Moderately thermostable phage Φ11 Cro repressor has novel DNA-binding capacity and physicochemical properties

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The temperate Staphylococcus aureus phage Φ11 harbors cl and cro repressor genes similar to those of lambdoid phages. Using extremely pure Φ11 Cro (the product of the Φ11 cro gene) we demonstrated that this protein possesses a single domain structure, forms dimers in solution at micromolar concentrations and maintains a largely α-helical structure even at 45°C. Φ11 Cro was sensitive to thermolysin at temperatures ranging from 55-75°C and began to aggregate at ~63°C, suggesting that the protein is moderately thermostable. Of the three homologous 15-bp operators (O1, O2, and O3) in the Φ11 cl-cro intergenic region, Φ11 Cro only binds efficiently to O3, which is located upstream of the cl gene. Our comparative analyses indicate that the DNA binding capacity, secondary structure and dimerization efficiency of thermostable Φ11 Cro are distinct from those of P22 Cro and λ Cro, the best characterized representatives of the two structurally different Cro families. [BMB reports 2009; 42(3): 160-165]

INTRODUCTION

The bacteriophage-encoded Cro proteins vary notably in structure, oligomerization ability and in thermal stability (1-6). While the Cro proteins of phages P22, 434, N15, and Xfaso 1 all bear similar α-helical folds, λ Cro and Phi 6 Cro harbor homologous α-helix/β-sheet folds. These two Cro protein structural families share 25-40% identity at the primary sequence level. Of the Cro proteins adopting α-helical folds, all except N15 Cro exist primarily as monomers in solution. N15 Cro, λ Cro and Phi 6 Cro all form dimers in solution but their dimerization efficiencies differ significantly. P22 Cro was shown to be more thermally stable than λ Cro. Interestingly, Cro proteins, which bind to operator DNAs as dimers, inhibit transcription from the operator early promoters (7). To date however, Cro proteins from most temperate phages have yet to be investigated at the structural and functional level.

RESULTS AND DISCUSSION

Φ11 Cro is a single domain protein

Thirteen trypsin and eight chymotrypsin recognition sites were distributed throughout the primary structure of native Φ11 Cro (data not shown). These enzymes only cleaved those sites that were located in flexible regions or those that were exposed on the surface of the folded Cro molecule. To map such flexible region(s) in native Cro, we performed limited proteolysis of histidine-tagged Cro (named Cro here) with both trypsin and

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Fig. 1. Physicochemical characterization of Φ11 Cro. Chymotrypsin (A) and trypsin (B) digested Cro fragments were analyzed by Tris-tricine SDS-15% PAGE followed by silver staining. Fragments I and II were generated by chymotrypsin (Chy), whereas, fragments III and IV were generated by trypsin (Try). The molecular masses (in kDa) of marker protein bands were as shown at the right side of the gel. (C) Far UV CD-spectra of the Cro repressor were recorded at 25°C and 45 °C according to Ganguly et al. (21). (D) SDS-15% PAGE analysis of thermolysin-cleaved Cro products. Proteolysis of Cro by thermolysin was performed essentially according to Hecht et al. (22). Briefly, each reaction mixture including 1 μg of Cro and 3.33 ng of thermolysin was incubated for 5 min at the indicated temperature followed by termination of the cleavage by the addition of EDTA. (E) Thermal aggregation of 5 μM Cro in Buffer C was performed according to Bandhu et al. (23). (F) Analytical gel filtration chromatography. Approximately 100 μl of 20 μM Cro in phosphate buffer was loaded onto a ∼14 ml HPLC gel filtration column equilibrated in phosphate buffer and the absorbance of the eluted fractions, determined at 220 nm, were expressed as Volts. The column was calibrated with BSA (66 kDa, III), β-Lactoglobulin (18.4 kDa, II) and Cytochrome C (12.3 kDa, II). The molecular weights were plotted against Ve/Vo, where Ve and Vo denote the elution volume and void volume, respectively. The void volume of column, as determined by the elution of blue dextran, was 5.5 ml. (G) Glutaraldehyde (GCHO)-treated (+)/untreated(-) Cro/β-lactoglobulin (LG) solutions were analyzed by SDS-15% PAGE followed by either Coomassie blue (Y and Z) or silver (X) staining. Lanes 1-4, 5-8 and 9-12 contained ∼0.65 μg, 2.5 μg and 1.8 μg protein, respectively. The concentrations of Cro and LG in the reactions were as indicated above the respective lanes. The molecular masses (in kDa) of marker protein bands are shown at both sides of the gel. An asterisk denotes dimeric Φ11 Cro.

Our data are presented in Fig. 1. Proteolysis of Cro by either trypsin (Fig. 1A) or chymotrypsin (Fig. 1B) yielded only one stable protein fragment (II or IV) of nearly 10 kDa. The data suggested that the majority of the enzymatic cleavage sites were buried in the interior of the folded Cro protein. The sequences of the first five N-terminal amino acid residues of fragments II and IV were determined to be NMQWN and GSEFN, respectively. This indicated that fragment II was generated by the cleavage of a peptide bond connecting amino acid residues 36 and 37, whereas, fragment IV was generated by digesting the peptide bond between residues 32 and 33. Because these cleavage sites were located in the histidine tagged region in Cro, our data suggested that native Cro is a single domain protein similar to that of P22 Cro, 434 Cro and λ Cro (7).

Secondary structure of Φ11 Cro
To find clues about the Cro secondary structure and to learn the effect of temperature on the conformation of Cro, CD spectra (200-260 nm) were recorded at different temperatures. Fig. 1C revealed that the spectrum of Cro at 25°C is very similar to that recorded at 45°C and that both spectra carry nearly identical negative elliptical peaks at both 208 and 222 nm. While the peaks obtained at 208 nm are nearly pointed, the peaks recorded at 222 nm are somewhat flattened. Although the presence of the histidine tag on Cro reduces its helical content
slightly as was evident from bioinformatic analysis of both native as well as histidine-tagged Cro (data not shown), the CD-spectrum of Φ11 Cro appears to be somewhere between those of P22 Cro and λ Cro (6).

**Moderate Φ11 Cro thermostability**

The CD-spectrum of Φ11 Cro remained unaffected by increasing the incubation temperature from 25°C to 45°C (Fig. 1C), suggesting that Cro is thermostable. To see whether Φ11 Cro is truly thermostable, we studied both the thermolysin-mediated proteolysis and the aggregation of Φ11 Cro at different temperatures. Nearly 80% of Φ11 Cro was digested by thermolysin by raising the incubation temperature of proteolysis from 30°C to 55°C or even higher (Fig. 1D). Interestingly, the aggregation of Φ11 Cro began at −63°C (Fig. 1E). Together, these data suggest that Φ11 Cro is a moderately thermostable protein and that this thermostability is greater than that observed for λ Cro, with a melting temperature close to 40°C (3).

**Oligomeric state of Φ11 Cro**

The Cro repressor encoded by Φ11 can dimerize spontaneously in solution as observed for λ Cro (3, 7) or can remain a monomer similar to that of P22 Cro (15). To understand the status of Cro in solution, we carried out both gel filtration chromatography and glutaraldehyde crosslinking of Cro at various concentrations. As shown in Fig. 1F, passage of a 20 μM solution of Cro through a gel filtration column produced a single peak that corresponded to monomeric Cro (~12.4 kDa) when compared to the elution profiles of protein standards. Similar analysis conducted with a 30 μM Cro solution also produced a single monomeric Cro-specific peak (data not shown), indicating that at micromolar concentrations Φ11 Cro exists as monomer in solution. Contrary to the results described above, glutaraldehyde-mediated crosslinking showed that Φ11 Cro predominantly forms a dimer at 1-20 μM concentrations. This dimerization appears to be specific because β-lactoglobulin did not form an oligomer under identical conditions (Fig. 1G). As observed from the CD-spectrum of Φ11 Cro (Fig. 1C), the dimerization efficiency of Φ11 Cro also appears intermediate to monomeric P22 Cro and dimeric λ Cro (3, 6). Previously, a mutant λ Cro harboring an Ala33Trp (as in P22 Cro) change, showed reduced dimerization efficiency and an increased thermal stability (3). Ala33 in λ Cro and Trp30 of P22 Cro were found to align with Leu32 of Φ11 Cro (data not shown), indicating that Leu32 may be responsible for the dimerization as well as the moderate thermal stability of Φ11 Cro.

**Φ11 Cro binds to a single site in the Φ11 cl-cro intergenic region**

To identify the possible Cro binding site(s) in the Φ11 cl-cro intergenic region, we performed DNase I footprinting analysis using Cro and radioactively labeled O DNA (Fig. 2A). The footprints of both the top and bottom strands of O DNA indicate that one extended region in O DNA was resistant to DNase I digestion in the presence of saturating amounts of Cro. More precisely, Cro protected the -128 to -47 and -127 to -54 regions of the top and bottom strand, respectively (Fig. 2B). The entire protected region harbors two putative 15 bp operator sites (O3 and O2), which are separated by 31 bp. Interestingly, the O1 site that showed binding affinity for Φ11 Cl was not protected by Cro. In contrast, the O3 site that did not interact with Φ11 Cl (our unpublished data) was protected by Cro. The DNase I hypersensitive sites on both strands of the cl-cro intergenic region (designated by open circles; Fig. 2B) indicate that O DNA undergoes a conformational change of upon Cro binding.

To see whether Cro truly binds to both O2 and O3, we carried out a gel shift assay using 32P labeled O DNA and varied the Cro concentration. As shown in the Fig. 2C, one prominent shifted complex (designated I) gradually formed with increasing amounts of Cro. Additional gel shift assays using synthetic 32P labeled DNAs (harboring either O1, O2, or O3) and varying the Cro concentration revealed that only O3 DNA bound appreciably to Cro (Fig. 2D). The Cro/O3 DNA binding plot indicated that at Cro concentrations producing 50% O3 DNA saturation, the apparent equilibrium dissociation constant was nearly 475 nM (Fig. 2E). We did not use Cro concentrations higher than 900 nM in any of our gel shift assays because non-operator cspC DNA showed weak binding to Cro at concentrations higher than 1,000 nM (data not shown).

Multiple Cro binding sites were shown to be present in the cl-cro intergenic regions in P22, 434, A2, Φg1e, Tuc2009 and λ (7, 10, 15, 16) phage. In contrast, the cl-cro intergenic region of S. aureus phage, similar to that of Φ11 or ΦSa3ms, bore only a single site supporting Cro binding (as mentioned above and in 13), indicating that the mechanism of Φ11 Cro or ΦSa3ms Cro action might be distinct from that observed for other lambdoid phages.

**MATERIALS AND METHODS**

**Bacterial and phage strains and their growth conditions**

*S. aureus* RN4220 and *E. coli* cells were routinely grown in Trypticase soy broth/agar (17) and Luria broth/agar (18), respectively. The growth media were supplemented with the appropriate antibiotics as needed. Phage Φ11 and its growth conditions were as described previously (17).

**Molecular biological, biochemical and biophysical techniques**

Plasmid DNA isolation, digestion of DNA by restriction enzymes, ligation of DNA fragments, transformation, polymerase chain reaction (PCR), estimates of the amount of protein and DNA, native and SDS-PAGE, polyacrylamide gel staining, sequencing of PCR generated DNA using Φ11 DNA fragments as templates, analytical gel filtration chromatography, glutaraldehyde crosslinking, the recording of CD spectra, partial proteolysis, thermal aggregation and N-terminal end sequenc-
Fig. 2. Operator DNA-Φ11 Cro interaction. (A) Autoradiograms showing the DNase I footprints with the top (T) and bottom (B) strands of O DNA. The + and - indicated the DNase I-cleaved fragments of 32P labeled O DNA, generated in the presence and absence of Cro, respectively. G and G+A are markers generated from the labeled O DNA as previously published (26). Angled lines and open circles denote the DNase-I resistant regions and the DNase I hypersensitive sites, respectively. (B) The DNA sequence of the Φ11 cl-cro intergenic region. Angled lines, circles and angled arrows indicate the DNase I-protected regions, the DNase I hypersensitive sites and the start codons of Cl and Cro, respectively. The 15-bp inverted repeats (designated O1, O2 and O3) are in the boxed areas. The first base of the start codon of Cro was considered +1 and the whole DNA sequence was numbered according to that +1 designation. (C) Autoradiogram of the gel shift assay exhibiting the interaction of the said amounts of Cro to ∼0.5 nM 32P-labeled O' DNA. I denotes shifted complex. (D) Autoradiogram of gel shift assay showing the binding of indicated concentrations of Cro to ∼0.5 nM 32P labeled O3 DNA. (E) Equilibrium binding of Cro to O3 DNA. The amounts of O3 operator bound by Cro were estimated by scanning the autoradiogram shown in Panel D and plotting the densitometric data against the corresponding Cro concentrations (300-900 nM).

Cloning, overexpression and purification of Φ11 Cro
To clone the Φ11 cro gene, a 252 bp Φ11 DNA fragment [PCR amplified using genomic Φ11 DNA as template and primers PCR4 and PCR5 (Table 1)] was inserted into a pGEMT-Easy vector (Promega, U.S.A.) according to the manufacturer’s protocol. One of the recombinant pGEM-T-Easy vectors carrying Φ11 cro with no mutation (as confirmed by DNA sequence analysis) was selected and designated pSAU1176. The Φ11 DNA insert, pSAU1176, was subcloned into an E. coli pET28a (Novagen, U.S.A.) expression vector to construct pSAU1259. The subcloning included 37 extra amino acid residues including six histidine residues at the N-terminal end of the Cro. After transforming pSAU1259 into competent E. coli BL21 (DE3) cells (Novagen, U.S.A.), a healthy transformant was selected, purified and stocked at -70°C.

To induce the expression of Cro, 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to an E. coli (pSAU1259) cell culture in log phase. The culture was then grown for another 3 h at 32°C. The induced cells were harvested, washed with 0.9% NaCl and resuspended in a 1:100 volume of lysis Buffer A [20 mM Tris-chloride buffer (pH 8.0), 300 mM NaCl, 5% glycerol, 10 mM imidazole and 10 μg/ml PMSF (phenylmethane sulfonylfluoride)]. After disrupting the
cells, Cro was purified from the crude extract by Ni-NTA column chromatography according to the method described for Φ11 Cl (9). The affinity chromatography-purified Φ11 Cro migrated as expected on SDS-12% PAGE (data not shown) and bound to Φ11- DNA in vitro (see above). The protein was about 97% pure as was evident from scans of the band intensity on the PAGE gel. The overall yield approached 0.5 mg Cro per liter of induced E. coli cell culture. Prior to our experiments, Cro in Buffer A was extensively dialyzed against Buffer B [10 mM Tris-Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 5% glycerol and 100 μg/ml BSA], Buffer C (Buffer B minus BSA) or against a phosphate buffer [50 mM Na-phosphate buffer (pH 7.0), 200 mM NaCl, 1 mM EDTA, 5% glycerol]. The phosphate buffer did not affect the DNA binding activity of Cro (data not shown). The concentration of Cro was calculated using the molecular mass of monomeric Cro.

**Synthesis of 32P labeled DNA fragments**

The 269 bp EcoRI-BamHI DNA fragment (harboring O1, O2 and O3) of pSAU1201 DNA (9) was renamed O DNA. O' DNA (also carrying O1, O2 and O3) was synthesized by PCR using pSAU1201 DNA and primers pHC1 and pCI15 (Table 1). Similarly, O3 DNA was amplified using primers PCR15 and Illd (Table 1) and pSAU1201 DNA. The S. aureus cspC DNA was amplified using primers CSP4 and CSP6 (Table 1) and S. aureus Newman DNA (24). The DNA fragments described above were purified from agarose gels according to a standard procedure. Oligos PCR11 & PCR21 and Illa & Illb (Table 1) were annealed to prepare O1 and O2 DNA, respectively. All DNA fragments were labeled with either [α-32P] dATP or [α-32P] dATP using standard procedures (18).

To label the top strand of O DNA with 32P, oligonucleotide pHCl was end labeled with [α-32P] dATP prior to amplification of O DNA by Taq DNA polymerase using pSAU1201 DNA and pHCl and labeled pHCl oligonucleotides. The resulting DNA fragment was purified from agarose gels. Plasmid pSAU1201 was successively treated with EcoRI, Klenow polymerase and [α-32P] dATP, and BamHI. Bottom strand labeled O DNA was purified from agarose gels.

**Gel shift assay**

The gel shift assay was performed by a standard procedure (9) using 300-900 nM of Cro in Buffer B and ~0.5 nM of 32P labeled DNA. Separate gel shift assays were carried out to investigate the binding of Cro to each of O', O1, O2, O3 and cspC DNAs. The S. aureus cspC DNA harboring no Φ11 operator site was found to bind Cro only weakly at concentrations of 1,100 nM and higher (data not shown).

**DNase I footprinting assay**

DNase I footprinting was performed using a standard procedure with certain modifications as described below (25). Briefly, ~60 μM of a labeled DNA fragment (~5,000 cpm) was incubated with 500 μM of Cro in 50 μl Buffer C for 20 min on ice. The reaction mixture was made to 1 mM with MgCl2 and treated with 0.15 units of DNase I for 4 min at room temperature. The addition of 90 μl of Stop solution [200 mM NaCl, 80 mM EDTA (pH 8.0), 1% SDS, 0.03% Glycerogen] terminated the reaction. After cleaning the cleaved DNA fragments by phenol chloroform (1:1) extraction and ethanol precipitation, the cleaved DNA fragments were resuspended in sequencing gel buffer (98% deionized formamide, 10 mM EDTA (pH 8.0), 0.025% bromophenol blue). Each labeled DNA was treated with DNase I in the absence of Cro and the recovered DNA fragments were used as controls. Finally, both experimental and control DNA fragments were analyzed by urea-6% PAGE along with G and/or A+G sequencing ladders from identically labeled DNA fragments generated by standard procedures (26).

**Acknowledgements**

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**Table 1. Primers used in the study**

<table>
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<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Purpose</th>
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<tr>
<td>PCR4</td>
<td>AAGCTTGCATTGTTATGTCTCCC</td>
<td>Synthesis of Cro ORF</td>
</tr>
<tr>
<td>PCR5</td>
<td>GAATTCACATGCCAATGGGATTATAAAG</td>
<td>Synthesis of Cro ORF</td>
</tr>
<tr>
<td>pHCl</td>
<td>GCTCTAAATTCCTTCTGTAGTAC</td>
<td>Synthesis of O' DNA</td>
</tr>
<tr>
<td>PCR11</td>
<td>GACTCAGTACAGTATGCTATAGTTTAA</td>
<td>Synthesis of O1 DNA</td>
</tr>
<tr>
<td>PCR21</td>
<td>AACCTACTATACACGATACGGTTGAGTCA</td>
<td>Synthesis of O2 DNA</td>
</tr>
<tr>
<td>Illa</td>
<td>ATCCACAAAATACTACGAAAAAGCTTATGTGACTCAATGTA</td>
<td>Synthesis of O3 DNA</td>
</tr>
<tr>
<td>Ilb</td>
<td>TACCTAGTCAACACATAAAAAAGTTTGGTTCTCTGTGTATTTTTTTTGTTGAAT</td>
<td>Synthesis of O and O' DNAs</td>
</tr>
<tr>
<td>PC151</td>
<td>GAACTTGGCTAATCTTTTTTATC</td>
<td>Synthesis of O3 and O' DNAs</td>
</tr>
<tr>
<td>Illd</td>
<td>TTTTTTTTGATTACCAAAAAATAATGGTTATATAG</td>
<td>Synthesis of O3 DNA</td>
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<tr>
<td>CSP4</td>
<td>CATGCGATGGTAGAATAACGGTACAG</td>
<td>Synthesis of S. aureus cspC DNA</td>
</tr>
<tr>
<td>CSP6</td>
<td>CTGAGCATTTTAACTACGTTTGG</td>
<td>Synthesis of S. aureus cspC DNA</td>
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Note: Most DNA fragments synthesized here were also used in other study (unpublished data).
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REFERENCES


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