Chapter 2

Changes in functional activity of Cro repressor mediated by various ions

2.1. **Introduction**

Temperate bacteriophages can adopt the lytic as well as the lysogenic mode of development based on the interaction of the phage encoded CI protein and the Cro protein with their cognate operator DNA sequences (Ptashne 1980; Susskind & Youderian 1983; Ptashne 1986; Ogawa et al 1988; Heinrich et al 1995; Kimsey & Waldor 2004; Koudelka et al 2004; Dodd et al 2005; Hatfull & Jacobs 2014). Repressor and Cro proteins are expressed by the temperate lambdoid phages during the early stages of infections. While repressor is involved in switching off the lytic pathway, Cro is involved in switching off the lysogenic pathway of temperate bacteriophages. Both Cro and CI repressor proteins of coliphage λ had been studied extensively at biochemical, biophysical, genetic and structural levels (Ptashne 1986). The *cI* and *cro* genes are particularly important in giving us a good insight about the gene regulation in the host bacteria as well as the infecting bacteriophages. Moreover, these regulatory proteins are also extensively utilized for construction of various expression vectors.

Bacteriophages of *S. aureus* include Phi11, phi 12, phi13, L45a, phi42 and phiSa3ms and it has been observed that most of these phages carry the *cI* and *cro* genes in divergent orientation in their genome, akin to that of lambdoid phages (Ptashne 1986). Phi11 is a member of serogroup B (Bachi 1980). The genome of Phi11 is a double stranded, linear DNA with a G+C content of 35-37% (Brown et al 1972). Additionally, the genome is circularly permuted (40% or more) (Kretschmer & Egan 1975; Lofdahl et al 1981) and is 5% (Lofdahl et al 1981) to 25% (Bachi 1980) terminally redundant.

Phi11 Cro is a 72 amino acid polypeptide and is 20-40% identical with Cro-like proteins of phages Tuc2009 (Kenny et al 2006), A2 (Ladero et al 2002) and so on. It is a moderately thermostable, single domain protein and binds to a single operator DNA (designated as *O*3) located in the *cI-cro* intergenic region (Das et al 2009).

The interaction of different regulatory proteins, such as lac repressor, bacteriophage repressor and Cro proteins, with their cognate operator DNA can be largely affected by different parameters such as ions, pH, temperature and so on (Mandal & Lieb 1976; Barkley et al 1981; Koblan & Ackers 1991; Relan et al 1997; Alberty & Bock 1953). Salts, in general, can play a very important role in influencing the biologically active conformation of proteins. The effects exerted by different salts upon different proteins can vary. Some salts might lead to aggregation, denaturation or unfolding of the protein and some might lead to minute changes in the domains of a multidomain protein. In

both cases the functional activity of the protein may or may not be affected (Bandhu et al 2009). However, there are a few ions such as magnesium cations which can stabilize double stranded DNA and help it to adopt its biologically active secondary and tertiary structures (Misra & Draper 1998). In fact, there are reports which state that stabilization of native DNA at room temperature require 100 times more concentration of NaCI as compared to $MgCl_2$ (Thomas 1954). It has also been reported that melting temperature of duplex DNA increases with increasing concentration of magnesium ions. A possible explanation for this observation could be that DNA unwinding is induced by the negatively charged phosphate groups which repel each other. The presence of counter ions like magnesium reduces this unwinding (Butzow & Eichhorn 1965). Whether such cations interfere with interaction of DNA binding proteins is yet to be established. The importance of ions upon repressor–operator interaction has already been reported for mycobacteriophage L1 (Bandhu et al 2009). Phi11 Cro repressor plays a very important role in the lytic regulation of Phi11; however, it has a very low affinity towards its cognate operator (*O* DNA, especially *O*3), as compared to lysogenic repressor CI (Das et al 2009). In spite of the importance of ions upon protein-DNA interaction, there are no reports regarding the effect exerted by different ions on the interaction of Phi11 Cro protein with its cognate operator DNA. In this regard, this chapter investigates the effect of different cations and anions on the structure and function of Phi11 Cro with special emphasis on its operator binding capacity. The results indicate that NH_4^+ and $C_2H_3O_2^-$ ions are required for optimal binding of Cro to its cognate operator DNA.

2.2. **Materials and Methods**

2.2.1. **Bacterial strains and growth conditions**

E. coli BL21 (λDE3) and *E. coli* XL1 Blue cells were grown at 37ºC in Luria broth (Sambrook & Russell 2001). The growth media were supplemented with kanamycin as per requirement. The *E. coli* strain BL21 (λDE3) harbouring recombinant *cro* was obtained from Prof. S. Sau (Bose Institute, India). The salts were purchased from Sigma, USA and SYBR Green II Stain-10,000X concentrate was purchased from ThermoFisher Scientific.

2.2.2. **Over-expression and purification of recombinant Cro**

Recombinant histidine tagged Cro was purified by Ni-NTA column using gravity flow technique as described previously (Das 2009). Briefly, an overnight primary culture of the *E. coli* strain BL21 (λDE3) harbouring recombinant *cro,* was grown in LB medium containing kanamycin (50µg/ml) by incubating in an incubator shaker at 37°C, 120 rpm. 10 ml of this primary culture was used to inoculate 500ml of LB medium containing kanamycin (50µg/ml) in two 1 litre conical flasks. Cells were allowed to grow at 37°C with shaking (at 120rpm) until an OD_{600} ~0.4-0.5 was reached. The culture was then transferred to a shaking incubator at 32°C and shaken at 120 rpm. After a 20 min cool-down period, IPTG was added to a final concentration of 0.5 mM. The induced cells were further allowed to grow for 3 h at 32°C and 140 rpm following which they were harvested by centrifugation (10 min, 6,500 rpm at 4°C). The cell pellet was washed with 0.9% NaCl and was stored at -80°C until use.

For purification of the recombinant Cro, the cell pellet was thawed and resuspended in 10 ml of ice-cold lysis buffer (Lysis buffer: 20 mM Tris-chloride buffer pH 8, 300 mM NaCl, 5% glycerol, 10 mM imidazole and 10 µg/ml of PMSF). The suspension was then lysed by sonication (Misonix Microson Sonifier Misonix XL2000 Sonicator System Ultrasonic Cell Disrupter) at 10 watts (RMS) for 12 min (Repeated cycles of 20 seconds with 2min intervals) at 4°C.Finally, the sonicated extract was centrifuged (45 min, 12,000rpm, 4°C) and the supernatant (cytoplasmic fraction) was separated from the pellet fraction. The recombinant Cro was purified from the supernantant fraction by Ni-NTA column chromatography according to manufacturer's protocol (ThermoFisher Scientific). The supernatant obtained after centrifugation was loaded onto Ni-NTA resin (pre-equilibrated with ice cold lysis buffer). The supernatant was allowed to pass through the column slowly to give adequate time for 6X histidine tagged Cro to bind to column material. This binding process was repeated thrice. After three rounds of binding, the flow-through was collected and stored at 4°C. The protein bound column was washed using 20 bead volumes of wash buffer (Wash buffer: 20mM Tris pH 8, 300mM NaCl, 25mM imidazole and 5% glycerol). Finally, the hexa His-tagged Cro was eluted

from the column using elution buffer (Elution buffer: 20mM Tris pH 8, 100mM NaCl, 300mM imidazole and 5% glycerol). The elution fractions were pulled together and kept for dialysis against dialysis buffer (Dialysis buffer: 20mM Tris pH 8, 200mM NaCl, 0mM imidazole, 5% glycerol) overnight at 4°C, to allow complete removal of imidazole. The dialyzed protein was stored at 4°C until further use.

2.2.3. **Qualitative estimation of the purified Cro using Tris-Glycine polyacrylamide gel electrophoresis (Tris-Glycine SDS-12% PAGE)**

The purified protein fractions were analyzed on Tris-Glycine SDS-12% PAGE (Sambrook & Russell 2001). The resolving gel (SDS-12% PAGE) was prepared by adding and mixing the different gel components (Table 2.1.) and the resultant solution was poured between the glass plates separated by spacers and allowed to polymerize. After allowing sufficient time for the resolving gel to polymerize, the 5% stacking gel mix (Table 2.1.) was prepared and added above the resolving gel with the insertion of a comb to form the lanes. Gel electrophoresis was carried out in presence of TGS buffer (5X TGS; tris-base 7.55gm, glycine 47 gm, 10% SDS 25ml and final volume 500ml by addition of water) initially at 90 Volts (V) and then at 120 V using 1X TGS running buffer till the optimal resolution was achieved.

Table 2.1. SDS poly-acrylamide gel percentage.

Components	**12% Resolving Gel[a]**	**15% Resolving Gel[a]**
H_2O	3.3ml	2.3ml
30% acrylamide	4ml	5ml
1.5M Tris HCl (pH 8.8)	2.5ml	2.5ml
10% SDS	0.1ml	0.1ml
10% APS	0.1ml	0.1ml
TEMED	0.012ml	0.012ml

Components	**5% Stacking Gel[b]**
H_2O	3.4ml
30% acrylamide	0.83ml
1M Tris HCl (pH 6.8)	0.63ml
10% SDS	0.05ml
10% APS	0.05ml
TEMED	0.005ml
[a] For the final 10ml. [b] For the final 5ml.	

2.2.4. **Staining of polyacrylamide gel using Coomassie Brilliant Blue-R250(CBB-R250)**

To stain the gel for visualising the protein bands, CBB-R250 has been used. The gel was taken out from the gel plates and washed with double distilled water. To visualize the protein bands in the gel, the gel was stained with CBB-R250 as described below (Ausubel et al 1998). Briefly, the washed polyacrylamide gel was soaked in freshly made CBB-R250 solution (Final volume 100ml: 40ml methanol, 10ml glacial acetic acid, 0.5gm CBB-R250, 50ml double distilled water) with rocking. After 20-30 min at room temperature, the staining solution was replaced with the destaining solution (given in appendix A). The gel was destained (at room temperature, on a rocker) until the gel background was clear and the protein bands were visible.

2.2.5. **Quantitative estimation of the purified Cro using Bradford method**

Bradford method was used for quantitative estimation of the purified protein (Bradford 1976). A 5X concentrated Bradford reagent (given in appendix A) was prepared and this preparation was kept at 4°C. To measure the concentration of a desired protein, the 5X Bradford reagent was diluted 1X with sterile double distilled water. Then a certain volume of the desired protein was added to the 1X Bradford reagent to make a final volume of 1 ml and the suspension was gently mixed. The mixture was allowed to stand at room temperature for 5 minutes till it turned blue in color. The intensity of the blue color was measured in a spectrophotometer at 595nm against an appropriate blank (1X Bradford reagent and sterile double distilled water). Initially a standard curve was constructed by using known concentrations of BSA (Bovine Serum Albumin). The unknown protein concentration was finally determined from the known standard curve. The molar concentration of Cro was calculated considering the theoretical molecular weight of Cro protein polymer from EMBOSS Pepstats (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/).

2.2.6. **Gel shift assay and K_D (Apparent equilibrium dissociation constant) determination by image analysis**

Gel Shift Assay was carried out by standard procedure (Das et al 2007) in which various concentrations of the purified Cro protein were incubated with 90ng of *O* DNA which harbors *O*3 region (Figure 2.1.), in a binding buffer (10 mM Tris-Cl [pH 8.0], 200 mM NaCl, 5% glycerol) as described previously (Das et al 2009). The effect of the various cations and anions on Cro-*O* DNA interactions was investigated by replacing the 200mM NaCl in dialysis buffer with 200mM of KCl, LiCl, NH_4Cl, $MgCl_2$, Na_2CO_3, $C_2H_3NaO_2$ or $Na_3C_6H_5O_7$. The pH values of all the buffers were checked and adjusted to pH 8.0, before use. The purified Cro protein was thoroughly dialyzed against each of the above buffers and used for binding reactions and CD-spectra analysis.

To carry out gel shift assay, a 6% non-denaturing PAGE was used. Individual reaction mixtures were loaded onto different lanes of the polymerized 6% non-denaturing PAGE. Electrophoresis was carried out in cold for 5h at 80 V. Finally, the gel was stained with SYBR Green II Stain-10,000X concentrate in DMSO (ThermoFisher Scientific) and the stained native gel pictures were captured by E-Gel® Imager System with UV Light Base used SYBR® Filter (Invitrogen, Life Technologies).

The gel pictures were analyzed by ImageJ version 1.51d (Schneider, C.A., et al 2012). Plot profile function was used to determine the percentage of Cro-operator bound complex and graphs were drawn using sigmoidal fitting function of OriginPro 2016 Version b9.3.2.303 (Academic). The fitting curves had R-square value above 0.9. The K_D values were calculated from the graphs. Each experiment has been repeated thrice and the standard deviation values have been presented in the figures.

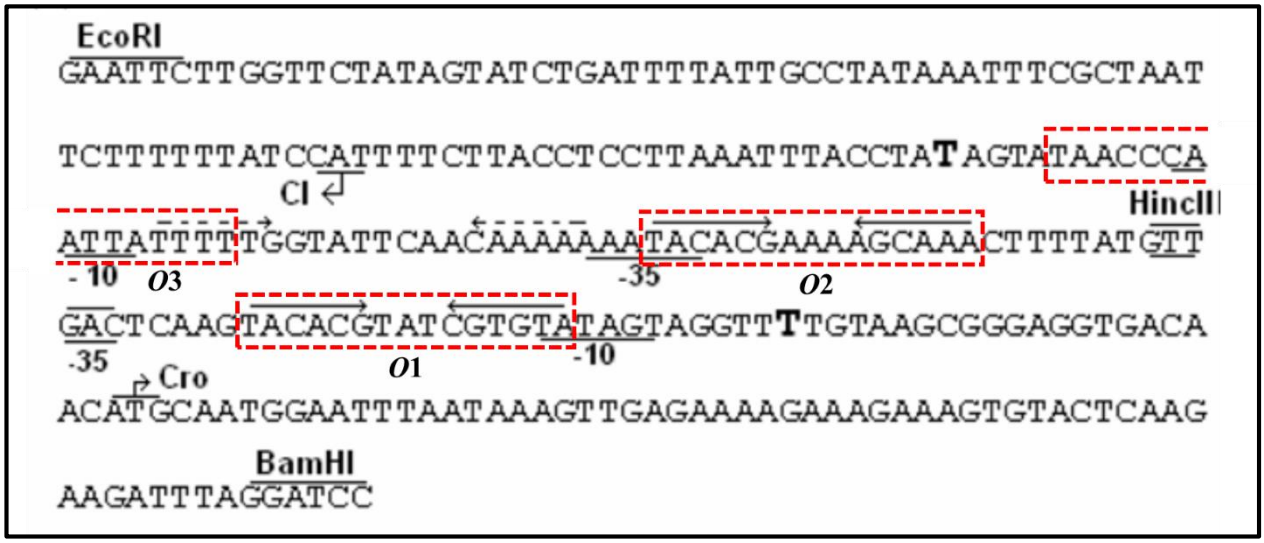

Figure 2.1. || Sequence of *cI-cro* intergenic region (designated as *O* DNA) of Phi11. The 15bp CI binding sites *O*1 and *O*2 are shown in red box. Cro binding site *O*3 (15bp) is shown in red box. Figure courtesy: (Das et al 2007).

2.2.7. **Secondary structure determination of Cro in presence of various ions by employing circular dichroism spectroscopy**

To investigate the effect of the various cations and anions on Cro-operator interactions, the 200mM NaCl in the binding buffer was replaced by 200mM of KCl, LiCl, NH_4Cl, $MgCl_2$, Na_2CO_3, $C_2H_3NaO_2$ or $Na_3C_6H_5O_7$. 30µM of Phi11 Cro was dialyzed in the desired buffers and the secondary structure of the protein in that particular buffer was analyzed by CD (200-240nm, an average of three scans were taken) by JASCO J-815 spectropolarimeter. Samples were transferred to a cuvette of 1mm path length prior to the recording of CD spectrum. The data were further analyzed by K2D2

web server (Perez-Iratxeta & Andrade-Navarro 2008) (http://cbdm-01.zdv.uni-mainz.de/~andrade/k2d2/).

2.2.8. **Oligomerization of Cro in presence of NaCl, $MgCl_2$ and Na_2CO_3**

To investigate the oligomeric status of Cro in presence of NaCl, $MgCl_2$ and Na_2CO_3, the aliquots of Cro (equilibrated with NaCl, $MgCl_2$ and Na_2CO_3) were run on a Tris-Glycine 12% PAGE without denaturation (Arndt et al 2012). To prevent denaturation, Cro (in the respective buffer) was mixed with 1X SDS sample buffer (without beta-mercaptoethanol) and loaded directly onto the gel without boiling. The gel was run at 50 V for approximately 8 hours in the cold room. The gel was then stained by CBB R-250, followed by destaining as described in section 2.2.4. To compare the oligomeric status of the native protein in comparison to its denatured state, the protein (in presence of $MgCl_2$ and Na_2CO_3) was run in native (Tris-Glycine 12% PAGE) as well as denaturing conditions (SDS-12% PAGE).

2.3 **Results**

2.3.1. **Cro retains its biological activity in presence of monovalent cations such as Na^+, K^+ and Li^+**

Gel shift assay was employed to measure the effect of monovalent cations Na^+, K^+ and Li^+ on the DNA binding activity of Phi11 Cro. Cro was equilibrated in NaCl, KCl or LiCl buffers and the equilibrium binding was studied. As is clear from the figures (Figure 2.2.A and 2.2.B, Figure 2.3.A and 2.3.B and Figure 2.4.A and 2.4.B), the apparent equilibrium dissociation constant, K_D for Cro-*O* DNA interaction in Na^+, K^+ and Li^+ buffers are 0.457, 0.437 and 0.427 micro molar (μM) respectively. This indicates that the three cations had similar effect upon the biological activity of Cro protein.

To further investigate the effect of Na^+, K^+ and Li^+ upon the structure of the Cro protein, CD spectral analysis (200-240nm) was carried out for Cro (equilibrated in NaCl, KCl and LiCl). The CD spectrum exhibited a plot of large negative ellipticity at 208nm for Cro equilibrated in all three buffers, namely, NaCl, KCl and LiCl (Figure 2.2.C, Figure 2.3.C and Figure 2.4.C respectively). K2D2 analysis of the CD spectrum for Cro in each of the three buffers indicated that the protein was approximately 68-75% α-helical, 1-2% β-stranded and 22-28% coiled in structure.

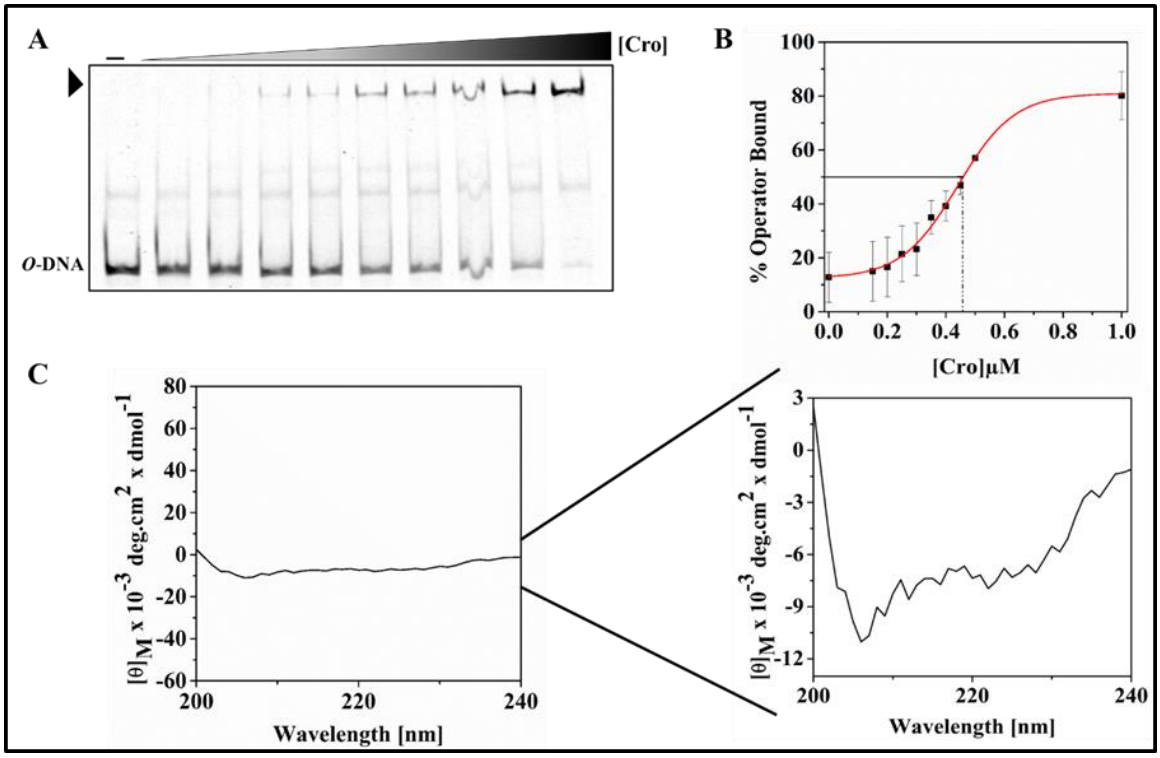

Figure 2.2. || Effect of NaCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with NaCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.15 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in NaCl buffer.

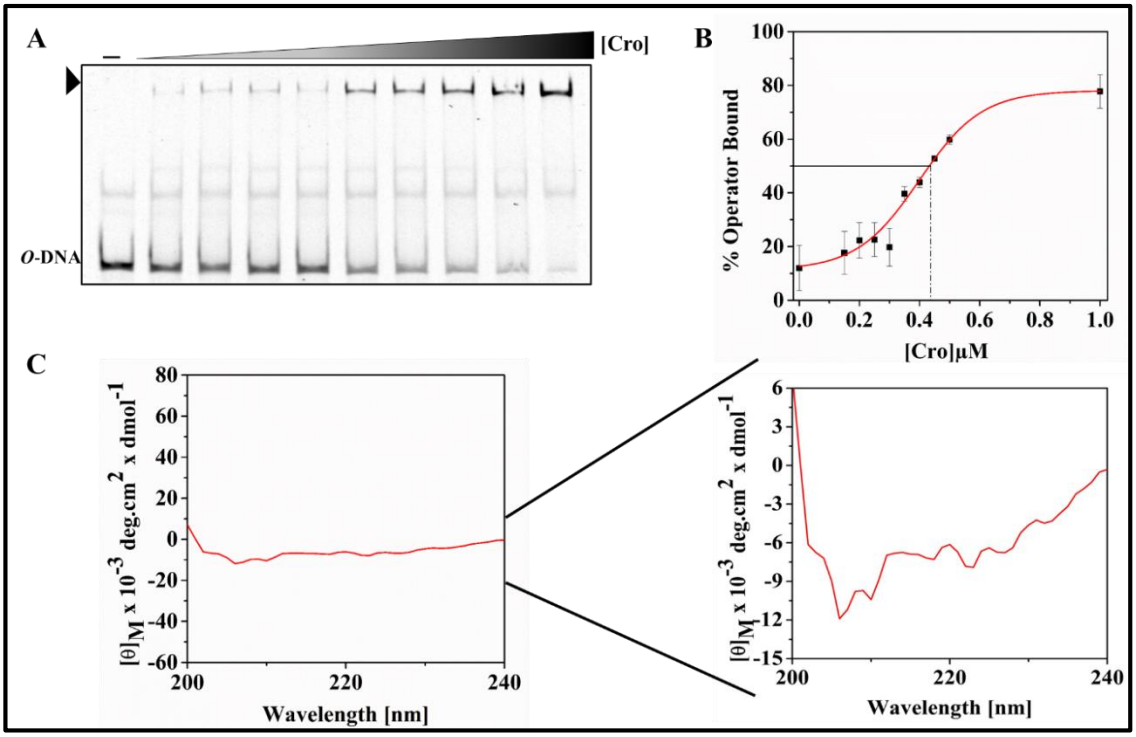

Figure 2.3. || Effect of KCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with KCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.15 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in KCl buffer.

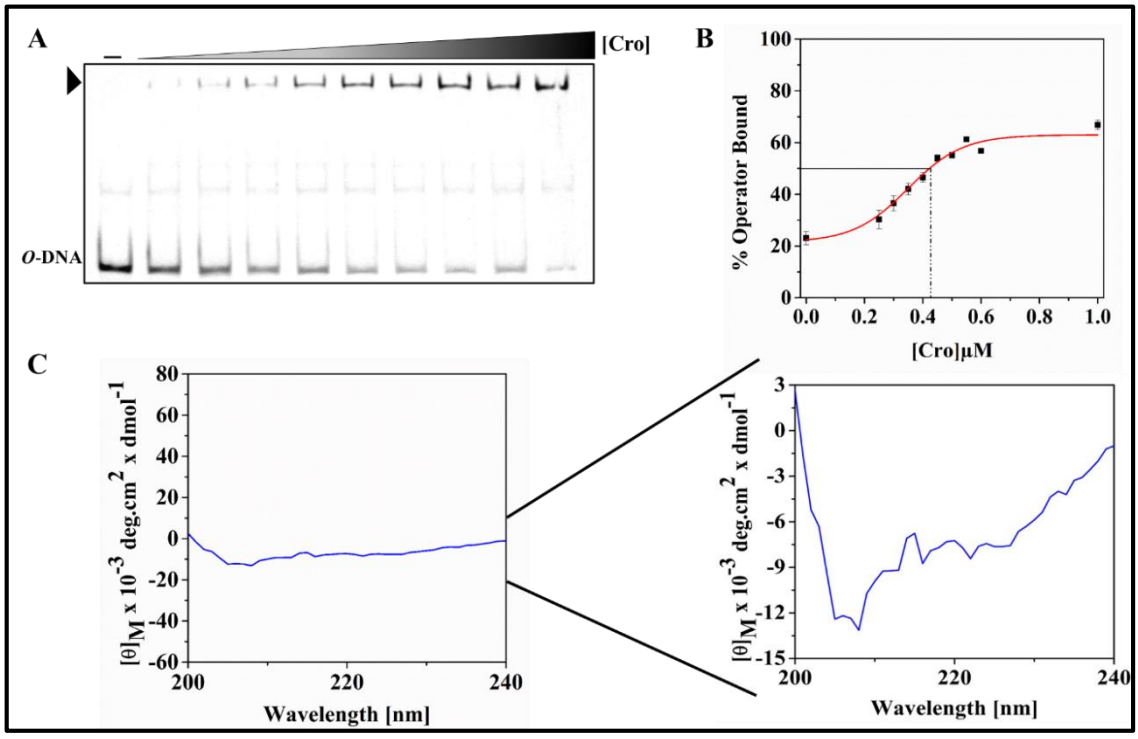

Figure 2.4. || Effect of LiCl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with LiCl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in LiCl buffer.

2.3.2. **The monovalent cation NH_4^+ has a stimulatory effect upon the binding of Cro to its cognate operator DNA**

Cro protein, equilibrated in NH_4Cl buffer, was allowed to bind to *O* DNA. The complex thus formed was analysed by gel shift assay and the K_D in this case was found to be 0.376 μM (Figure 2.5.A and 2.5.B), which is lower than the K_D obtained for the previous cations.

To investigate the effect of NH_4^+ on the structure of the Cro protein, NH_4Cl buffer equilibrated Cro was subjected to CD spectral analysis (200-240nm). K2D2 analysis of CD spectrum obtained, however, indicated a spectrum similar to the other three cations. NH_4Cl buffer equilibrated Cro was found to be 63.05 % α-helical, 3.29% β-stranded and a 33.66 % coiled. In this case, the CD spectrum also exhibited a similar peak at 208nm as compared to that of the previous three cations (Figure 2.5.C).

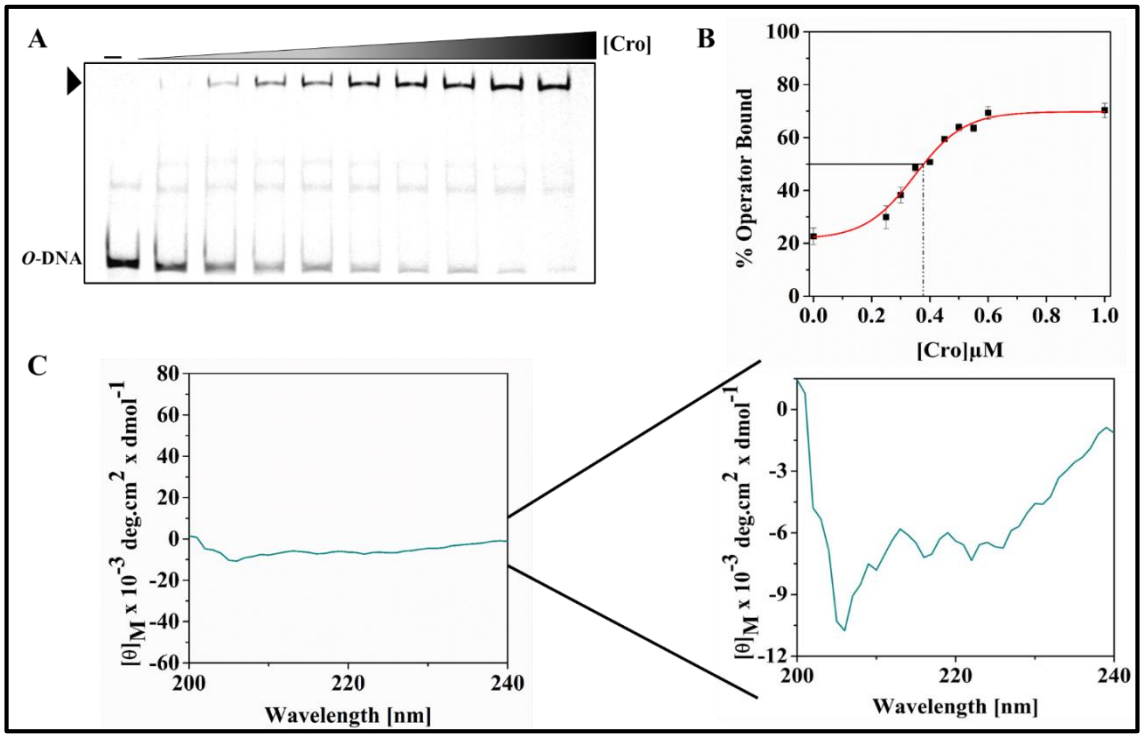

Figure 2.5. || Effect of NH_4Cl on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with NH_4Cl). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-1 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in NH_4Cl buffer.

2.3.3. **Mg^{2+} does not unfold Cro but has an inhibitory effect upon the binding of Cro to its cognate operator DNA**

Cro protein, equilibrated in $MgCl_2$ buffer, was allowed to bind to *O* DNA. The complex thus formed was analyzed by gel shift assay and the K_D in this case was found to be 0.821 μM (Figure 2.6.A and 2.6.B).

However, the CD spectrum of Cro equilibrated with $MgCl_2$ buffer showed a plot of large negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 75.57 % α-helical, 1.72 % β-stranded and 22.71% coiled in structure (Figure 2.6.C).

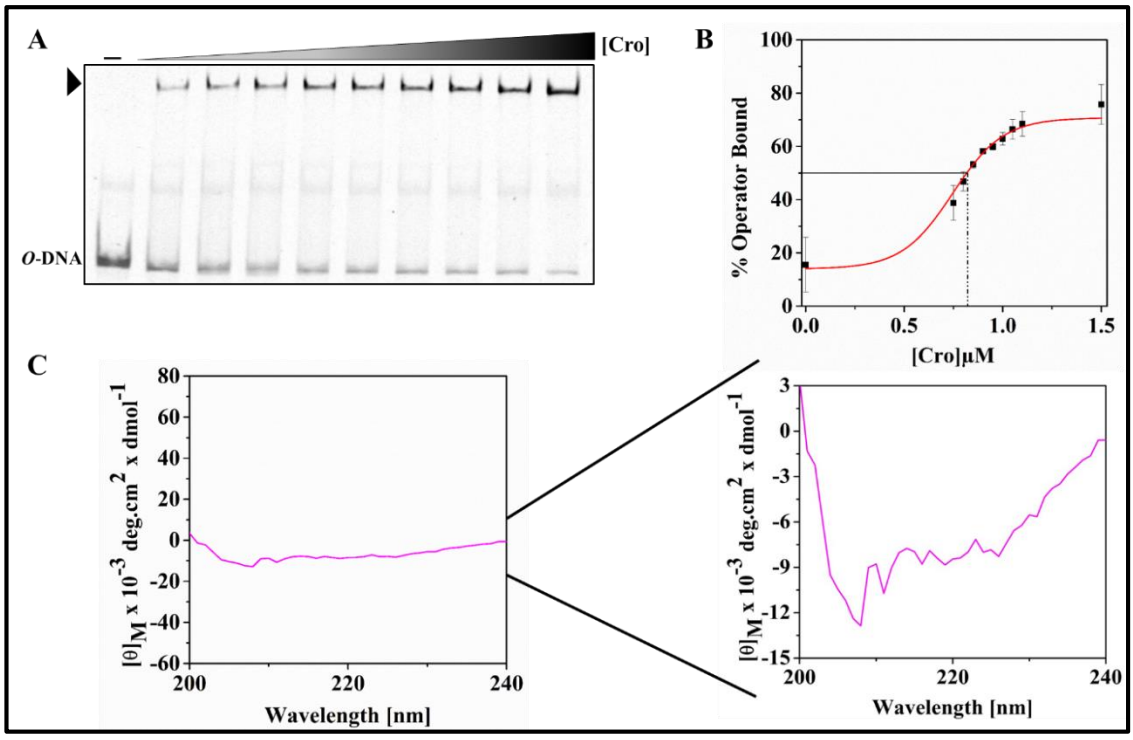

Figure 2.6. || Effect of $MgCl_2$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(O) and Phi11 Cro (equilibrated with $MgCl_2$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.75 μM-1.5 μM. (B) The % operator bound to Cro in the O DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $MgCl_2$ buffer.

2.3.4. **Effects of $C_2H_3O_2^-$ and CO_3^{2-} on Cro**

The K_D value of Cro-*O* DNA (Cro equilibrated with Na_2CO_3, $C_2H_3NaO_2$ buffers) interaction was studied using gel shift assay. It was found that the K_D value for Cro-operator interaction was 0.429μM in $C_2H_3NaO_2$ buffer (Figure 2.7.A and 2.7.B) and 0.753 μM in Na_2CO_3 buffer (Figure 2.8.A and 2.8.B). The CD spectrum of Cro equilibrated with Na_2CO_3 buffer showed a plot of large negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 75.57 % α-helical, 1.72 % β-stranded and 22.71% coiled in structure (Figure 2.8.C).

On the other hand, the CD spectrum of Cro equilibrated with $C_2H_3NaO_2$ buffer showed a plot of comparatively smaller negative ellipticity at 208nm and K2D2 analysis of the spectrum indicated that the protein was 67.45 % α-helical, 3.24 % β-stranded and 29.26% coiled in structure (Figure 2.7.C).

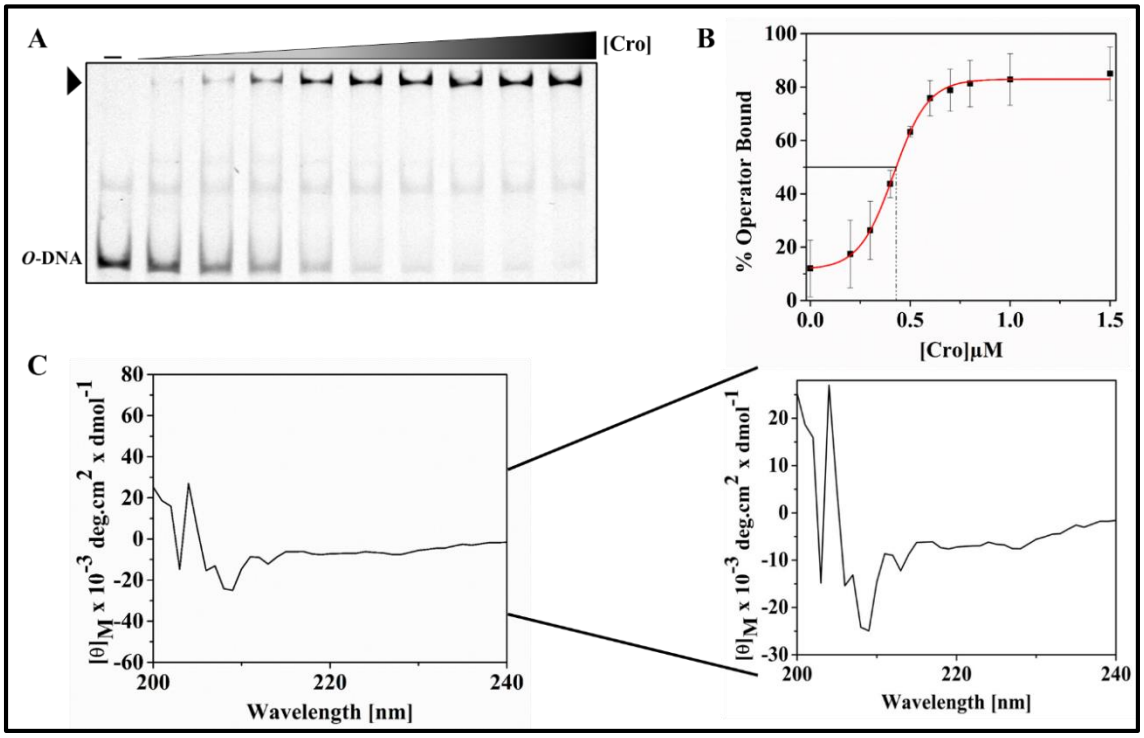

Figure 2.7. || Effect of $C_2H_3NaO_2$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between Operator DNA(*O*) and Phi11 Cro (equilibrated with $C_2H_3NaO_2$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.2 μM-1.5 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $C_2H_3NaO_2$ buffer.

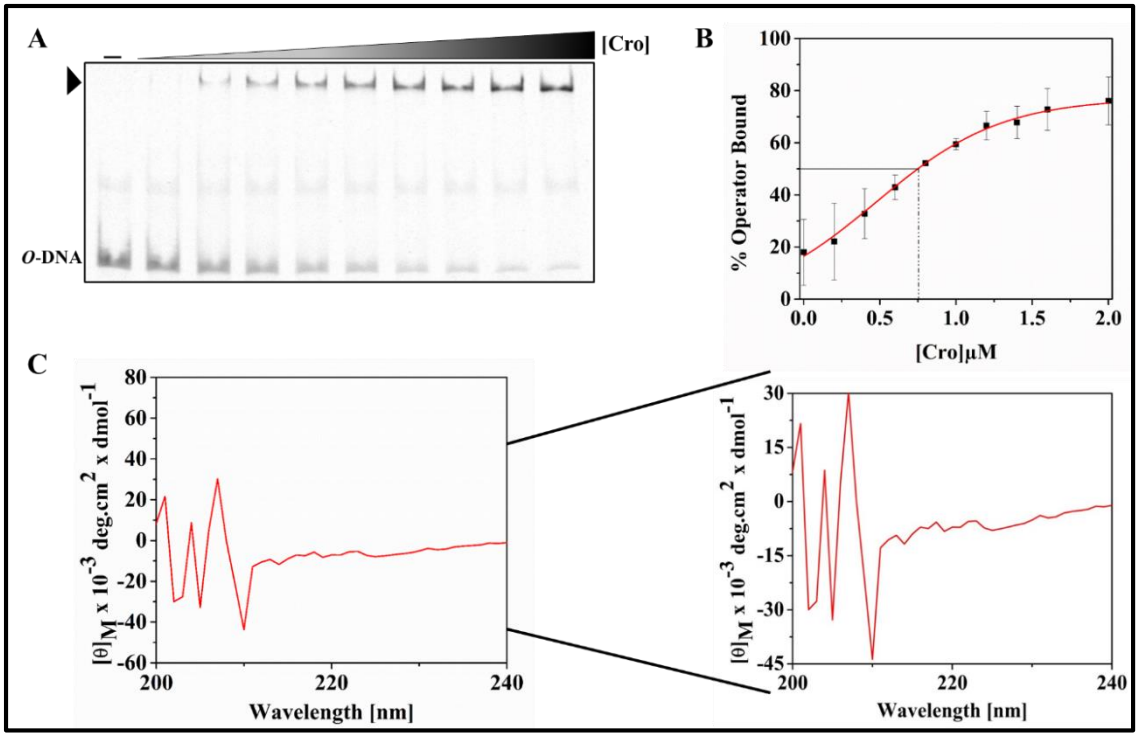

Figure 2.8. || Effect of Na_2CO_3 on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between operator DNA(*O*) and Phi11 Cro (equilibrated with Na_2CO_3). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.2 μM - 2 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in Na_2CO_3 buffer.

2.3.5. $C_6H_5O_7^{3-}$ has a profound effect on the structure and function of Phi11 Cro

Cro protein, after equilibration in $Na_3C_6H_5O_7$ buffer, was allowed to interact with *O* DNA and the K_D value of this interaction was found to be 1.846 μM (Figure 2.9.A and 2.9.B). To investigate the effect exerted by $C_6H_5O_7^{3-}$ upon the structure of Phi11 Cro, the CD spectra of $Na_3C_6H_5O_7$ equilibrated Cro was studied. It was found that Cro exhibited a much reduced peak at 208nm and was 7.96 % α-helical, 21.23 % β-stranded and 70.81% coiled in structure (Figure 2.9.C).

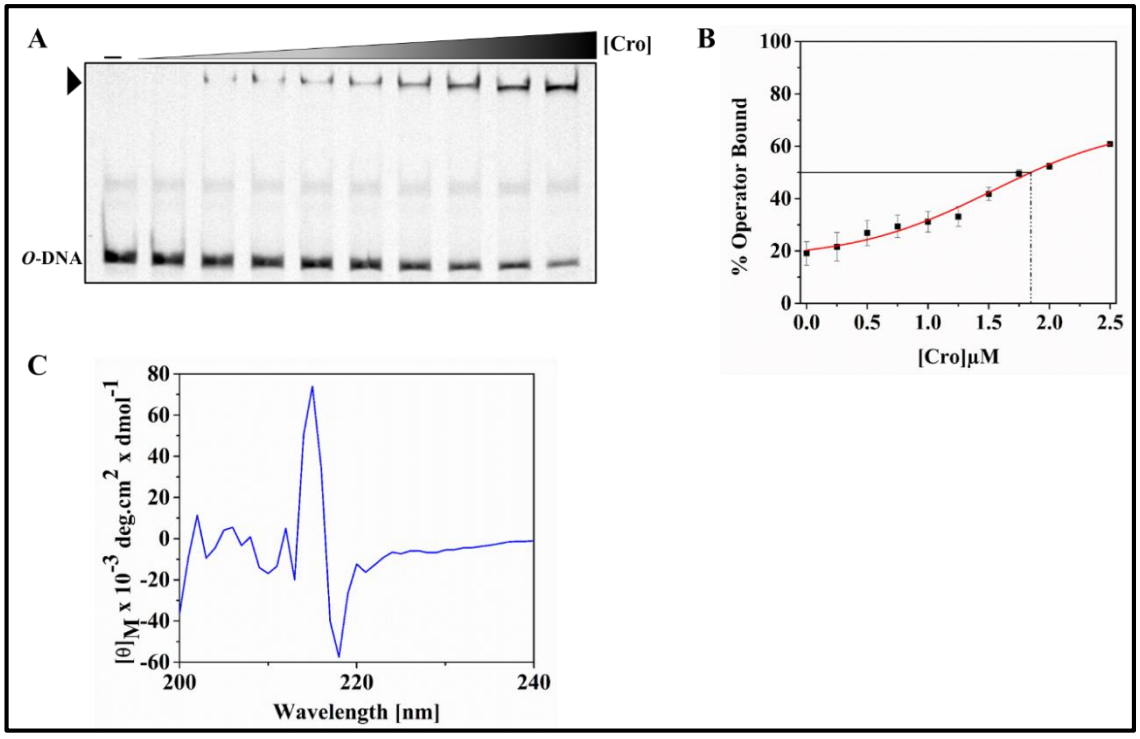

Figure 2.9. || Effect of $Na_3C_6H_5O_7$ on Phi11 Cro.
(A) 6% non-denaturing PAGE showing the interaction between operator DNA(*O*) and Phi11 Cro (equilibrated with $Na_3C_6H_5O_7$). Arrowhead represents the *O* DNA-Cro complex. The concentration of Cro ranges from 0.25 μM-2.5 μM. (B) The % operator bound to Cro in the *O* DNA-Cro complex (Measured by scanning the gel shift assay gel, presented in panel A which was plotted against the corresponding concentration of Cro). (C) CD-spectra (200-240nm) of 30 μM Cro in $Na_3C_6H_5O_7$ buffer.

2.3.6. **Mg^{2+} and CO_3^{2-} induce oligomerization in Phi 11 Cro**

The oligomeric status of Phi11 Cro in presence of $MgCl_2$ buffer or Na_2CO_3 buffer was investigated. It was found that Cro, when equilibrated with $MgCl_2$ buffer or Na_2CO_3 buffer, actually existed as higher oligomeric species, unlike Cro equilibrated with NaCl buffer whereby it exists as a monomeric species (Figure 2.10.).

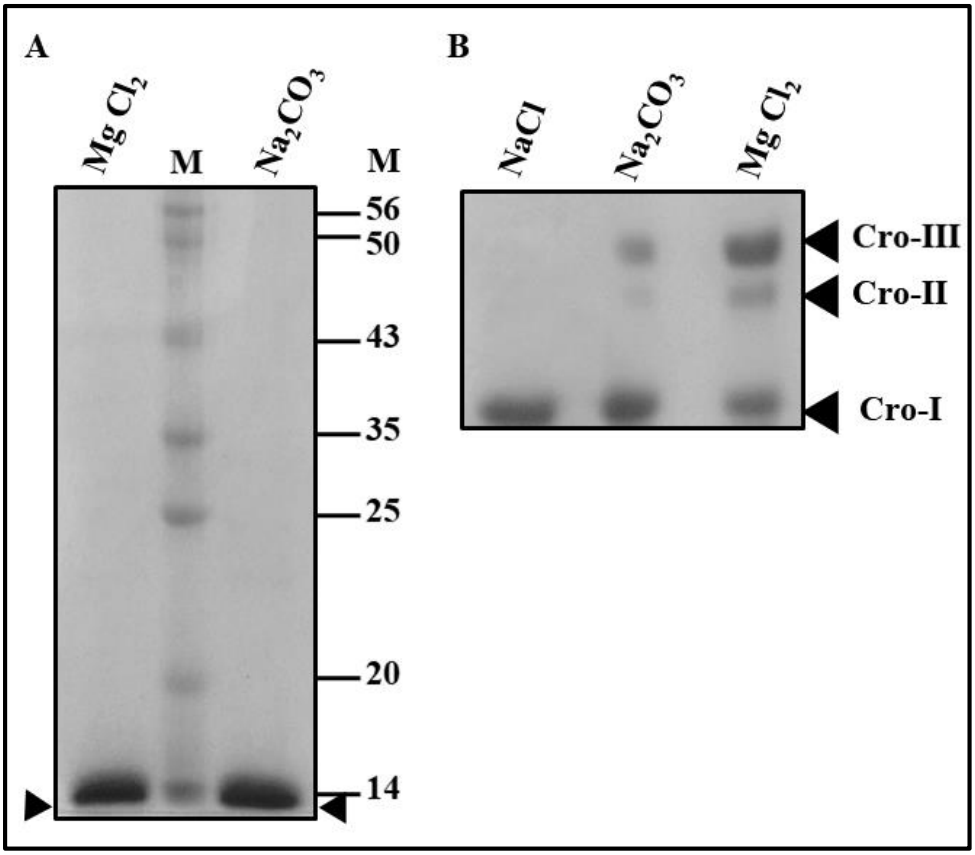

Figure 2.10. || Oligomeric status of Phi11 Cro in presence of buffers containing NaCl, $MgCl_2$ or Na_2CO_3.
(A) Denatured Cro (equilibrated with $MgCl_2$ and Na_2CO_3) run on SDS-12% PAGE. M stands for standard protein marker (size indicated on the right side of the gel). Arrowheads indicate the position of Cro. (B) Oligomerization of unboiled, Cro (in NaCl, $MgCl_2$ or Na_2CO_3) analysed by electrophoresis in 12% polyacrylamide gel (Materials and methods). Arrowhead Cro-I indicates the position of monomeric Cro. Arrowheads Cro- II and Cro-III represent the higher oligomers of Cro.

2.4. **Discussion**

Salts can play a very important role in influencing the biologically active conformation of proteins. The effects exerted by different salts upon different proteins can vary. Some salts might lead to aggregation, denaturation or unfolding of the protein and some might lead to minute changes in the domains of a multi-domain protein. In both cases the functional activity of the protein may or may not be affected (Bandhu et al 2009). In this study, we have employed gel shift assay to study the effect exerted by various anions and cations upon the functional activity of Phi11 Cro. We have also carried out CD spectral analysis (200-240nm) of Phi11 Cro (equilibrated with different salt buffers) to investigate the effect of the various salts upon the structure of the protein. We also attempted to investigate the structure-function relationship of Cro.

From the data obtained, it is clear that Phi11 Cro showed almost similar operator binding activity in presence of each of the three monovalent cations (Na^+, K^+ and Li^+) used. This indicates that the three cations had similar effect upon the biological activity of Cro protein. However, the K_D value of Cro-*O* DNA interaction in NH_4Cl buffer was found to be 0.376 μM, which is slightly lower than the K_D values obtained with NaCl, KCl and LiCl buffers. The data is indicative of the fact that in presence of NH_4^+, the interaction between Cro and its cognate operator is stronger as compared to Na^+, K^+ and Li^+. NH_4^+ possibly induces some modifications in the structure of either the Cro protein or *O* DNA or both, thereby strengthening the interaction.

The divalent ions, Mg^{2+} and CO_3^{2-}, were however found to severely hamper the Cro-*O* DNA interaction as indicated by their K_D values (approximately 2 times higher than the K_D obtained for the monovalent ions Na^+, K^+, Li^+ or $C_2H_3O_2^-$).

To investigate if the above salts had any effect upon the conformation of the protein, CD spectral analysis (200-240nm) was employed to check the conformation of Cro in presence of each of the ions. Surprisingly, it was found that when incubated in $MgCl_2$ buffer or Na_2CO_3 buffer, Cro exhibited the least randomness and maximum α-helical content. To explain this data, we assumed that Mg^{2+} and CO_3^{2-} might lead to multi-merisation of the protein which may partially block its operator binding site (Winter et al 2012). Previously, it has been had reported that micro molar concentrations Phi11 Cro exists as monomer in solution (Das et al 2009). In fact, our recent experiments showed that Cro equilibrated with $MgCl_2$ buffer or Na_2CO_3 buffer actually existed as higher oligomeric species, unlike Cro equilibrated with NaCl buffer whereby it exists as a monomeric species (Figure 2.10.).

CD spectra of Na^+, K^+, Li^+, NH_4^+ and $C_2H_3O_2^-$ equilibrated Cro showed similar peak at 208nm, indicating that these monovalent ions possibly maintain the biologically active conformation of Cro.

$C_6H_5O_7^{3-}$ ions led to a decrease in the affinity of Cro for its cognate operator, as indicated from the K_D value. Moreover, the CD spectral analysis indicates that $C_6H_5O_7^{3-}$ severely unfolded Cro. This unfolding of Cro in $Na_3C_6H_5O_7$ buffer strongly interfered with its functional activity.

Considering all the above data, it is clear that the structure of Phi11 Cro was maintained by NH_4^+ and $C_2H_3O_2^-$ allowed it to bind optimally to its cognate operator.

In spite of the changes of different cationic and anionic salt, the Cro repressor binding ability to *O* DNA has not changed significantly. Specially, cellular cations (Na^+ and K^+) does not shown effective changes in DNA-binding nature of lytic repressor Cro. Using the previous knowledge of low affinity of Cro repressor to its cognate operator DNA compare to CI repressor, we focused on a downstream gene (ORF7) of Cro (early express gene) for better understanding the involvement of ORF7 gene (designated as antirepressor in NCBI; NC_004615.1) product on Phi11 regulatory development. This chapter work has been published.

2.5. **References**

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