificity. It is only as a complex with the ligand cAMP that CRP is active. Binding of cAMP induces an allosteric change in CRP making it transcriptionally "competent" [4,5]. From the crystal structures of cAMP–CRP and cAMP–CRP–DNA, it has been found that the cAMP binding site and the DNA binding sites are in different domains of the protein [6–8]. Changes in several biochemical as well as physicochemical properties distinguish CRP from the cAMP–CRP complex [4,5,9–11]. Additionally, it has been observed that the level of cAMP rather than its mere presence or absence decides the transcriptional competence of CRP [12,13]. In *E. coli*, it has been reported that CRP at low cAMP concentration (binds only at the N-terminal domain) has a different conformation than at high cAMP concentration (binds both at N- and C-terminal domains) [12,13]. When cAMP is added to CRP at increasing concentrations, a biphasic change in the conformational and biological properties of CRP is observed. Moreover, the cAMP–CRP complex only at low cAMP concentration is the transcriptionally active form [13]. *crp* genes have also been identified in other systems [14–19], but CRP from non-*E. coli* systems have not been studied much at the protein level. The cAMP–CRP system has been shown to play an important role also in the regulation of virulence gene expression and pathogenesis in *Vibrio cholerae* [20,21], where the cAMP–CRP complex negatively regulates the coordinate expression of cholera toxin (CT) and toxin-coregulated pilus (TCP) responsible

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[1] *Abbreviations used:* cAMP, cyclic 3′-5′-adenosine monophosphate; CRP, cyclic AMP receptor protein; VcCRP, CRP from *Vibrio cholerae*; EcCRP, CRP from *Escherichia coli*; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); TNB, 2-nitro-5-thiobenzoate; CT, cholera toxin; TCP, toxin-coregulated pilus.

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for virulence and pathogenesis [21]. It also regulates other *V. cholerae* genes (as a positive regulator in ompT [22] and hemaglutinin/protease [23]). The similarities between the phenotypes of the *V. cholerae cya* and *crp* mutants and those of *E. coli* [20] suggest that cAMP–CRP functions in an analogous manner in the two bacteria. Previous studies, however, had failed to demonstrate a relationship between cAMP levels and CT production [24]. The *V. cholerae* CRP (VcCRP) protein consists of 209 amino acid residues, the same number as in *E. coli*, and has 95 and 81% identities with respect to the amino acid sequence and nucleotide sequence, respectively, with *E. coli* CRP (EcCRP) [20]. Although it was found to functionally complement EcCRP, there is no report of purification of VcCRP. In this paper, we have examined VcCRP for its response to increasing concentrations of cAMP in vitro. In the present work, the *crp* gene from *V. cholerae* has been cloned as an N-terminal histidine tagged protein in *E. coli*, and expressed and purified by a Ni–NTA column. Biochemical experiments including in vitro transcription and DNA binding studies as a function of cAMP concentration have been carried out with this recombinant protein. A biphasic dependence is observed for VcCRP, similar to that found earlier for the *E. coli* protein.

Materials and methods

Materials

The expression vector pET28A (Pharmacia), *V. cholerae* classical strain O395 (gift from Prof. A.C. Ghose, Department of Microbiology, Bose Institute), *E. coli* BL21 (DE3) strain (Prof. D.J. Chattopadhyay, Department of Biochemistry, Calcutta University), and *E. coli* Δcrp strain pp47 harboring the plasmid pHA7 containing the *E. coli crp* gene as well as the plasmid pSA509 containing *E. coli galactose* promoter (Dr. Sankar Adhya, NCI, NIH, USA) were obtained from the sources indicated.

BSA, chymotrypsin, DTNB, cAMP, IPTG, and NTPs were purchased from Sigma. *E. coli* RNA polymerase was purchased from Epicenter, USA, as a 100% sigma-saturated holoenzyme. All other enzymes were from Roche Molecular Biochemical, Germany. [α-^{32}P]UTP was from BRIT, India, and Ni–NTA–agarose was from Pharmacia.

Cloning, expression, and purification of CRP from V. cholerae

Chromosomal DNA was isolated from *V. cholerae* O395 by the bacterial DNA isolation protocol [25]. From the purified chromosomal DNA, *crp* gene was PCR-amplified using the primers VC1 (5′-GCAGCCGGATCCATTAT GGTTCTAGGTAA-3′) and VC2 (5′-ATTCACCTCGA GTTAGCGAGTGCCGTAAA-3′). The PCR product was digested with *Bam*HI and *Xho*I and ligated into the corresponding sites of the expression vector pET28a and transformed into BL21 (DE3) strain. Following an overnight induction at 16 °C with 0.3 mM IPTG, the expressed protein was purified through a Ni–NTA–agarose column (pre-equilibrated with buffer A: 20 mM potassium phosphate, pH 7.4, 200 mM KCl, and 0.2 mM DTT). Elution was done by the addition of 500 mM imidazole at pH 8. The protein obtained was 98% pure as estimated from a 13.5% SDS–PAGE following coomassie staining. cAMP binding activity was measured by ammonium sulphate precipitation using [^{3}H]cAMP following Anderson et al. [26].

Size exclusion chromatography

Size exclusion chromatography was performed in an AKTA FPLC system using a Superdex 75 HR 10/30 column. The R_f value was calculated from $R_f = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume, V_0 = void volume, V_t = total volume of the column. Five hundred micrograms of protein was injected at a time. Buffer A was used as the eluent.

Circular dichroism spectroscopy

CD spectra were recorded at pH 8, 25 °C, in a JASCO J600 spectropolarimeter, in the far-UV (190–260 nm) region. The spectra were deconvoluted using the CDNN software [27] to estimate the secondary structures of the proteins.

Chymotrypsin digestion

Proteolysis of VcCRP was carried out in 40 mM Tris–HCl (pH 8.0) with 0.2 M KCl, 0.1 mM DTT, 10 mM $MgCl_2$, and 0.2 mM EDTA by incubating 8 μg protein with chymotrypsin (0.8 μg) at 28 °C for 30 min in the presence of various cAMP concentrations. Digested products were run on a 13.5% SDS–PAGE followed by Coomassie blue staining and analyzed by densitometric scanning with a GS700 Densitometer (Bio-Rad).

Determination of the accessibility of –SH groups

The accessibility of Cys residues in CRP at different cAMP concentrations was determined by treating 900 μl protein solution (in 100 mM potassium phosphate buffer, pH 8.0) with 100 μl DTNB at 25 °C. Final concentrations of protein and DTNB were 3 and 300 μM, respectively. The number of free Cys residues was determined from the changes in absorbance at 412 nm as the number of TNB molecules released in the reaction using the molar extinction coefficient 13,600 $M^{-1}\,cm^{-1}$ at 412 nm for TNB [28].

Intersubunit crosslinking of Cys residues by DTNB

Ten micromolar protein was treated with 100 μM DTNB for 20 min at 22 °C to induce S–S crosslinking between the Cys 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.