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Stability and Structural Change of cAMP Receptor Protein at Low and High cAMP Concentrations

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Abstract Proteolytic digestion and CD measurement of wild-type and mutant cyclic AMP receptor proteins (CRPs) were performed either in the presence or absence of cyclic nucleotide. Results indicated that transition of a structural change to the hinge region by the binding of cAMP to the *anti* site was required for the binding of cAMP to the *syn* site near the hinge region and, although the occupancy of cAMP in the *anti* site increased the protein stability, CRP adopted more a stable conformation by the binding of cAMP to the *syn* site.

Key words: cAMP receptor protein, protein stability, melting temperature

Cyclic AMP receptor protein (CRP) is a well-known transcription factor and involved in the regulation of more than 150 gene expressions in *E. coli* [4, 16]. CRP exists as a homodimer composed of 209 amino acid residues per subunit. The CRP:DNA structure [14] and equilibrium dialysis [21] suggest that the first cAMP molecule binds to the cyclic AMP binding pocket in the N-terminal domain (also called the *anti* binding site) at micromolar cAMP concentration and, at millimolar cAMP, the second cAMP binds to the regions between the hinge region and the helix-turn-helix motif (also called as the *syn* binding site) in each subunit. Binding of cAMP to CRP in the *anti* binding site induces conformational changes in the N-terminal domain, and its structural changes are transmitted to the helix-turn-helix motif in the C-terminal domains for the recognition of specific base sequences and DNA binding [16].

Many investigations have been carried out to determine the interactions between cAMP and amino acids in the *anti* binding site [1, 7, 22], to measure cAMP-induced conformational change of CRP [6, 7, 11], and to elucidate the allosteric mechanism of transcription regulation in *E. coli* [3, 5, 20]. Lee *et al.* [10] reported that substitution of glycine for threonine 127 (T127G) in the *anti* binding site produced a form of CRP that did not activate *lac*P in the presence of either cAMP or cGMP, although T127G CRP showed protease sensitivity in the presence of cAMP, similar to that of the wild-type CRP:cAMP complex. Cysteine substitution for threonine 127 (T127C) produced a mutant CRP that activated *lac*P in the presence of either cAMP or cGMP and formed a disulfide bond across the subunit interface. It appeared that threonine 127 plays a role in transmitting the allosteric signal from the *anti* binding site by providing the subunit-subunit realignment [10].

In this study, wild-type and mutant CRPs (T127G and T127C) were investigated to examine the effect of amino acid 127 in CRP on the protein structure through the subunit-subunit realignment for the binding of cAMP to the *anti* site at micromolar cAMP concentration and also to measure the additional effect on CRP structure for cAMP binding to the *syn* binding site at millimolar cAMP concentration.

CRP was purified from the strain CA8445/pRK248(cI^{ts})/pRE2crp grown at 30°C on LB medium that contained 50 μg/ml ampicillin and 10 μg/ml tetracycline. The culture was shifted to 42°C from 30°C at a culture density of A_{600}= 0.6 and grown for 4 h [8, 13, 23]. The purification protocol included chromatography on phosphocellulose, hydroxyapatite, and CM-Sephadex. CRP concentrations were determined using the extinction coefficient of 3.5×10^4 $M^{-1}cm^{-1}$ at A_{280} [10, 19, 24].

Protease digestion was carried out in 30 μl of final volume at 37°C in transcription buffer [40 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 100 mM KCl, 5% glycerol]. The ratio of CRP to chymotrypsin was 200:1 by weight. Chymotrypsin digestion of CRP produced a 16 kDa

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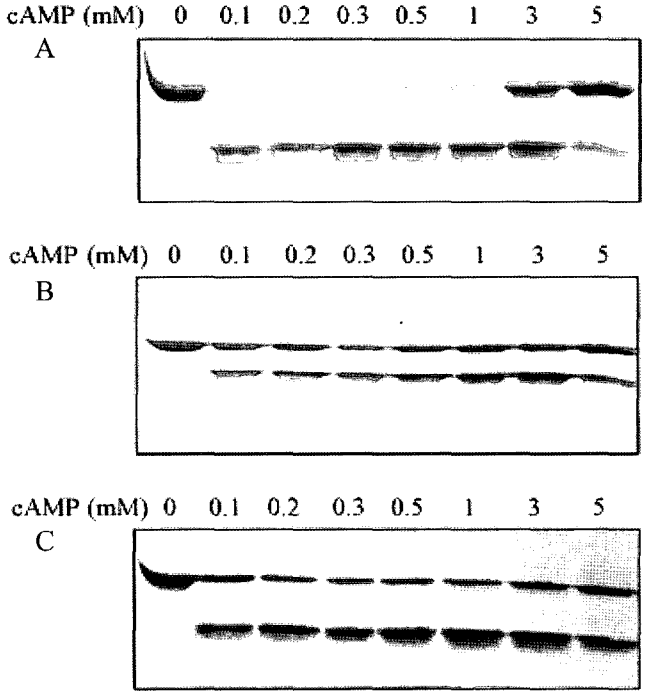

Fig. 1. Proteolytic digestion of wild-type (A), T127G (B), and T127C (C) CRPs.
Reactions were carried out at different cAMP concentrations shown in the figure.

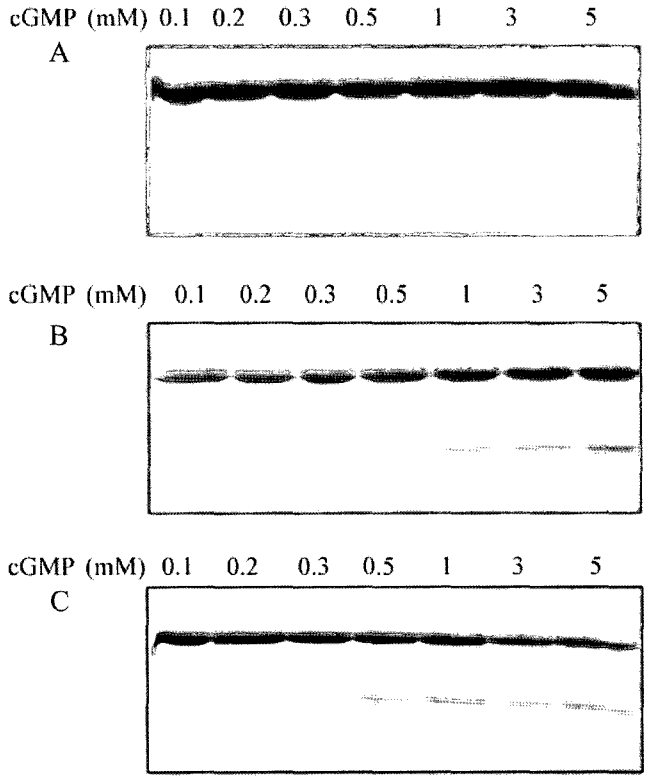

Fig. 2. Proteolytic digestion of wild-type (A), T127G (B), and T127C (C) CRPs with various concentrations of cGMP.

fragment that was hydrolyzed at amino acid 135 of CRP. As shown in Figs. 1 and 2, digested fragments of wild-type, T127G, and T127C CRPs were gradually increased as the cAMP concentration increased. Although the three forms of CRP were all sensitive to protease in the presence of cAMP, the conformation of T127G CRP was different from that of either T127C or wild-type CRP. It was demonstrated that only T127G CRP did not activate transcription of *lac*P in the presence of cAMP [10]. In Fig. 3, the digested fraction of CRP was plotted against either cAMP or cGMP concentration. Each of the band intensities in Figs. 1 and 2 were measured using a scanning densitometer. Digestion of wild-type CRP was decreased abruptly below 1 millimolar concentration of cAMP (Fig. 3A). This is consistent with the results on the proteolytic digestion of CRP at millimolar cAMP concentration [12]. If cAMP binds to the *syn* binding site near the hinge region, protease cannot attack

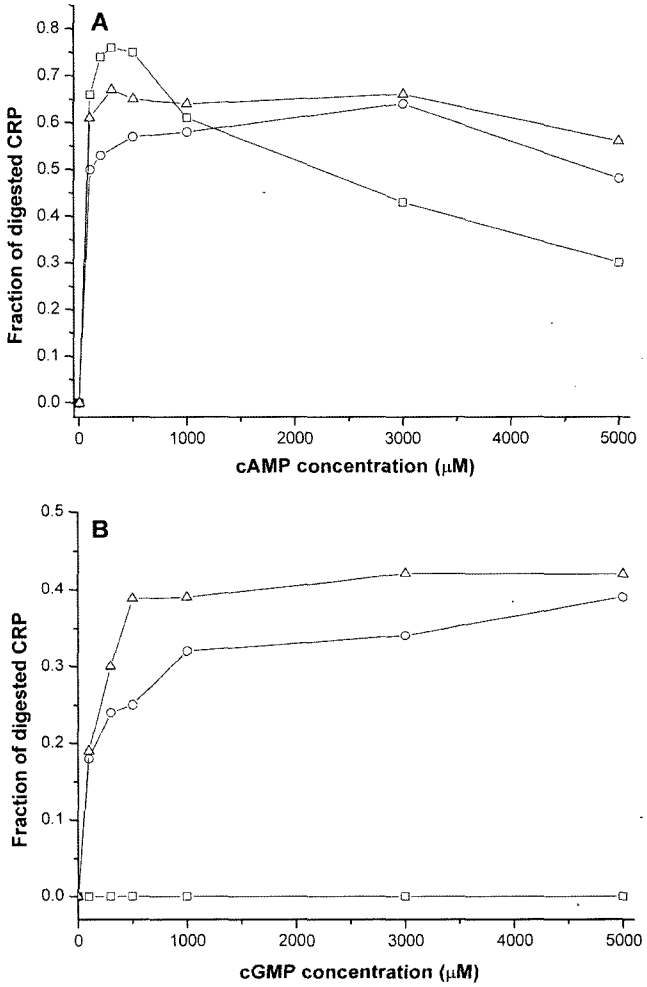

Fig. 3. Plots of the digested fraction of CRP against cAMP (A) or cGMP (B) concentrations.
Reactions were conducted with either wild-type (□), T127G (○), or T127C (△) CRPs.

the hinge region owing to inaccessibility of protease. With T127G and T127C CRPs, the digested CRP was decreased above 3 mM cAMP (Fig. 3B), indicating that cAMP binding to the *anti* binding site (i.e. cAMP-induced structural changes) is required for the binding of cAMP to the *syn* binding site. Figure 2 shows the results of chymotrypsin digestion of three CRPs in the presence of cGMP, showing that T127G and T127C CRPs were sensitive to the protease in the presence of cGMP, similar to the wild-type CRP:cAMP complex. Although T127G CRP was as sensitive to chymotrypsin as T127C CRP in the presence of cGMP, T127G CRP does not have the conformation that activated transcription of *lac*P in the presence of cGMP [10]. Thus, substitution of glycine for threonine 127 produced a mutant CRP whose structure changed upon binding of cAMP or cGMP, but failed to induce the structural changes required for the transcription activation by CRP.

Circular dichroism (CD) was measured with a Jasco J715 CD/ORD spectropolarimeter (Korea Basic Science Institute, Daejon, Korea). All measurements were carried out in thermostated curvettes with a 0.1 cm path length at 230 nm. Data obtained from CD measurement were analyzed by the nonlinear regression method with Origin 6.0 (Microcal Software, Inc.). Figures 4–6 show the changes in CD signal at 230 nm as a function of temperature. By using the equation described in Fig. 4 [17], melting temperatures (T_m) were determined to be 70.5, 69.5, and 73.0°C for wild-type, T127G, and T127C CRPs, respectively, in the absence of cAMP. T127C CRP showed a higher T_m value than that of wild-type CRP, because of the strengthened intersubunit association by a disulfide bond formation across the subunit interface [18]. This would be consistent with the observation that the binding of cAMP to CRP reduces the intersubunit dissociation of CRP at low concentration [2]. In the presence of 100 μM cAMP, the T_m of wild-type CRP was largely increased by 8.5°C, compared with those of T127G and T127C CRPs that were increased by 2.3°C and 2.5°C, respectively. T127C and T127G CRPs showed small increases in T_m, compared with that of wild-type CRP. Because those CRPs contain conformations close to that of wild-type CRP:cAMP, cAMP binding to them resulted in additional protein conformation change into an efficient protease-sensitive structure near the hinge region, as shown in Fig. 1. However, T127G CRP failed to activate the transcription of *lac*P in the presence of cAMP, thus showing that those CRPs contain different protein conformations by binding of cAMP [10]. These results indicate that threonine 127 in CRP affects the structure of CRP in and around the hinge region and the structural stability of CRP by the binding of cAMP to the *anti* site.

As shown in Figs. 4C, 5C, and 6C, we could not accurately determine T_m at a high cAMP concentration because of the absence of denatured baseline intercept and slope (y_N and m_N in the equation mentioned in Fig. 4).

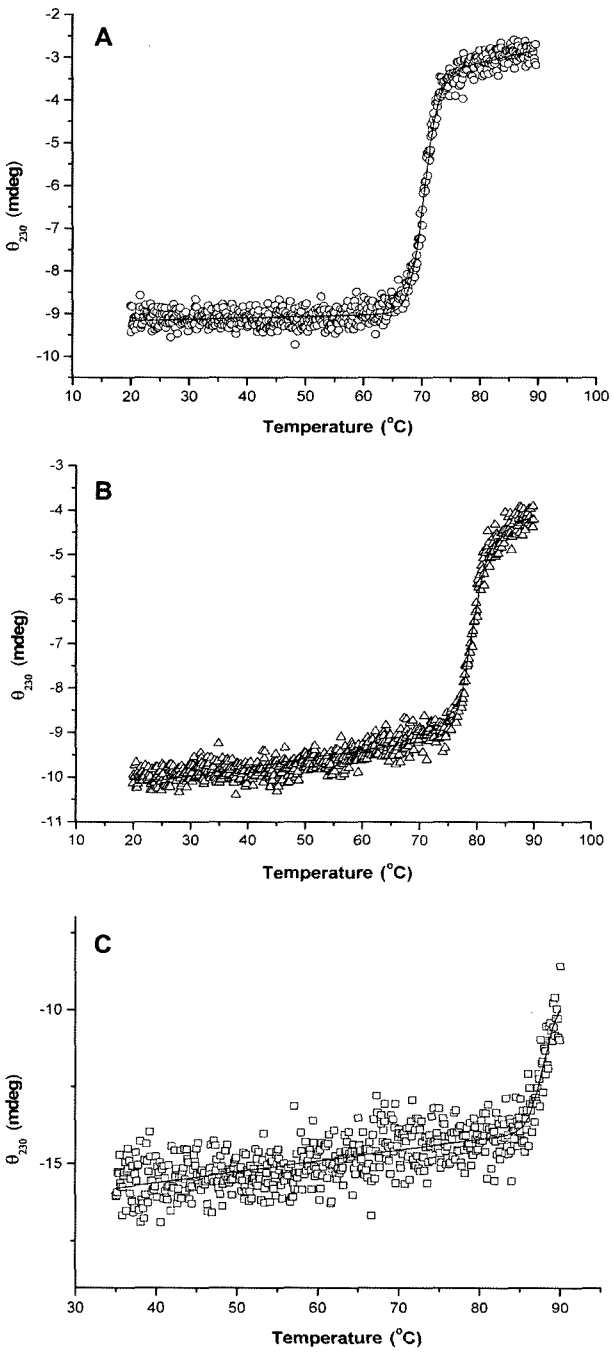

Fig. 4. Change in CD signal at 230 nm with no cAMP (A), 100 μM cAMP (B), and 5 mM cAMP (C). CRP concentration used in this experiment was 0.3 mg/ml in transcription buffer. The following equation [17] was used, based on the assumption that CRP denatures from native form (N) to denatured form (D).

$$\mu=\frac{\mu_b m_N T+(\mu_D+m_D T)\exp|\Delta H_{vh}/R(1/T_m-R(1/T_m-1/T))|}{1+\exp|\Delta H_{vh}/R(1/T_m-1/T)|}$$, where y= observed signal; R=gas constant; y_N and y_D are native and denatured baseline intercept, respectively; m_N and m_D are native and denatured baseline slope, respectively; T=temperature; T_m=temperature of transition midpoint; ΔH_{vh}=van't Hoff enthalpy. T_m values were estimated as follows: 70.5°C for no cAMP (A), 79.0°C for 100 μM cAMP (B). T_m at 5 mM cAMP (C) was not determined because of the lack of data in the denatured condition.

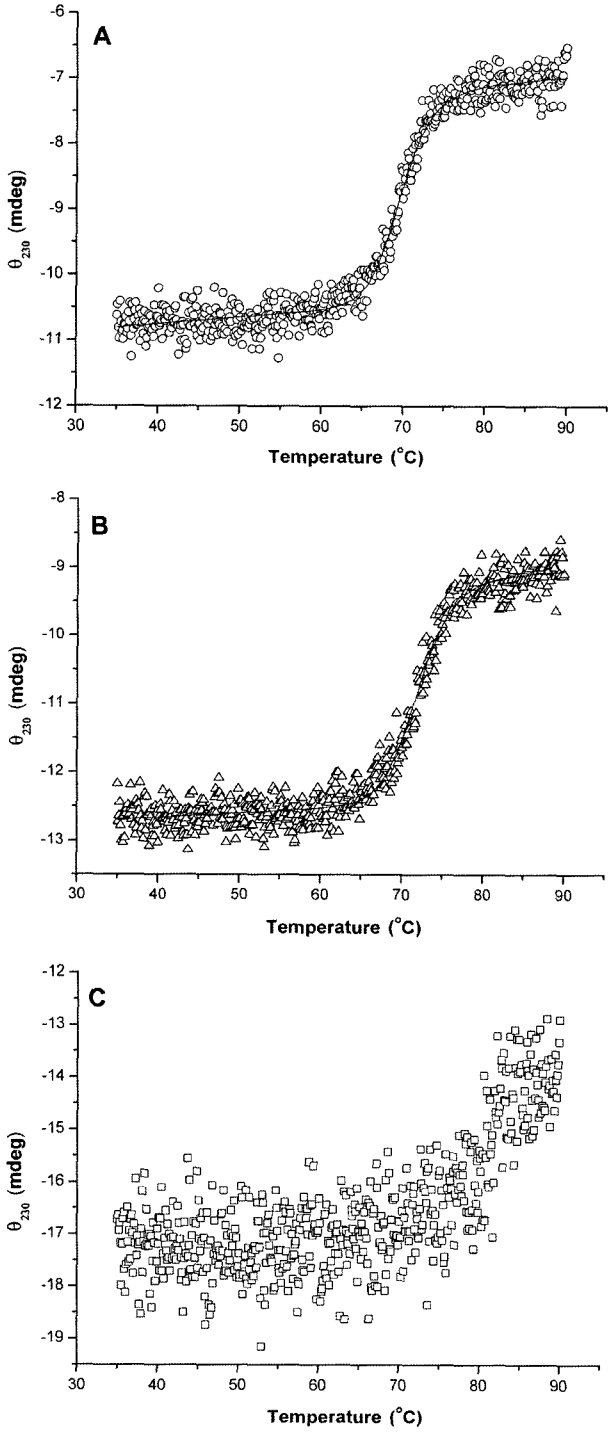

Fig. 5. Change in CD signal at 230 nm with no cAMP (A), 100 μM cAMP (B), and 5 mM cAMP (C). T127G CRP concentration was 0.3 mg/ml in transcription buffer.
T_m values were estimated as follows: 69.5°C for no cAMP (A), 71.8°C for 100 μM cAMP (B).

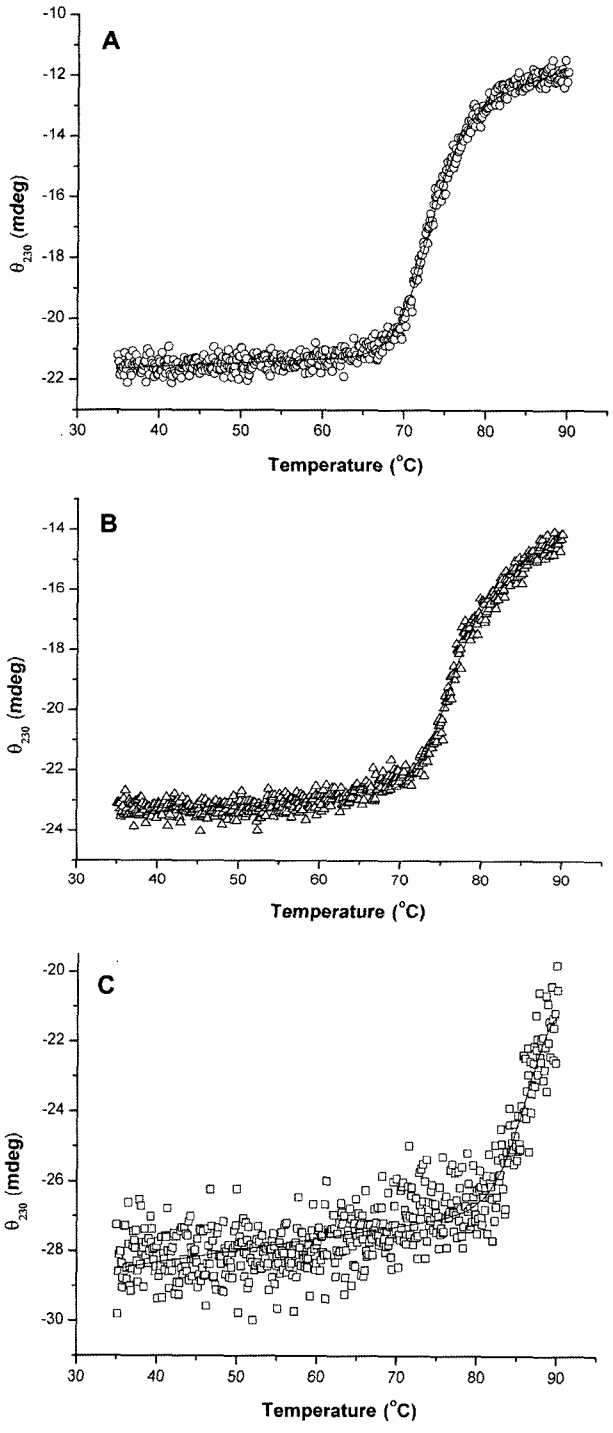

Fig. 6. Change in CD signal at 230 nm with no cAMP (A), 100 μM cAMP (B), and 5 mM cAMP (C). T127C CRP concentration was 0.3 mg/ml in transcription buffer.
T_m values were estimated as follows: 73.0°C for no cAMP (A), 75.5°C for 100 μM cAMP (B).

Instead of approximate analysis of data, the protein stability was compared by just looking at the temperature to sharply increase the CD signal: It showed that wild-type CRP was the most stable among the three CRPs at millimolar cAMP concentration. Furthermore, in T127G and T127C CRPs, the increased protein stabilities were

observed at millimolar cAMP concentration, compared with CRPs at micromolar cAMP concentration. Analytical gel chromatography [9] indicated that there was little elution volume change at a low cAMP concentration, but large increased elution volume was observed at a high cAMP concentration. Additional studies should be conducted to elucidate what kinds of structural changes affect T_m through the binding of cAMP to the *syn* binding site.

In summary, our results of proteolytic digestion and CD measurements indicated that transition of a structural change to the hinge region by the binding of cAMP to the *anti* site was necessary for the binding of cAMP to the *syn* site near the hinge region. The protein stability was increased by the binding of cAMP to the *anti* site at micromolar cAMP concentration and also additional protein stability by cAMP binding to the *syn* site at millimolar cAMP concentration.

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