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Stability and Structure of S128A Mutant cAMP Receptor Protein

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Abstract

Cyclic AMP receptor protein(CRP) is involved in the activation of many genes corresponding to catabolite enzymes in *Escherichia coli*. In this study, mutant CRP(S128A) was used to elucidate the effect of Ser 128 on the cAMP-induced structural change. Based on the protease digestion and thermal analysis, serine 128 in CRP affects the cAMP binding capability and then structural change of CRP protein. In addition, CD spectra in near UV region revealed that S128A CRP retained the sensitive conformation to thermal effect relative to that of wild-type CRP, in spite of identical Tm values in the absence of cAMP.

Key words : cAMP Receptor Protein, Thermal Analysis of CRP, Transcription Activator

1. Introduction

The 3', 5' cyclic adenosine monophosphate (cAMP) receptor protein (CRP) activates the transcription of many genes coding for the catabolite enzymes in *Escherichia coli*[1,2]. CRP is a homodimeric protein composed of 209 amino acid residues. Each subunit is folded into the large N-terminal domain with cAMP binding site, the small C-terminal domain with DNA binding site, and the intervening region called the hinge region. The binding of cAMP to CRP communicates the conformational change of cAMP binding pocket with DNA binding domain through the hinge region of CRP[3,4,5]. CRP:cAMP complex binds specifically to CRP binding site around the promoter region. The ternary complex of CRP: cAMP:DNA leads to the formation of an active transcription initiation complex in the presence of RNA polymerase.

Crystal structure[6] showed that CRP bound the second cAMP molecule in the *syn* binding site around the hinge region in addition to *anti* binding site (N-terminal domain). It was indicated that, at micromolar cAMP concentration, CRP was occupied with two molecules of cAMP in *anti* binding site and complexes was existed as CRP:$(cAMP)_2$. This complex is involved in the activation of many genes expressions. However, at millimolar concentration of cAMP, the complexes with second cAMP in *syn* binding site showed the loss of affinity and sequence specificity for DNA and regulatory function of gene[5,7].

Lee *et al*.[8] reported that S128A CRP reduced affinity for cAMP and produced only 15% of the open complex observed for wild-type in the presence of cAMP. It suggested that serine 128 seems to play a crucial role in CRP structural change for the activation of corresponding gene expression. This study was designed to monitor the mutational effect on the stability and structure of S128A CRP in the presence or absence of cAMP by the thermal analysis.

2. Materials and Methods

Subtilisin (EC 3.4.21.62), 3',5'-cyclic adenosine monophosphate (cAMP), 3',5'-cyclic guanosine monophosphate (cGMP), and PMSF (phenylmethanesulfonyl fluoride) were purchased from Sigma-Aldrich.

2.1. Protein Purification

S128A CRP was purified from the strain CA8445/pRK248 (cI^{ts}) harboring recombinant pRE2*crp** expression vector according to the modified methods by Harman *et al*.[9,10]. The Affi-gel Blue gel chromatography (Bio-Rad) was used further to eliminate the high molecular weight contaminants. Protein was more pure than 95% purity by 12.5% SDS-PAGE. CRP concentration was determined using the extinction coefficient

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3.5×10^{-4} $M^{-1}cm^{-1}$ at A_{280}[10,11].

2.2. Proteolytic Digestion

Proteolytic digestion reactions were carried out as described by publications[12] at a volume of 30 μl at 37°C in transcription buffer (40 mM Tris, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 5% glycerol). The ratio of CRP to protease was 200:1 by weight. Proteins were incubated for 30 min either in the presence of cAMP or cGMP, and digestion reactions were carried out for 20 min at 37°C. Reactions were stopped by addition of PMSF to final concentration of 5mM. Reactions were applied to 20% SDS-PAGE and shown by staining with Coomassie brilliant blue. Band intensity was measured with Kodak Image analysis (ver. 3.5). Fraction of digested protein to CRP was plotted against cAMP concentration with Origin 6.0 (Microcal Software, Inc.).

2.3. Circular Dichroism Measurement

Circular dichroism (CD) ellipticity was measured with J715 CD/ORD spectropolarimeter (Korea Basic Science Institute). All measurements were performed 5 times in thermostated curvet with a 0.1 cm path length at 222 nm. 0.3 mg/ml of CRP was used either in the presence or absence of cAMP. Molar ellipticity data were plotted and analyzed with Origin 6.0. And also CD data were collected from 180 to 340 nm wavelength to figure out structural change of CRP at 25 and 55°C.

3. Results and Discussion

Proteolytic digestion is a useful technique to measure the structural change of protein by the binding of ligand. Fig. 1 showed the result of proteolytic digestion of S128A CRP in the presence of cAMP (A) and cGMP (B). It has been reported that the protease digestion of CRP showed the biphasic pattern in the presence of cAMP[1]. The *anti*-binding site of CRP was occupied at micromolar concentration of cAMP and the *syn*-binding site was filled with cAMP at millimolar concentration of cAMP. In this study, digested α-CRP is gradually increased as cAMP concentration was reached to 1 mM. Fig. 2 shows that α-CRP is decreased over 1 mM cAMP concentration. Occupancy of cAMP to the *syn*-binding site causes protease not to access the hinge region and not to hydrolyze the hinge region of CRP. In case of

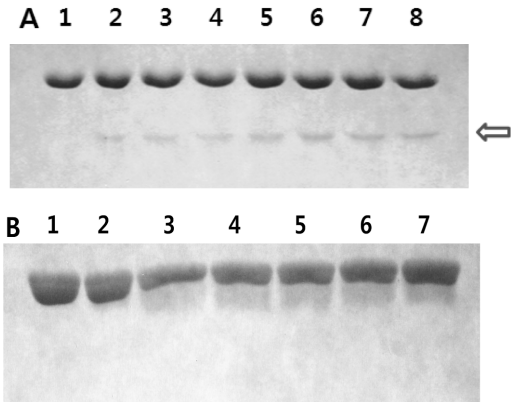

Fig. 1. Proteolytic digestion of S128A CRP with cAMP (A) and cGMP (B).
Lane 1 contains only S128A CRP. Lane 2~8 contain 0.1, 0.2, 0.3, 0.5, 1, 3, and 5 mM cAMP, respectively. Arrow indicates the digested CRP (α-CRP).

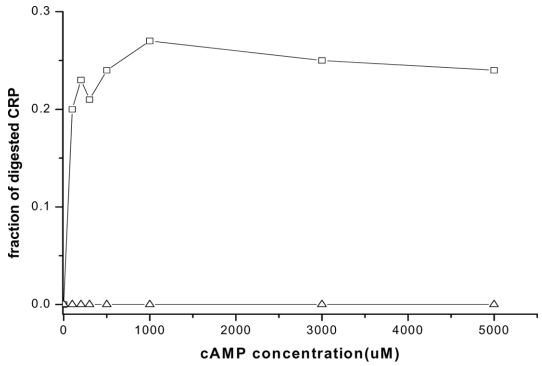

Fig. 2. Plot of the digested fraction of CRP against cAMP (□) and cGMP (△) concentrations. Digested fractions were estimated from the ratio of digested band to the digested and undigested bands of Fig. 1 by using Kodak Image analysis (ver. 3.5).

wild-type CRP, however, resistance to protease is revealed below 0.5 mM cAMP[12]. Based on the digested fraction and protease resistance, serine 128 in CRP affects the cAMP binding capability and then structural change.

It shows that the *anti*-binding site of S128A CRP is occupied with cAMP like wild-type CRP but *syn*-binding site was not occupied to the extent of wild-type CRP due to the decreased binding affinity of S128A CRP to cAMP[13]. In addition, Fig. 1B shows that S128A CRP does not complex with cGMP like wild-type CRP. It implies that hydroxyl group of Ser 128 is not involved

in distinguishing base moiety of cyclic nucleotide at cAMP binding site of CRP.

Fig. 3 shows the plots of molar ellipticity at 222 nm (θ_{222}) as a function of temperature (T). For the analysis, plotted data are fitted by the non-linear regression method according to the following equation[14], based on the assumption that CRP denatures from native form (N) to denatured form (D).

$$\theta = \frac{(\theta_N + m_N T + (\theta_D + m_D T)\exp[\Delta H_v / R(1/T_m - R(1/T_m - 1/T))])}{1 + \exp[\Delta H_v / R(1/T_m - R(1/T_m - 1/T))]}$$

Where θ is observed signal of molar ellipticity at a given temperature; R is gas constant; θ_N and θ_D are native and denatured baseline intercepts, respectively; m_N and m_D denote native and denatured baseline slop, respectively; Tm is melting temperature of protein; ΔHv represents van't Hoff enthalpy.

Tm is determined by the nonlinear regression analysis with the equation described above. Tm value of S128A was 71°C which is similar to wild-type CRP (70.5°C)[12]. It suggests that S128A CRP shows similar stability in comparison with wild-type CRP, although amino acid is substituted in cAMP binding site. This result is consistent with the GuHCl-induced chemical denaturation result of S128A CRP[15]. In the presence of 100 μM cAMP, Tm value of S128A is increased to 74.1°C due to the cAMP-induced conformational change. Tm of S128A CRP is lower than that of wild-type CRP (79°C) in the presence of 100 μM cAMP. It indicates that S128A CRP can complex with cAMP but S128A CRP:cAMP complex reveals not fully active conformation relative to that of wild-type CRP:cAMP. Kinetic study[15] showed that the mutations in T127I and S128A in CRP affected the kinetics of carboxyl and amino-terminal domain communication as well as subunit-subunit interactions.

At 5 mM cAMP concentration (see Fig. 3C), Tm is not able to be estimated due to lack of data at high temperature. Tm is presumably higher than 85°C which is similar to that of wild-type CRP. The binding of cAMP to *syn*-binding site results in the increased stability of CRP to the thermal effect. Even though small structural change was monitored at CRP:$(cAMP)_4$ complex by fluorescence energy transfer study[16], CRP:$cAMP_4$ complex retains obviously different conformation according to the protease resistance and the decreased promoter complex formation[1].

Fig. 4 shows CD spectra of S128A CRP recorded in the far (A) and near (B) UV regions at 25 and 55°C. CD spectra in far UV region are influenced by the geometry of the polypeptide backbone and reflective of the different types of secondary structures present. These spectra indicates that there is no dramatic change in the secondary structure. Based on CD spectra in the

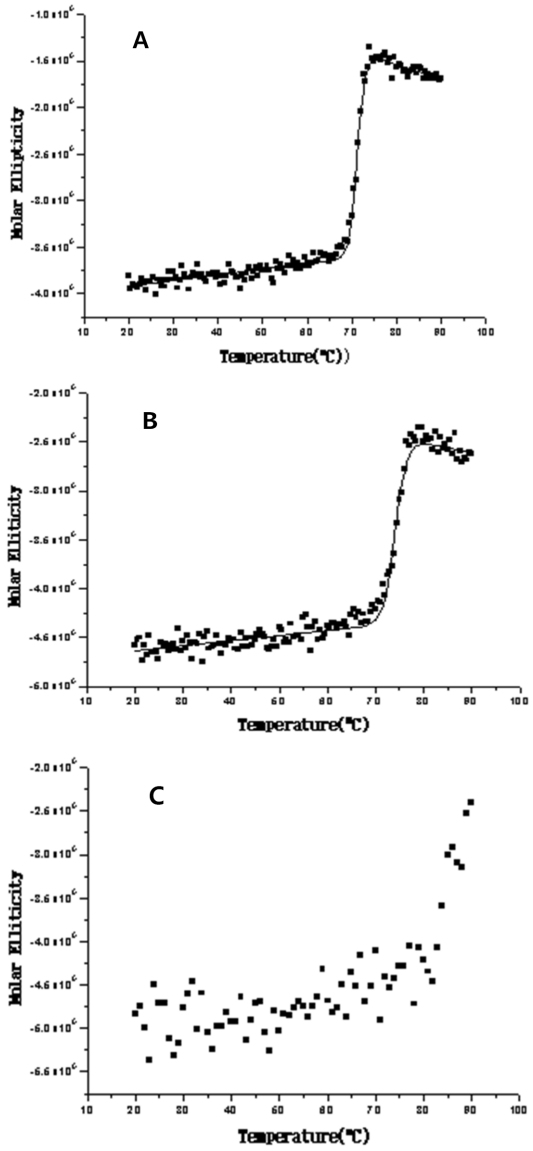

Fig. 3. Molar ellipticity of S128A CRP at 222 nm under constant increase of temperature from 20 to 90°C. 0.3 mg/mL of S128A CRP was used either without cAMP (A), or with 100 μM cAMP (B) or 5 mM cAMP (C).

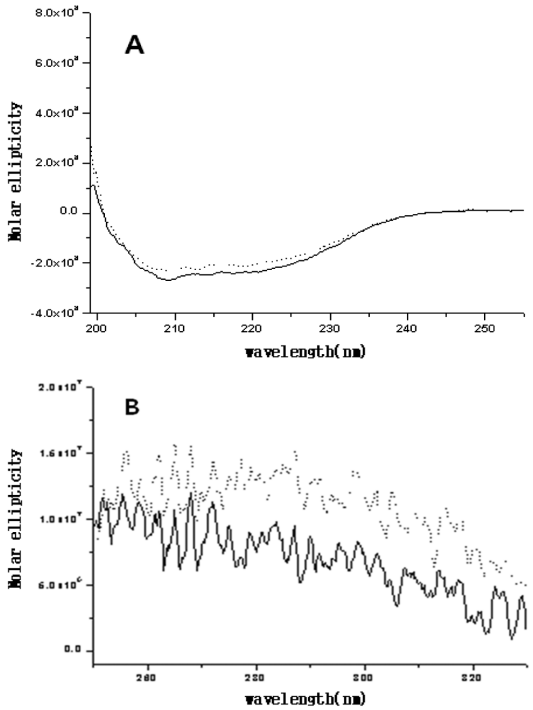

Fig. 4. CD spectra around far UV region of 128A CRP at 25 (——) and 55°C (----). CD spectra within near UV region (B) of S128A CRP at 25 (——) and 55°C (----). CRP used in this experiment was 0.3 mg/mL.

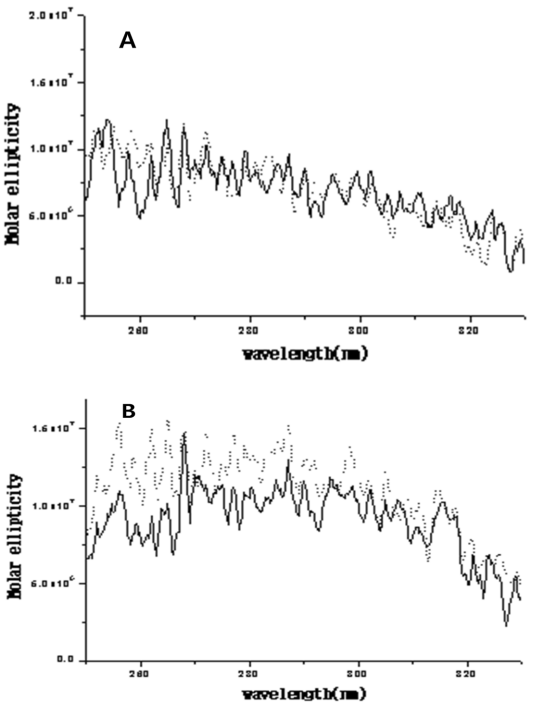

Fig. 5. CD spectra within near UV region of wild-type (——) and S128A CRP (----) at 25°C (A). CD spectra of wild-type CRP (——) and S128A CRP (----) at 55°C (B).

near UV region, however, the aromatic side chains like tryptophan and tyrosine is in different environment at 55°C relative to that at 25°C[17].

In Fig. 5, CD spectra within near UV region indicates that S128A CRP has the same conformation like wild-type CRP[17,18] at 25°C. But it shows high intensities and different pattern in comparison with wild-type CRP at 55°C. It implies that S128A CRP retains the sensitive conformation to thermal effect relative to that of wild-type CRP, although two CRPs have same Tm value. And thermal stability of S128A CRP is increased depending on the bound cAMP molecules.

4. Conclusion

Tm values of S128A CRP was measured by the thermal denaturation analysis. Its values are 74.1°C and > 85°C in the presence of 0.1 and 5 mM cAMP, respectively. Protease digestion shows that S128A CRP is more resistant to protease in the presence of cAMP than wild-type CRP. This study indicates that serine 128 in CRP play an important role of the cAMP binding capability and cAMP-mediated CRP structural change and protein stability.

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