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The Preliminary Study on the Structure of Cop Protein by CD and NMR

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Abstract: Cop protein is the transcription repressor protein in rolling circle replication plasmid. With antisense RNA, Cop protein controls the copy number of plasmid. Cop family proteins have been found in various plasmids. Among Cop family proteins, Cop studied in this paper consists of 55 amino acids (Mw.6,400), and was known to have trimer structure. Since no structural facts are elucidated, we have carried out preliminary experiments aimed at the elucidation of its three dimensional structure. The secondary structure of Cop is studied by CD and NMR. To solve the aggregation of Cop at high concentration, we tested various detergents and salts. The addition of detergents and salts could not solve the aggregation problem. However, we found that concentration is important in solving the aggregation problem. We knew that 0.18mM in 50mM potassium phosphate without any other ingredients is maximum concentration not to aggregate. We also investigated the pH dependence of Cop protein, and knew that Cop protein is more stable in acid state. At various temperatures, ^{15}N-^{1}H HSQC spectra were measured in order to find the optimal experimental condition. To enhance the peak resolution, 3D NOESY-HSQC spectrum is acquired. Since there are NOE peaks in the NH-NH region, we knew that Cop protein has α-helical content, which was also confirmed by CD.

INTRODUCTION

The pLS 1 is a member of small plasmids that replicate by using a rolling circle intermediate. Rolling circle-replicating plasmid is one of small, promiscuous multicopy replication groups[1]. It can replicate independently in gram positive and gram negative host. The replication of pLS1 family plasmid is controlled by antisense RNA and transcriptional repressor protein, Cop[2]. Cop is primarily discovered in pLS1 and called Cop G (formerly Rep A), the 45 amino acid repressor. The Cop protein has also been shown to be synthesized

pLS1	MKKRLTITLSESVLEN	LEKMAREMGLSKSAMISVAL	ENYKKGQEK
pWV01	MVISESKKRVMISLTKEQDKK	LTDMAKQKGFSKSAVAALAI	EEYARKESEQKK
pFX2	MVISESKKRVMISLTKEQDKK	LTDMAKQKGFSKSAVAALAI	EEYARKESESQKK
pE194	MVVDRKEEKKVAVTLRLTTEENEI	LNRIKEKYNISKSDATGILI	KKYAKEEYGAF
pLB4	MVEVEKKKITLSIPVETNGK	LEELAQKYGMTKSGLVNFLV	NQVAEAGTIYRQ
pADB201	MDKLVTLRLDEQQHEK	LKEQANKLGMTISGYVRYLV	LKSSEMKTKESKKKL
pKMK1	MDKKITFKIDEEHFER	IKEKGDELGMTMGGYVRYLV	LKSSEIKGRGNKKKKPE

Fig. 1. The family of Cop repressors. Amino acid residues are aligned with respect to the putative HTH motif.

in *B. subtilis* minicells, harboring pE 194[3]. This Cop protein consists of 55 amino acids. Fig.1 is the list of known Cop proteins.

Cop proteins in Fig. 1 share several features[4] : (i) Cop proteins are small (45-55 residues), and have two conserved K residues at the N-terminal; (ii) all Cop proteins show a putative α -helix-turn-α -helix (HTH) motif, typical of many DNA-binding proteins; (iii) *cop* and *rep* genes are probably co-transcribed from a single promoter (P_{cr}), as is the case for pLS1, and (iv) there is a symmetric DNA element in the vicinity of this promoter (probably the Cop- operator).

The Cop protein is already known to have a trimer-like structure which was confirmed by high-performance size exclusion chromatography.[5] Trimer structure seems to be unstable and affected by pH, and protein concentration. We studied the physical condition on the structural stability of Cop, and finally found the best condition for NMR structure experiments.

MATERIALS AND METHOD

Bacterial strain and plasmids

The synthetic gene of Cop was cloned into pET3a (we designated the plasmid as pJHK102). The pJHK102 was transforred into *Escherichia coli* strain BL21 (DE3) (Novagen).[5]

Growth medium and overexpression of Cop protein

The BL21 (DE3) harboring pJHK102 was cultured in LB medium with antibiotics, ampicilin (200 $\mu g/m\ell$). M9 medium is used for preparing ^{15}N full-labeled Cop and $^{15}NH_4Cl$ or $^{15}(NH_4)_2SO_4$ was used as the nitrogen source of medium. The expression of Cop was induced by 0.5mM isopropyl β -D-thiogalactoside at an OD_{600} of 0.8. After induction,

E.coli was grown for an additional 3hr before harvesting by centrifugation. The pellet was stored at -80℃.

Purification of Cop protein

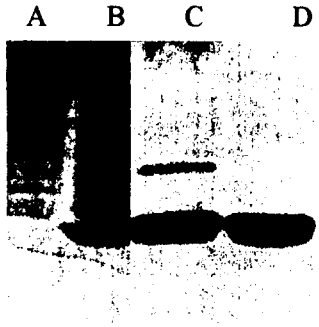
The cell pellet was washed in ice with 50mM potassium phosphate buffer (pH 6.5) containing 10 % glycerol. The cell pellet was sonicated and then centrifuged at 8,000g for 20min. The supernatant was collected and diluted with 50mM potassium phosphate buffer (pH 6.5) (Buffer A). Diluted supernatant was applied to Bio-Rex 70 cation exchange column (Bio-Rad) equilibrated with buffer A. After the sample was eluted with linear gradient of NaCl (0 - 2M) in buffer A, fractions were analyzed for the presence of Cop by SDS-tricine polyacrylamide gel electrophoresis. Peak fractions containing Cop protein were pooled and dialyzed against 50mM potassium phosphate buffer (pH 7.0). The sample was applied to the Bio-Rex70 cation exchange column equilibrated with 50mM potassium phosphate buffer (pH 7.0), eluted and analyzed by SDS-tricine PAGE. At this stage, some fractions had the high purified Cop protein. After collecting fractions containing purified protein, protein solution was dialyzed against proper buffer for each spectroscopic experiment and concentrated by Centriprep3 (Amicon).

Protein Stability Measurement

CD spectra over the frequency range of 190 - 260nm were recorded on a JASCO-715 spectropolarimeter. Measurements were made using a cylindrical fused quartz cell with a 0.1cm path length. CD spectra were measured on Cop protein with various salts, detergents and pHs.

NMR spectroscopy

All NMR spectra were acquired by Bruker DRX 500MHz in College of Pharmacy, Seoul National University. For NMR measurement, the samples were prepared to a final volume of 280 $\mu\ell$ in 50mM potassium phosphate buffer (pH 3.0, pH 3.5, pH4.0) containing 1mM NaN_3, Shigemi micro NMR tube was used for these samples. The final concentration of Cop was 0.18mM. 2D HSQC and 3D NOESY-HSQC spectra were respectively recorded.



A : Before IPTG induction
B : After IPTG induction
C : First Bio-rex column purification
D : Second Bio-rex column purification

Fig. 2. 15% Tricine-SDS PAGE

RESULTS AND DISCUSSION

The secondary structure prediction

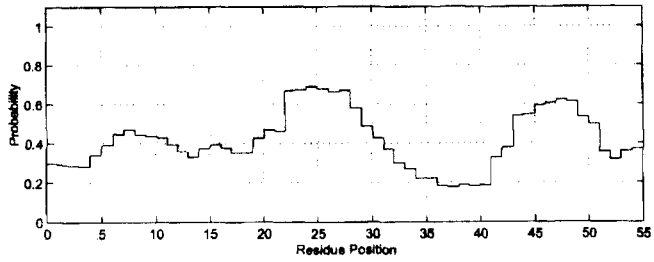


Fig. 3. Computer-derived predictions on secondary structure of Cop

At 25℃, the Cop had a CD spectrum with negative minimal at ~222nm and ~209nm that is characteristic for proteins with predominantly α-helical structure (Fig. 4A).[6] Secondary structure prediction in web site (www.expasy.ch) was also carried out by PSA sequence Analysis. When we consider that the number of amino acid is not over 100 residues, the result of secondary structure prediction is acceptable. The prediction result is shown in Fig. 3. As expected, Fig. 3 shows the predominant α-helical structure. The *y* axis of Fig. 3 represent α-helix probability. As shown in this figure, they show typical HTH characteristics[7].

pH stability test

(A) (B)

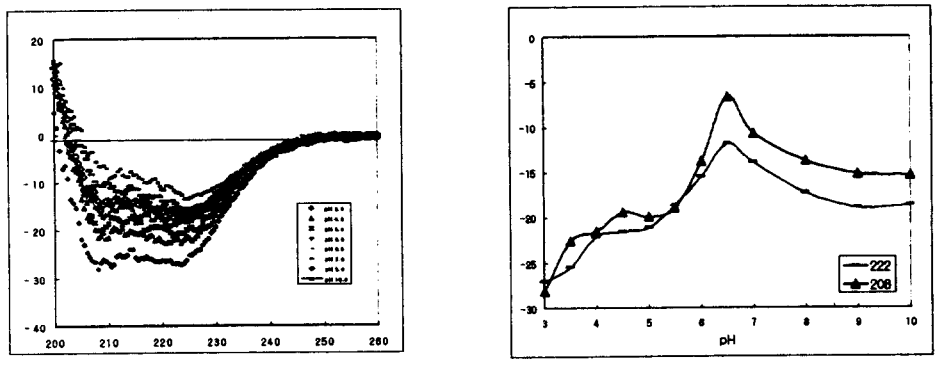


Fig. 4. (A) CD spectrum of Cop as a function of pH. Experimental conditions are pH 3.0, pH 4.0, pH 5.0, pH 10.0, pH 8.0, pH 6.0, pH 7.0, pH 6.5.
(B) The plot of peak intensity against pH. Peak intensities were acquired atarla 208nm and 222nm wavelength.

In order to investigate the pH dependence of Cop protein, CD experiments were also conducted on the samples between pH 3 and pH 10 in potassium phosphate buffer. The result of CD experiment was shown in Fig. 4. As shown in this figure, the pH change to neutral significantly made the structure of Cop protein lose, and the pH increase to alkali made the structure of Cop protein recover. Especially in Fig. 4B, peak pattern is sigmoidal. It can be assumed that Cop passed 2 different step in denaturing process by pH. The first step is dissociation of trimer to monomer, and the second step is de-naturation of monomer. When we consider that pI of Cop is 8.96 and HSQC spectrum of pH 3.5 is better than HSQC spectrum of pH 3.0, increasing α-helixity is not only the absolute parameter of stabilization. To make the trimer, structrue of monomer may be relaxed. Because Cop is trimer, the structure of multimer is more important than the secondary structure of monomer.

Concentration test

The typical α-helical pattern of CD was changed upon increasing the concentration, namely, the minima at 208 nm disappeared and the minima at 222 nm shifted toward longer wavelength (Fig. 5). This phenomenon is well explained by the aggregation of proteins.[10] We think therefore that Cop aggregates at higher concentration above 0.18 mM. From the NMR experiments, we could confirm this result is due to the aggregation of Cop.

The ^{1}H-^{15}N HSQC spectra of Cop clearly show that the spectrum at 0.18 mM is resolved well compared with the spectrum at 0.7 mM (Fig. 6). Generally, the overlap of NMR spectra could be explained by degradation or aggregation of proteins. Since Cop protein was not degraded after NMR experiments which was confirmed by electrophoresis analysis, we think that Cop protein aggregates at the concentration higher than 0.18mM.

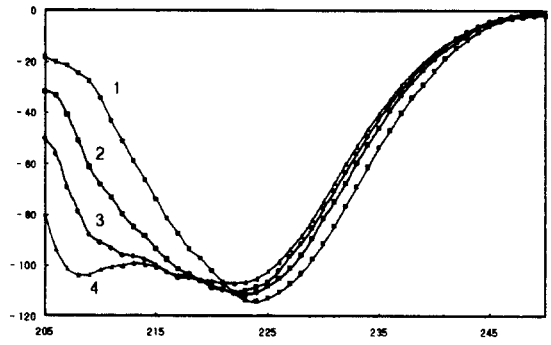


Fig. 5. CD spectrum of Cop as a function of concentration 1. 0.30mM ; 2. 0.22mM ; 3. 0.19mM ; 4. 0.18mM

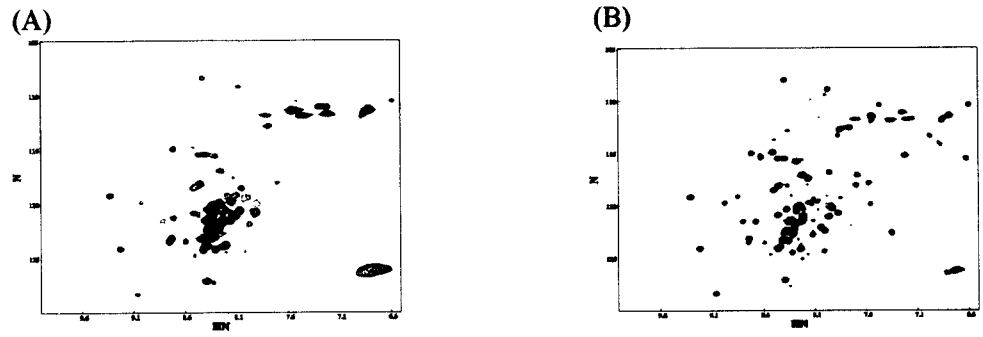


Fig. 6. HSQC spectrum of Cop protein at concentration 0.7mM (A) and 0.18mM (B)

Detergent and Salt test

In many cases, the problem of protein aggregation caused by intermolecular hydrophobic interaction is solved by addition of detergents. We tested therefore many detergents such as CHAPS, Deoxycholic acid, Dodecylmaltoside, Triton X-100, and Tween 20 to solve the aggregation of Cop. However we could not detect any improvement of CD sepctra in all cases (Fig. 7A). Since the aggregation could be induced by the electrostatic interaction, we also tested the effect of salts. However any significant spectral changes were not detected (Fig 7B).[8]

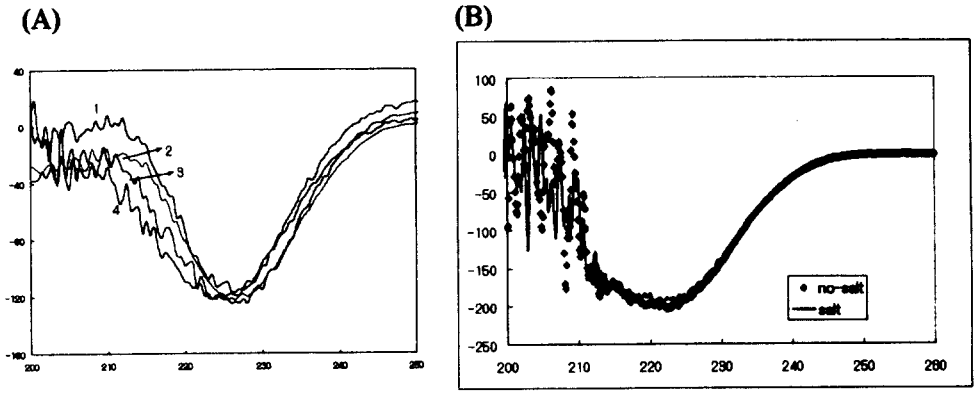


Fig. 7. CD psectra of Cop in various detergents(A. 1. 20mM CHAPS; 2. 20mM Deoxycholic acid; 3.20mM Dodecylmaltoside; 4. 20mM Triton X-100) and in various salt concentration(B)

NMR experiments

We found the appropriate pH value for NMR sample from CD data. Because the solubility of Cop is significantly decreased as increasing pH, the pH must be lower than pH 4.0. Therefore, we tested the temperature dependence of NMR spectrum, at pH 3.0, pH 3.5, and pH 4.0. From a lot of 2D ^{1}H-^{15}N HSQC spectra, the best conditions of NMR measurements were at pH 3.5. The peak of HSQC spectrum at pH 3.5 was well dispersed compared with that at other pH. HSQC spectrum was acquired at 298K, 303K, 308K, 313K, 318K and 323K by using sample at pH 3.5.

Because the spectrum at 318K has good peak dispersion, the optimal temperature for NMR experimnets is 318K. The protein is de-natured at 55℃ as the CD temperature study. Consequently, we knew that optimal condition is 0.18mM Cop protein in 50mM potassium phosphate buffer (pH 3.5) at 318K.

To enhance the resolution, 3D NOESY-HSQC spectrum is acquired (Fig. 9). Since ther are NOE peaks in the NH-NH region, we knew that Cop protein has α -helical content which was also confirmed by CD.

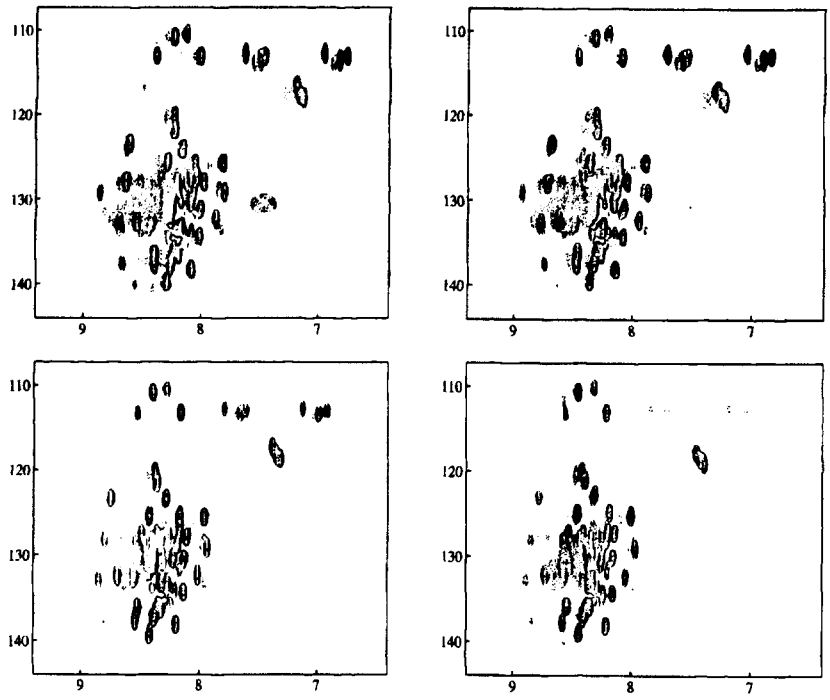


Fig. 8. HSQC spectra of Cop protein acquired at various temperature. (A) 308K (B) 313K (C) 318K (D) 323K

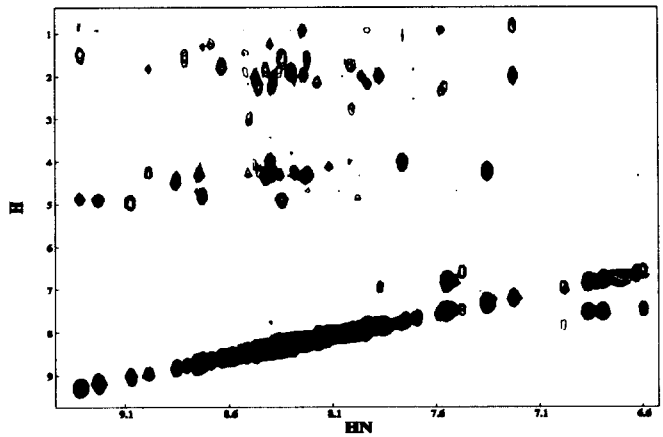


Fig. 9. 3-dimensional NOESY-HSQC spectrum of Cop (200ms mixing time) at 308K. Buffer condition is 50 mM potassium phosphate buffer, pH 3.0 and sample concentration is 0.18 mM.

Despite of these serial experiments, the concentration is too low to be studied by conventional NMR spectroscopy. However, the problem could be overcome by using recently developed 800MHz NMR equipment and new NMR techniques that improved the sensitivity and resolution.

Acknowledgement

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