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Purification of bacteriophage lambda repressor

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Abstract

Bacteriophage lambda repressor controls the lysogeny/lytic growth switch after infection of *E. coli* by lambda phage. In order to study in detail the looping of DNA mediated by the protein, tag-free repressor and a loss-of-cooperativity mutant were expressed in *E.coli* and purified by (1) ammonium sulfate fractionation, (2) anion-exchange chromatography and (3) heparin affinity chromatography. This method employs more recently developed and readily available chromatography resins to produce highly pure protein in good yield. In tethered particle motion looping assays and atomic force microscopy “footprinting” assays, both the wild-type protein and a C-terminal His-tagged variant, purified using immobilized metal affinity chromatography, bound specifically to high affinity sites to mediate loop formation. In contrast the G147D loss-of-cooperativity mutant bound specifically but did not secure loops.

Introduction

Regulation of gene expression at the transcriptional level is a fundamental mechanism which is evolutionarily conserved from viruses to humans. It is becoming increasingly clear that large, cooperative assemblies of proteins that wrap and/or loop genomic DNA may operate epigenetically to shift configurational equilibria to transcriptionally determine developmental pathways (1). Such is the case of the repressor protein (CI) encoded by bacteriophage lambda (λ) that infects *Escherichia coli* and, probably, of most temperate bacteriophages which may adopt either quiescent (lysogenic) or virulent (lytic) growth. CI controls the expression of viral genes as part of the lysogenic/lytic growth switch. In lysogeny, CI prevents lytic growth by directly repressing promoters P_L and P_R which encode proteins involved in lysis (2, 3). These promoters are associated with two triplets of DNA binding sites (operators), *OL1*, *OL2*, *OL3* and *OR1*, *OR2*, *OR3*, to which CI dimers bind cooperatively (4, 5). CI regulates its own synthesis by activating and repressing its own promoter, P_{RM} . At low CI concentrations, CI enhances transcription at P_{RM} , but at high CI concentrations it represses P_{RM} (2, 6, 7). A model incorporating data from biochemical, biophysical, and genetic experiments shows that CI participates in long-range interactions between the widely separated operator triplets by generating a loop in the intervening DNA (4, 8). It has been shown that DNA loops generated by oligomerization of CI bound to

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its operators influence the regulation of P_{RM} (2, 9). This epigenetic switch is not only the basis of our understanding of λ phage lysogeny (10), but is a paradigm for transcriptional regulation.

The CI repressor protein consists of two domains connected by a linker: an N-terminal DNA-binding domain, residues 1–92, that mediates interactions with its specific operators and RNA polymerase, and a C-terminal protein-protein interaction domain, residues 132–236. The C-terminal domain of the repressor mediates dimerization as well as a dimer–dimer interaction that results in the cooperative binding of two repressor dimers to adjacent operators (11). The interactions that mediate this cooperativity were revealed in x-ray crystallography studies in which two dimers were found to associate about a 2-fold axis of symmetry (12). *In vitro* transcription assays showed that P_{RM} repression was not observed in the presence of two point-mutant CI proteins (G147D or D197G) that bind to operators as dimers but fail to interact further cooperatively, suggesting that interactions among C-terminal domains of operator-bound CI dimers are critical for proper transcriptional regulation (2, 13–15).

The CI repressor of bacteriophage λ is a classic example of a cooperative protein assembly that regulates transcription. In order to be able to study CI in more detail, wild-type and the G147D mutant CI were expressed in *E. coli* and purified in a two or three step procedure consisting of ammonium sulfate fractionation, anion-exchange chromatography, and finally heparin affinity chromatography. This method employs more recently developed and readily available chromatography resins to produce highly pure protein in good yield. Proteins produced in this way were compared to C-terminally His-tagged CI protein purified from *E. coli* using immobilized metal affinity chromatography (IMAC). Pull-down, tethered particle motion, and atomic force microscopy assays were used to demonstrate the DNA binding and looping activity of the purified CI proteins.

Material and Methods

XL1-blue competent cells were purchased from Stratagene (Santa Clara, CA). 1ml, pre-packed HiTrap Q HP, HiTrap Heparin HP and HisTrap HP columns were purchased from GE Healthcare Life Science (Piscataway, NJ). Streptavidin-coated, paramagnetic microspheres (Dynabeads MyOne™ Streptavidin C1) were purchased from Invitrogen (Grand Island, NY). SPHERO™ Streptavidin-coated polystyrene microspheres were purchased from Spherotech (Lake Forest, IL). Plasmid pEA305 containing the coding sequence for λ - repressor CI was provided by Dr. Krishnananda Chattopadhyay (Indian Institute of Chemical Biology). The G147D CI point mutant was created from plasmid pEA305 using the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix, Santa Clara, CA) and cloned in strain DH5 α (Invitrogen, Grand Island, NY). Plasmid pT7 λ CI His6 (16) was a gift from Prof. Ann Hochschild (Harvard Medical School). The 735 bp DNA fragments for the pull down assays were obtained by PCR on plasmid template pDL300 (17) using an unlabeled forward (5'ggagacccacactaccat or 5' ggattctgcccttcttcagg) and a biotin-labeled reverse (5'bio-tatgcccgagaagatgtt) primer. The DNA fragment for tethered particle motion assays were obtained by PCR using a biotin-labeled forward and a digoxigenin-labeled reverse primer on plasmid template pDL2317 (18).

Expression and Purification of CI protein without His-tags

Plasmid pEA305 encoding wild-type CI or the G147D point mutant were transformed into XL1-blue competent cells, plated, and incubated overnight at 37°C on LB medium containing 100 μ g/ml ampicillin. One liter of LB medium with 100 μ g/ml ampicillin was inoculated with a colony from the overnight culture. CI expression was induced with 1 mM of isopropyl- β -1-thiogalactopyranoside (IPTG) once the optical density of the cell culture at

595 nm reached 0.5. After shaking for 3 hrs and 6 hrs at 37°C, the cells were harvested by centrifugation at 4721g. 50 ml of lysis buffer (100 mM Tris-HCl pH 8.0, 1 mM EDTA, 2 mM CaCl₂, 10 mM MgCl₂, 200 mM KCl, 5% glycerol, 0.1 mM βME) and 1 mM (final concentration) PMSF were added and then cells were lysed by sonication [15 watts power, 35 cycles, 20 sec pulses with 30 sec intervals]. The lysate was centrifuged at 23,631g for 50 mins at 4°C and the supernatant was collected. Expression of CI in the soluble fraction was confirmed using SDS-PAGE analysis and coomassie blue staining.

Ammonium sulfate was added to the supernatant (40 g/100 ml) and dissolved under constant stirring at 0°C for 1 hrs to give a 60 % saturated solution. The pH was maintained at 8.0 by adding 3N NaOH. The solution was centrifuged at 27,734g for 50 mins at 4°C. The whole pellet was resuspended and dialyzed at 0°C with two changes of 1000 ml of the standard buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 2 mM CaCl₂, 5% glycerol, 0.1 mM βME) during 48 hr. The dialyzed protein was centrifuged at 12,000 rpm for 20 mins, 4°C. The supernatant was collected and purified by ion exchange on a 1 ml HiTrap Q fast flow column.

The HiTrap Q column was equilibrated with 10–20 ml of standard buffer and then the supernatant was loaded onto the column. After washing the column with 10 ml standard buffer, the CI protein was eluted in about 22 ml of standard buffer with a linear potassium salt gradient from 50 to 600 mM KCl. Fractions were assayed for the protein of interest using 12% SDS-PAGE followed by coomassie blue staining (Figure 3).

The CI-containing fractions from the ion exchange chromatography (Figure 2 fractions 22 and 23) were pooled and further purified by affinity chromatography on a 1 ml Heparin fast flow column. The heparin column was equilibrated with 10–20 ml of standard buffer and then the CI protein-containing fraction was loaded onto the column. After washing the column with 10 ml standard buffer, the CI protein was eluted in about 15 ml standard buffer with a potassium salt gradient from 50 to 600 mM KCl. Fractions were assayed for the protein of interest using 12% SDS-PAGE followed by coomassie blue staining.

CI containing fractions eluted from the Heparin column (Figure 2, fractions 19 and 20) were pooled and transferred to a dialysis bag (10,000 MW cut off) and dialyzed at 4°C with two changes of 500 ml of the standard buffer during 24 hr.

Expression and purification of his-tagged CI protein

E coli strain BL21 plysS carrying the pT7 CI His6 construct was grown overnight in LB medium containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol at 37°C. The next day, 2.5ml of this culture was used to inoculate 500mL of the same media, which was grown with shaking (175–200 rpm) at 37°C. Once the optical density at 600nm had reached 0.55, the culture was induced with IPTG to a final concentration of 0.5 mM, and growth continued for a further 3 hours at 37°C. Cells were harvested by centrifugation (5000 rpm, 10 min, 4°C), washed once with 50 mM Tris-HCl, 0.1 mM EDTA, 150 mM NaCl, 10% glycerol, pH 7.5 (TEG150) and the cell pellet stored at –20°C until use.

For protein purification, the cell pellet was resuspended in 20mL of buffer A (20 mM sodium phosphate, pH 7.2, 500 mM NaCl) which also contained 20mM imidazole. The resuspended cell pellet was sonicated (Branson sonifier) on ice (4 × 10 pulses, 50% duty cycle). Cell debris was removed by centrifugation (12 000g, 30 min, 4°C). The supernatant was filtered through a 0.45 µm filter and loaded onto a freshly charged 5 ml His Trap HP column using a disposable 10 mL syringe.

The column was then washed with at least 12 column volumes of buffer A containing 20mM imidazole, followed by 12 column volumes of buffer A containing 100mM imidazole, and the lambdaCI protein eluted with two column volumes of buffer A containing 500 mM imidazole.

CI-containing fractions were pooled and dialysed at 4°C against 3 × 500ml of TEG150 over 16hrs. Final protein concentration was measured by absorbance at 280nm, using an extinction coefficient for lambda CIHis6 of 26390 M⁻¹ cm⁻¹. Purity of the final protein was examined by SDS PAGE on 12% pre-cast gels (Invitrogen) with MOPS running buffer. Typical yield was ~20 mg per liter of culture. Protein was stored at -80°C.

Tethered particle motion assays

Details of the TPM technique have been published previously (19–21). One end of a DNA fragment is tethered to a glass surface and the other end is attached to a microscopic bead which can be seen under an optical microscope. The Brownian motion of this bead is recorded by using a CCD camera and serves as a reporter of the DNA length. Thus if the DNA undergoes a conformational change, such as CI-mediated loop formation, which reduces the effective tether length, the Brownian excursions of the bead will diminish(18). The TPM measurements were performed on DNA derived from plasmids pDL2317, which contains the complete CI regulatory region. The motion of DNA-tethered beads was monitored in the presence of CI concentrations varying from 0 to 100 nM. Ten to fifteen tethered beads were tracked in each microchamber in λ buffer (10 mM Tris-HCl pH 7.4, 200 mM KCl, 5% DMSO, 0.1 mM EDTA, 0.2 mM DTT and 0.1 mg/ml casein) for 10–20 min. The procedure was repeated in order to monitor the motion of 30–40 DNA-tethered beads for each CI concentration. The *p* values obtained in the control experiments (no CI) and those corresponding to the looped state in the presence of CI were as expected from a calibration curve assembled with the same-sized beads attached to DNA fragments of various lengths.

Atomic force microscopy

1555 bp DNA fragments were produced by PCR amplification of a segment of plasmid pDL944 using 5'-CGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCAGGC-3' and 5'-GCATTGCTTATCAATTTGTTGCAACGAACAGGTCCTATCAGTC-3' as forward and reverse primers. This fragment contained wild-type lambda operator regions (OL and OR) and including the associated promoters PL, PRM and PR. The distance between the midpoints of operator sites OL3 and OR3 was 393 bp. Wild-type CI, CI-His, or G147D CI protein were diluted to the desired final concentration (100 or 200 nM dimer) in the presence of 1 nM DNA in a buffer containing 50 mM HEPES, 150 mM NaCl and 0.1 mM EDTA (pH 7.0). All steps were conducted at room temperature. The mixture was incubated for 20 min. Shortly before deposition, a 10 µl drop of 0.01 µg/ml poly-L-ornithine (1 kDa MW, Sigma-Aldrich, St. Louis, MO) was incubated on freshly cleaved mica for one minute. The poly-L-ornithine-coated mica was then washed with 0.4 ml HPLC water and dried with compressed air. Then, 10 µl of the solution containing DNA and protein were deposited on the poly-L-ornithine-coated mica and incubated for one minute. The droplet was rinsed with 0.4 ml HPLC water and dried gently with compressed air. The sample was left to dry overnight before imaging.

Images were acquired with a NanoScope IIIa MultiMode AFM microscope (Digital Instrument, Santa Barbara, CA) operated in tapping mode using uncoated, etched silicon tips (NSC18, MirkoMasch, San Jose, CA). The oscillation amplitude was 50–60 mV with a resonance frequency of 75 kHz. Areas of 1×1 µm² were scanned at a rate of 1.2 Hz and with

a resolution of 512×512 pixels using the automated scan function of the version 5.30r3sr3 NanoScope control software to collect approximately 100 images during 24 hours.

After filtering images to remove scan line offsets and bowing, DNA molecules were interactively traced with NeuronJ (22), a plug-in function for ImageJ (23). As previously reported, traced lengths were interpreted using 0.32 nm/bp with a measurement error of 2.2% (24).

Results

Expression and purification of CI without His-tags

The amount of CI protein produced as a function of time was investigated by SDS-PAGE analysis in order to optimize expression conditions. The amount of CI expressed at 37 °C 3 hours or 6 hours after induction with IPTG are shown in Fig. 1. The amount of CI was similar for 3 hours and 6 hours, so 3 hours was selected as an optimal expression time.

Recombinant CI in crude extracts was fractionated with ammonium sulfate before ion exchange chromatography. The precipitated protein was recovered by centrifugation and the pellet was dissolved in fresh buffer. Then, the partially purified CI was loaded on an anion-exchange (HiTrap Q) column and eluted in a linear gradient of KCl (Fig.2A). SDS-PAGE showed that some other proteins were present in the elution fraction (Fig.3A), so an additional step of purification was needed.

Since CI binds to DNA, a second fractionation on a heparin column was performed. Many DNA-binding proteins will bind to heparin which is a negatively charged, highly sulfated glycosaminoglycan. Thus the CI protein-containing fractions from the ion exchange chromatography were pooled and further purified by affinity chromatography on a Heparin fast flow column and elution in a linear gradient of KCl (Fig.2B). Following this step, an SDS-PAGE assay showed a single band migrating at 26 kDa, the expected MW of CI (Fig. 3A). After buffer exchange by dialysis, about 5 mg purified protein, estimated to be at least 95% pure, was obtained from a 1 L bacterial culture.

Expression and purification of His-tagged CI

C-terminally His-tagged CI expressed well following 3 hrs of induction with IPTG (Figure 3B). His-tagged CI was loaded onto the Ni-NTA column in the presence of 20 mM imidazole, and contaminating proteins were efficiently removed with wash buffer containing 100 mM imidazole. Use of a wash buffer containing 150 mM imidazole resulted in significant loss of CI from the column. CI was eluted with buffer containing 500 mM imidazole and buffer exchanged into storage buffer. Purity was estimated as at least 95%, and about 20 mg of CI-His was obtained per liter of culture.

Tethered particle motion assays

Lysogenic infection of *E. coli* by bacteriophage λ is extremely stable and spontaneously switches to lysis only once in 10,000 bacterial cell divisions. The lysogenic transcriptional state is maintained by CI, which represses lytic functions and regulates its own synthesis. At low cellular concentrations, CI enhances its own transcription from PRM; when high, CI represses PRM. The mechanistic details of regulation are based on electron and atomic force microscopy and genetic experiments showing that a CI tetramer cooperatively bound to OR1-OR2 interacts with a tetramer cooperatively bound to OL1-OL2 creating a loop in the intervening DNA (1, 4, 18, 25). Also genetic experiments indicate that transcriptional repression at PRM occurs when an OR1-OR2/OL1-OL2 loop with a CI dimer bound to OL3 stabilizes a CI dimer bind at OR3 through a cooperative interaction (4).

To investigate the loop-forming activity of our purified CI protein, tethered particle motion experiments were performed, and CI-mediated looping of single DNA molecules containing λ repressor binding sites was quantitatively analyzed. A 3500 bp DNA fragment containing both triplets of operators separated by the wild-type distance was produced by PCR. This DNA fragment was labeled by biotin on one end and digoxigenin on the other end in order to link one end of the DNA to glass coverslips and tether polystyrene microspheres at the other end. The motion of DNA-tethered beads was monitored in the presence of CI concentrations varying from 0 to 100 nM. In the absence of CI protein the range of XY excursions, $\langle \rho \rangle$ was constant in a narrow range (Fig. 4A). In the presence of CI, excursions transiently fell to a lower level that corresponded to the effective length of the looped DNA, determined from a calibration curve (19) (Figure S1). The frequency of the loop state increased and the probability of unloop state decreased as CI concentrations were increased from 25 to 100 nM (Fig. 4A). The fraction time in the looped conformation versus CI concentration was calculated from histograms including all measurements for a given condition and is reported in Fig. 4B. At the higher concentration of CI, looping efficiency was very high, reaching nearly 80%. These looping frequencies were very similar to those obtained in reference 17, which used CI purified by an older method (26).

Atomic force microscopy

Atomic force microscopy has been used to image DNA molecules containing the *OL* and *OR* operator sites separated by 398 base pairs (1). However, only one concentration of wild-type repressor was employed previously, and therefore new experiments aimed at detecting the amount of looping as a function of repressor concentration were undertaken. DNA was incubated for ten minutes with wild-type, His-tagged, or G147D mutant CI protein and a 10 μ l droplet was deposited on poly-L-ornithine-coated mica. The surface was imaged after rinsing with 0.4 ml of NanoPure water and drying with a gentle stream of gas from an aerosol duster. One to two hundred molecules in which the entire contour of the DNA could be traced were analyzed for each sample (Figure 5A). At 100 nM, the wild-type protein promoted looping in 14.5% of the molecules (Table 1). This percentage rose to 38.5% when the concentration of repressor was 200 nM. Similar values were tabulated for the His-tagged CI, 16 and 42% for 100 and 200 nM respectively. Furthermore, since the proteins divided the DNA fragments into segments that corresponded exactly to those expected based on the known positions of the operators in the DNA fragment (Figure 5B, Supplementary figure S2), only specific binding was observed. The same was true for the CI G147D loss-of-cooperativity mutant which was observed exclusively positioned at operators. However, the mutant protein did not secure any loops at all in identical DNA molecules at either concentration (Figure 5A; Table 1). At the 200 mM concentrations used in the AFM footprinting assay, all three proteins exhibited only specific binding.

Discussion

Many protein-purification schemes rely on affinity tags appended to proteins to allow efficient separation from crude biological homogenates. These include chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST) (27, 28). The poly (His) tag is also a widely-used protein tag that binds to metal matrices (29). However, highly charged tags, such as His₆, can influence the biochemical activity and specificity of proteins (30). In this work, an efficient strategy was developed to efficiently produce tag-free, full length CI protein. Since individual proteins often exhibit unique combinations of affinity and ion exchange equilibria, even quite similar proteins may be separated on these principles (31). In the case of the CI protein, ion exchange on Q sepharose followed by heparin affinity chromatography was selected for the purification from over-expressing clones.

Before chromatography, ammonium sulfate precipitation was used to eliminate a broad range of more soluble, poorly precipitating proteins. The precipitated proteins including CI were isolated by centrifugation and resuspended in fresh buffer for the next stage of purification.

Different estimates of the isoelectric point of the CI protein indicated a value around 5.0. Therefore anion-exchange chromatography was used to remove negatively charged proteins from the cell extract. The eluent from ion exchange was then applied to a heparin column. Heparin interacts with proteins either as a ligand with specific affinity or electrostatically with cations due to its high content of anionic sulfate groups (32). Since it mimics the polyanionic structure of the nucleic acid, it was expected to interact strongly with CI which binds nucleic acids. After a final dialysis, about 5 mg of purified protein was obtained from a 1 L bacterial culture. In pull-down assays the purified CI protein exhibited affinity for DNA (supplementary information), and in tethered particle motion assays it mediated loops between specific regulatory elements with activity similar to that reported previously (18) and was shown to bind specifically to operator sequences by AFM footprinting. In that assay, the wild-type and his-tagged lambda repressor proteins produced the same amount of looping. While the His-tagged protein has also been shown to be active in *in vitro* transcription assays (16), further investigation is necessary to determine whether the His-tagged protein behaves identically to the wild-type protein in the context of a lambda phage.

Conclusion

The purification of bacteriophage lambda CI repressor expressed in *E.coli* bacterial cells was carried out in three steps: (i) ammonium sulfate fractionation, (ii) anion-exchange chromatography, and (iii) heparin affinity chromatography. This simple protocol produced unmodified CI of high purity that was active in pull down and tethered particle motion assays and bound specifically to operators as shown by atomic force microscopy footprinting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The purification of lambda repressor using recently developed chromatography media is described.
- Ion exchange followed by heparin affinity was used to obtain lambda repressor in high purity.
- Lambda repressor looping and binding specificity was assayed using single molecule techniques.
- Lambda repressor looping and binding specificity was similar with or without a C-terminal His-tag.

Fig. 1. CI expression. Protein expression from XL1-blue cells after mock induction (–) or induction (+) with 1 mM IPTG for 3 or 6 hours was analyzed by Coomassie blue staining following SDS-PAGE. 30–50 µg total protein was loaded in each lane.

Fig. 2. Purification of wild-type CI. Elution profiles for chromatography with ion exchange and heparin affinity resins are shown. (A) An elution profile (blue) from the HiTrap Q ion exchange column is shown in which KCl concentration (yellow) increases from 50 to 600 mM. (B) An elution profile (blue) from the Heparin fast flow column is shown in with the same KCl gradient.

Fig. 3. SDS-PAGE analysis of CI purification. (A) Wild-type CI Lane 1: homogenate supernatant, Lane 2: resuspended ammonium sulfate fraction, Lane 3 and 4: eluted fractions 22 and 23 (see Figure 2) from the HiTrap Q column, Lane 5 and 6: eluted fractions 19 and 20 (see Figure 2) from the Heparin fast flow column. (B) His tagged CI Lane 1: homogenate supernatant, Lane 2, flow through from HisTrap HP column, Lane 3 pooled and dialyzed elution fractions from HiTrap HP column.

Fig. 4.

Single molecule assay of CI activity. The fraction of looping of DNA tethers with 2317 bp between OL and OR was measured as a function of CI dimer concentration (12, 25, 50, 100 nM). (A) Histograms of the average XY excursion of microspheres were assembled from real-time analysis of video for 30–40 tethered beads (300–400 total minutes). (B) Estimates of the fraction of looping observed for the whole ensemble of beads were calculated by integrating the area in the histograms above and below a user determined threshold.

Fig. 5. Atomic force microscopy of CI-mediated DNA looping. DNA molecules in which *OL* and *OR* were separated by 398 bp were imaged after incubation with either 100 or 200 nM concentrations of wild-type, His-tagged, or G147D mutant CI repressor. (A) Loops formed using either the wild-type or the His-tagged CI repressor but not with the G147D mutant (see Table 1 also). (B) At 200 nM concentrations, all proteins bound specifically to operator sequences judging by their positions along the DNA fragment (see supplementary Figure S2).

Table 1

Percentages of looped molecules observed using AFM. DNA containing the *OL* and *OR* operator regions separated by 398 base pairs was incubated with the indicated concentrations of the wild-type, His-tagged, or G147D loss-of-cooperativity mutant CI proteins. The percentage of looped molecules found among the number of molecules examined (enclosed in parentheses) is listed.

[protein] (nM)	CI	His-CI	G147D
100 nM	14.5% (278)	16% (200)	0% (174)
200 nM	38.2% (123)	42% (107)	0% (156)