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TITLE:

CD Spectroscopy to Study DNA-Protein Interactions

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KEYWORDS:

CD spectroscopy, ATP-dependent chromatin remodeling, DNA-protein interaction, chromatin dynamics, chromatin remodeling, transcriptional regulation.

SUMMARY:

The interaction of an ATP-dependent chromatin remodeler with a DNA ligand is described using CD spectroscopy. The induced conformational changes on a gene promoter analyzed by the peaks generated can be used to understand the mechanism of transcriptional regulation.

ABSTRACT:

Circular dichroism (CD) spectroscopy is a simple and convenient method to investigate the secondary structure and interactions of biomolecules. Recent advancements in CD spectroscopy have enabled the study of DNA-protein interactions and conformational dynamics of DNA in different microenvironments in detail for a better understanding of transcriptional regulation *in vivo*. The area around a potential transcription zone needs to be unwound for transcription to occur. This is a complex process requiring the coordination of histone modifications, binding of the transcription factor to DNA, and other chromatin remodeling activities. Using CD spectroscopy, it is possible to study conformational changes in the promoter region caused by regulatory proteins, such as ATP-dependent chromatin remodelers, to promote transcription. The conformational changes occurring in the protein can also be monitored. In addition, queries regarding the affinity of the protein towards its target DNA and sequence specificity can be addressed by incorporating mutations in the target DNA. In short, the unique understanding of this sensitive and inexpensive method can predict changes in chromatin dynamics, thereby improving the understanding of transcriptional regulation.

INTRODUCTION:

Circular dichroism (CD) is a spectroscopic technique that relies on the inherent chirality of biological macromolecules that leads to differential absorption of right-handed and left-handed circularly polarized light. This differential absorption is known as circular dichroism. The technique, therefore, can be used to delineate the conformation of biological macromolecules, such as proteins and DNA, both of which contain chiral centers[1,2].

Electromagnetic waves contain both electric and magnetic components. Both the electrical and the magnetic fields oscillate perpendicular to the direction of wave propagation. In the case of unpolarized light, these fields oscillate in many directions. When the light is circularly polarized, two electromagnetic fields are obtained at 90° phase difference to each other. Chiral molecules show circular optical rotation (birefringence) such that they will absorb the right-handed circularly polarized light and the left-handed circularly polarized light to different extents[3]. The resulting electrical field will be traced as an ellipse, a function of the wavelength. The CD spectrum is, thus, recorded as ellipticity (q), and the data are presented as Mean Residue Ellipticity as a function of wavelength.

In the case of proteins, the Cα of amino acids (except glycine) is chiral, and this is exploited by CD spectroscopy to determine the secondary structure of this macromolecule[4]. The CD spectra of protein molecules are typically recorded in the Far UV range. α-helical proteins have two negative bands at 222 nm and 208 nm and one positive peak at 193 nm[4]. Proteins with anti-parallel β-sheet secondary structure show a negative peak at 218 nm and a positive peak at 195 nm[4]. Proteins with disordered structures show low ellipticity near 210 nm and a negative peak at 195 nm[4]. Thus, the well-defined peak/bands for different secondary structures make CD a convenient tool to elucidate the conformational changes occurring in the secondary structure of the proteins during denaturation as well as ligand binding.

Nucleic acids have three sources of chirality: the sugar molecule, the helicity of the secondary structure, and the long-range tertiary ordering of DNA in the environment[5,6]. The CD spectra of nucleic acids are typically recorded in the 190 to 300 nm range[5,6]. Each conformation of DNA, just like proteins, gives a characteristic spectrum, although the peaks/bands can vary by some degrees due to solvent conditions and differences in DNA sequences[7]. B-DNA, the most common form, is characterized by a positive peak around 260–280 nm and a negative peak around 245 nm[6]. The peaks/bands of B-form DNA are generally small because the base pairs are perpendicular to the double helix, conferring weak chirality to the molecule. A-DNA gives a dominant positive peak at 260 nm and a negative peak around 210 nm[6]. Z-DNA, the left-handed helix, gives a negative band at 290 nm and a positive peak around 260 nm[6]. This DNA also gives an extremely negative peak at 205 nm[6].

In addition to these conformations, DNA can also form triplexes, quadruplexes, and hairpins, all of which can be distinguished by CD spectroscopy. The parallel G-quadruplex give a dominant positive band at 260 nm, while the anti-parallel G-quadruplex gives a negative band at 260 nm and a positive peak at 290 nm, making it easy to distinguish between the two forms of quadruplex structures[6]. Triplexes do not give a characteristic spectrum[8]. For example, the spectra of a 36 nucleotide-long DNA with the potential to form an intramolecular triple helix containing G.G.C and T.A.T base pairs in the presence of Na^+ show a strong negative band at 240 nm and a broad positive peak. The broad positive peak shows contributions at 266, 273, and 286 nm. The same oligonucleotide in the presence of Na^+ and Zn^+ shows four negative bands (213, 238, 266, and 282 nm) and a positive peak at 258 nm. Thus, the spectra of triplex DNA can vary depending upon salt conditions[8].

In addition to these conformations, CD spectra have enabled the identification of another form of DNA called X-DNA. X-DNA is formed when the DNA sequence contains alternate

adenine and thymine residues. The CD spectra of X-DNA contain two negative peaks at 250 and 280 nm. Very little information is available about X-DNA, although it has been speculated to function as a sink for positive supercoiling[6,9]. Changes in CD spectra can also reveal details about ligand-protein interactions and, therefore, have been added to the arsenal of molecular methods for detecting drug-protein interactions[10–14]. CD spectra have also been used to monitor the changes in the secondary structure of proteins during the folding process[15]. Similarly, CD spectra can also be used for probing ligand-DNA interactions[16,17].

CD spectroscopy, thus, is an easy, inexpensive method to distinguish between the different forms of DNA conformation, provided there is access to not-so-inexpensive equipment and software. The method is exceedingly sensitive and quick. It only requires a small amount of DNA, giving it an edge over the alternate technique of nuclear magnetic resonance (NMR) spectroscopy. Titrations with ligands and substrates are also easy to perform. The major constraint is that the DNA should be highly pure. It is advisable to use polyacrylamide gel electrophoresis (PAGE)-purified DNA.

The information obtained by CD spectra has been mainly used to deduce protein structural features and to identify distinct DNA conformers. In this study, CD spectra have been used to integrate the results obtained from an *in vivo* Chromatin Immunoprecipitation (ChIP) experiment to delineate whether the protein of interest/predicted transcription factor can bring about a conformational change in the promoter region of its effector genes. This collaboration aids in the progress of traditional CD spectroscopic techniques by predicting the mechanism of transcription regulation by the predicted transcription factor on and around the transcription start site (TSS) of a promoter.

Chromatin remodeling is a well-defined mechanism known to regulate DNA metabolic processes by making the tightly packed chromatin accessible to various regulatory factors such as transcription factors, components of DNA replication, or damage repair proteins. The ATP-dependent chromatin remodelers, also known as the SWI/SNF family of proteins, are key remodeler proteins present in eukaryotic cells[18,19]. Phylogenetic clustering has categorized the SWI/SNF family of proteins into 6 sub-groups[20]: Snf2-like, Swr1-like, SSO1653-like, Rad54-like, Rad5/16-like, and distant. SMARCAL1, the protein of interest in this study, belongs to the distant sub-group[20]. This protein has been used to investigate its mode of transcriptional regulation using CD spectroscopy.

Most of the members of the ATP-dependent chromatin remodeling proteins have been shown to either reposition or evict nucleosomes or mediate histone variant exchange in an ATP-dependent manner[21,22]. However, members of this family have not been shown to remodel nucleosomes, e.g., SMARCAL1. Even though studies have shown that SMARCAL1 associates with polytene chromosomes, experimental evidence regarding its ability to remodel nucleosomes is lacking[23]. Therefore, it was postulated that SMARCAL1 may regulate transcription by altering the conformation of DNA[24]. CD spectroscopy provided an easy and accessible method to validate this hypothesis.

SMARCAL1 is an ATP-dependent chromatin remodeling protein that primarily functions as an annealing helicase[25–27]. It has been postulated to modulate transcription by remodeling the DNA conformation[24]. To test this hypothesis, the role of SMARCAL1 in regulating gene

transcription during doxorubicin-induced DNA damage was studied. In these studies, SMARCAL1 was used for *in vivo* analysis and ADAAD for *in vitro* assays[28,29]. Previous studies have shown that ADAAD can recognize DNA in a structure-dependent but sequence-independent manner[29,30]. The protein binds optimally to DNA molecules possessing double-strand to single-strand transition regions, similar to stem-loop DNA, and hydrolyzes ATP [30,31]. *In vivo* experiments showed that SMARCAL1 regulates the expression of *MYC*, *DROSHA*, *DGCR8,* and *DICER* by binding to the promoter regions[28,29]. The region of interaction was identified by ChIP experiments[28,29]. The ChIP technique is used to analyze the interaction of a protein with its cognate DNA within the cell. Its goal is to determine whether specific proteins, such as transcription factors on promoters or other DNA binding sites, are bound to specific genomic areas. The protein bound to DNA is first cross-linked using formaldehyde. This is followed by isolation of the chromatin. The isolated chromatin is sheared to 500 bp fragments either by sonication or nuclease digestion, and the protein bound to DNA is immunoprecipitated using antibodies specific to the protein. The cross-linking is reversed, and the DNA is analyzed using either polymerase chain reaction (PCR) or quantitative real-time PCR.

The ChIP results led to the hypothesis that SMARCAL1 possibly mediates transcriptional regulation by inducing a conformational change in the promoter regions of these genes. QGRS mapper and Mfold software were used to identify the potential of these promoter regions to form secondary structures[28,29]. QGRS mapper is used for predicting G-quadruplexes[32], while Mfold[33] analyzes the ability of a sequence to form secondary structures such as stem-loops.

After secondary structure analysis, further *in vitro* experiments were performed with recombinant 6X His-tagged Active DNA-dependent ATPase A Domain (ADAAD), the bovine homolog of SMARCAL1, purified from *Escherichia coli*[30,31,34]. ATPase assays were performed using ADAAD to establish that the identified DNA sequences could act as effectors[28,29]. Finally, CD spectroscopy was performed to monitor the conformational changes induced in the DNA molecule by ADAAD[28,29].

To prove that the ATPase activity of the protein was essential for inducing a conformational change in the DNA molecule, either ethylenediamine tetraacetic acid (EDTA) was added to chelate Mg^{+2} or Active DNA-dependent ATPase A Domain Inhibitor Neomycin (ADAADiN), a specific inhibitor of the SWI/SNF protein, was added[35,36]. This CD spectroscopic technique can be utilized with any purified protein that has been demonstrated by ChIP or any other relevant assay to bind to a predicted genomic region of a promoter.

PROTOCOL:

1. Working concentration of the reaction components

1.1. Prepare the working concentrations of buffers and other reaction components freshly (see **Table 1**) and keep them at 4 °C before setting up the reactions.

NOTE: For the reactions described in this paper, the working concentrations of components are as follows: Sodium phosphate buffer (pH 7.0) 1 mM, ATP 2 mM, DNA 500 nM, Protein 1 µM, $MgCl_2$ 10 mM, EDTA 50 mM, ADAADiN 5 µM.

2. ATPase activity

2.1. Before CD spectroscopy, establish the ATPase activity of the protein in the presence of the DNA molecules to ensure that the protein used in the CD spectroscopy is active and to identify the DNA molecules that are optimally effective in eliciting ATP hydrolysis.

2.2. Measure the ATPase activity of the protein in the presence of different DNA molecules by an NADH-coupled oxidation assay consisting of the following two reactions.

2.2.1. Mix 0.1 mM ADAAD, 2 mM ATP, 10 nM DNA, and 1x REG buffer in a 96-well plate to a final volume of 250 mL.

NOTE: The pyruvate kinase enzyme uses the ADP and Pi to convert phosphoenolpyruvate to pyruvate, thus regenerating ATP. This ensures that ATP is always in a saturating concentration in the reaction. In the second reaction, the pyruvate formed by the action of pyruvate kinase is converted by lactate dehydrogenase to lactate. In this reaction, one NADH molecule is oxidized to NAD^+. The consumption of NADH is measured by measuring the absorbance of the molecule at 340 nm.

2.2.2. Incubate for 30 min at 37 °C in an incubator.

2.2.3. Measure the amount of NAD^+ at 340 nm using a microplate reader.

2.2.4. To measure the amount of NAD^+, use the software provided along with the microplate reader.

2.2.4.1. Click on the **NADH assay** to measure the absorbance at 340 nm.

2.2.4.2. Place the 96-well plate on the plate holder in the instrument. Click on the **Read Plate** button to record the absorbance.

NOTE: The concentration of NAD^+ is calculated using the molar extinction coefficient of NADH as 6.3 mM^{-1} by using eq (**1**).

$$A = \varepsilon c l \qquad (1)$$

Here, A = Absorbance
ε = Molar extinction coefficient
c = Molar concentration
l = Optical path length in cm

3. Choosing and preparation of CD cuvettes

3.1. Collect CD spectra in high-transparency quartz cuvettes. Use rectangular or cylindrical cuvettes.

NOTE: A CD quartz cuvette (nominal volume of 0.4 mL, path-length of 1 mm) was used for all the reactions described in this paper.

3.2. Use a cuvette cleaning solution to clean the cuvette. Add 1% cuvette cleaning solution in water to make 400 µL of the solution, pour it in the cuvette, and incubate it at 37 °C for 1 h.

3.3. Wash the cuvette with water several times to clean the cuvette. Take a scan of the water or buffer in the cuvette to check whether it is clean.

NOTE: The water or buffer must give a reading in the 0 to 1 mdeg range.

4. Preparation of proteins and DNA oligonucleotide

4.1. Keep the volume of the protein below 50 µL in the reaction to minimize the amounts of the buffer components that sometimes cause the formation of ambiguous peaks. Keep the protein on the ice throughout the experiment to avoid any degradation.

4.2. Use PAGE-purified DNA oligonucleotides in the reactions.

NOTE: In the reactions described here, DNA was used both in native as well as heat-cooled forms (fast-cooled (FC) and slow-cooled (SC)). Fast cooling promotes intramolecular bonding in the DNA, yielding more secondary structures. In contrast, slow cooling promotes intermolecular bonding in the DNA, resulting in fewer secondary structures.

4.3. For fast-cooling, heat DNA at 94 °C for 3 min on the heating block and immediately cool it on ice. For slow-cooling, heat DNA at 94 °C for 3 min and allow it to cool to room temperature at a rate of 1 °C per minute.

5. Setting up control experiments to record the baseline spectra

5.1. Keep the reaction volume at 300 µL in all the reactions. Set up a total of 5 baseline reactions in 1.5 mL centrifuge tubes, one by one, as follows: i) Buffer + Water; ii) Buffer + $MgCl_2$ + ATP + Water; iii) Buffer + $MgCl_2$ + ATP + Protein + Water; iv) iii + EDTA or ADAADiN; v) Buffer + Protein +Water.

6. Setting up the experiments to record CD spectra

6.1. Set up a total of 5 reactions, one by one, in 1.5 mL centrifuge tubes as follows: i) Buffer + DNA + Water; ii) Buffer + DNA + $MgCl_2$ + ATP + Water; iii) Buffer + DNA + $MgCl_2$ + ATP + Protein + Water; iv) iii + EDTA or ADAADiN; v) Buffer + DNA + Protein +Water.

7. Recording scan

7.1. Turn on the gas and switch on the CD spectrometer.

7.2. Switch on the lamp after 10–15 min. Switch on the water bath and set the holder temperature at 37 °C.

7.3. Open the **CD spectrum** software.

7.3.1. Set the **temperature** to **37 °C**.

7.3.2. Set the **wavelength range** at **180–300 nm**.

7.3.3. Set the **time per point** to **0.5 s**.

7.3.4. Set the **scan number** to **5**.

7.3.5. Click on **Pro-Data Viewer**, make a new file, and rename it with details about the experiment and date.

7.4. Keep all the reaction components on ice to avoid any degradation. Make the baselines and reactions, one by one, in centrifuge tubes and mix them by pipetting. Transfer the reaction mix to the cuvette carefully, ensuring that there are no air bubbles.

7.5. If performing a time-course experiment, incubate the reactions at 37 °C for the required time and take the scan. Add EDTA to the buffer containing the DNA, ATP, Mg^{+2}, and protein to stop ATP hydrolysis.

7.6. Increase the concentration of EDTA and its incubation time to inhibit ATPase activity completely.

7.7. Subtract the baselines from the corresponding reactions in the software (e.g., subtract reaction 1 from baseline 1). Smoothen the data either in the CD spectrum software or in the data plotting software. Plot the data in the data plotting software.

NOTE: Subtracting the baselines from the corresponding reactions will give the net CD spectra of only DNA.

8. Data analysis and interpretation

8.1. Use the formula given by eq (**2**) to convert the values obtained in millidegrees to mean residue ellipticity.

$$[\theta] = (S \times mRw)/(10cl) \qquad (\mathbf{2})$$

Here, S is the CD signal in millidegrees, c is the DNA concentration in mg/mL, mRw is the mean residue mass, and l is the path length in cm.

8.2. Plot a graph against wavelength and mean residue ellipticity using the data plotting software and analyze the peaks.

8.3 To plot the graph, select the mean residue ellipticity on the Y-axis and wavelength on the X-axis and plot a straight line graph.

NOTE: This graph will provide the characteristics peaks of different forms of DNA. The forms of DNA corresponding to the peaks can be identified using existing literature[6].

REPRESENTATIVE RESULTS:

ADAAD stabilizes a stem-loop like structure on the *MYC* promoter

Previous experimental evidence showed that SMARCAL1 is a negative regulator of *MYC*[29]. Analysis of the 159 bp long promoter region of the *MYC* gene by QGRS mapper showed that the forward strand had the potential to form a G-quadruplex (**Table 2**). Mfold showed that both strands of the *MYC* DNA could form a stem-loop-like structure (**Table 2**). A 34 bp long DNA sequence containing the G-quadruplex (G_EC_E) was synthesized. The Mfold structures of the forward and the reverse sequence of the G_EC_E oligonucleotide are shown in **Figure 1A,B**.

ATPase assays using 6X His-ADAAD showed that fast-cooled G_EC_E was a better effector than the native and the slow-cooled forms. Therefore, fast-cooled G_EC_E was used to record the CD spectra in the absence and presence of ATP and ADAAD. The CD spectra showed that ADAAD induces two positive peaks—one at 258 nm with a shoulder at 269 nm and a larger peak at 210 nm in the DNA (**Figure 1C**). A dip towards the negative around 240 nm was also observed. This spectrum was similar to the one obtained when a synthetic stem-loop DNA, the optimal effector of ADAAD, was incubated with the protein and ATP (**Figure 2A,B**). Triplex DNA can give a similar spectrum[37], leading to the hypothesis that the protein could be inducing such a structure in this case. ATP forms a coordination complex with Mg^{+2}, and this cation is essential for ATP hydrolysis. The addition of EDTA chelates Mg^{+2}, leading to the inhibition of ATP hydrolysis[38]. Therefore, EDTA was added to the reaction mix to understand whether ATP hydrolysis by ADAAD was important for conformational change. The addition of EDTA to the reaction abrogates this conformation. The CD spectra now have a negative 210 nm peak and a broad positive band with peaks at 230 and 250 nm (**Figure 1C**).

The importance of ATPase activity was also confirmed using an ATPase-dead mutant of ADAAD. The K241A mutation occurs in the conserved GKT box of motif I, and this mutant has been shown previously to lack the ability to hydrolyze ATP in the presence of DNA. The mutant protein was expressed with a GST tag and purified using glutathione affinity chromatography. The conformational change induced in *MYC* DNA by this mutant was different from that induced by the wild-type ADAAD. The CD spectrum of the *MYC* DNA in the presence of the mutant protein possessed a positive 210 nm peak and a negative 260 nm peak (**Figure 1D**).

ADAAD induces A-form of conformation in *DROSHA* promoter

The promoter regions of *DROSHA*, *DGCR8,* and *DICER* too were analyzed by QGRS mapper and Mfold software. Both QGRS and MFold showed that the promoter regions possess the potential to form G-quadruplex and stem-like structures (**Table 2**). The Mfold structures of the forward and reverse oligonucleotides are shown in **Figure 3A,B**. The ATPase activity showed that the native and heat-cooled DNA behaved similarly. Therefore, the slow-cooled form of these DNA sequences was used for CD studies. The CD spectra showed that ADAAD induces a negative peak at 210 nm and a positive peak at 260 nm in the *DROSHA* promoter (**Figure 3C**). This spectrum is a characteristic of A-DNA[6].

ADAAD induces B-X transition and G-quadruplex formation in the *DGCR8* promoter

The Mfold structures of the forward and the reverse strands of the oligonucleotides used are shown in **Figure 4A,B**. A positive peak at 210 nm and a broad negative peak at 260 nm were observed for *DGCR8* pair 1 (**Figure 4C**). This spectrum is characteristic of B-X transition[6]. The Mfold structures of the forward and the reverse strands of the oligonucleotides used are shown in **Figure 4D,E**. The CD spectra of *DGCR8* pair 7 showed a strong positive peak at 210 and 270 nm and a negative peak at 250 nm (**Figure 4F**). This spectrum is characteristic of parallel G-quadruplex DNA structures[6].

ADAAD induces A-X transition in *DICER* promoter

The Mfold structures of the forward and reverse oligonucleotides are shown in **Figure 5A,B**. A positive peak at 210 nm and two negative peaks—one at 230 nm and the other at 260 nm peak—were observed for the *DICER* pair 1 (**Figure 5C**). These peaks are characteristic of A-X DNA transition[6,9]. All the CD spectra peaks and the forms of DNA having specific roles in the transcription process have been summarized in **Table 3**.

FIGURE AND TABLE LEGENDS:

Figure 1: ADAAD alters the conformation of G_EC_E DNA. Mfold structures were predicted for the (**A**) forward strand and (**B**) reverse strand. (**C**) CD spectra of G_EC_E alone (black), G_EC_E incubated with ATP and ADAAD before (red) and after adding EDTA (blue). (**D**) CD spectra of G_EC_E incubated with ATP and GST-tagged ADAAD before (black) and after adding EDTA (red) as well as CD spectra of G_EC_E incubated with ATP and GST-tagged K241A mutant (blue). This figure has been modified from [29]. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; CD = circular dichroism.

Figure 2: ADAAD alters the conformation of slDNA. (**A**) Mfold structure predicted for the stem-loop DNA. (**B**) CD spectra of slDNA alone (black), slDNA incubated with ATP and ADAAD (red). This figure has been modified from [29]. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; slDNA = stem-loop DNA; CD = circular dichroism.

Figure 3: ADAAD alters the conformation of *DROSHA* pair 5 DNA. Mfold structure predicted for the (**A**) forward strand and (**B**) reverse strand. (**C**) CD spectra of *DROSHA* pair 5 DNA alone (black), *DROSHA* pair 5 DNA incubated with ATP and ADAAD (red). This figure has been modified from [26]. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; CD = circular dichroism.

Figure 4: ADAAD alters the conformation of *DGCR8* pair 1 and 7 DNA. Mfold structures predicted for the (**A**) forward strand and (**B**) reverse strand of DGCR8 pair 1 oligonucleotide. (**C**) CD spectra of *DGCR8* pair 1 alone (black), *DGCR8* pair 1 incubated with ATP and ADAAD (red). Mfold structures predicted for the (**D**) forward strand and (**E**) reverse strand of DGCR8 pair 7 oligonucleotide. (**F**) CD spectra of *DGCR8* pair 7 alone (black), *DGCR8* pair 7 incubated with ATP and ADAAD (red). This figure has been modified from [26]. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; CD = circular dichroism.

Figure 5: ADAAD alters the conformation of *DICER* pair 1 DNA. Mfold structure predicted for the (**A**) forward strand and (**B**) reverse strand of DICER pair 1 oligonucleotide. (**C**) CD spectra of *DICER* pair 1 alone (black), *DICER* pair 1 incubated with ATP and ADAAD (red). This figure

has been modified from [26]. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; CD = circular dichroism.

Table 1: Buffer components.

Table 2: Oligonucleotide sequences. All the sequences are in the 5'-3' direction. Abbreviations: slDNA = stem-loop DNA.

Table 3: CD spectra peak corresponding to different forms of DNA with their role in transcription. Abbreviations: ADAAD = Active DNA-dependent ATPase A Domain; CD = circular dichroism.

DISCUSSION

The purpose of this article is to introduce the CD spectroscopy technique as an approach to study the conformational changes occurring in the DNA in the presence of ATP-dependent chromatin remodeling proteins and to link these conformational changes to gene expression. CD spectroscopy provides a fast and easily accessible method to study the conformational changes in DNA.

A crucial point to be considered for this technique is the purity of the DNA and protein. It is advisable to ensure that both DNA and protein are >95% pure. PAGE-purified oligonucleotides must be used in the assay, and the protein should be preferably affinity-purified to >95% purity. The other critical parameter is that the cuvette should be clean such that the baseline reading does not exceed 1 mdeg. The buffers should be made using autoclaved water, and the baseline reading of the buffer should not exceed 1 mdeg. To study the conformation of the promoter, it is essential to identify the regions where the protein binds. Therefore, it is advisable to perform ChIP experiments using the protein of interest as this process helps to identify DNA sequences present in the promoter region of the effector gene bound by the protein. Once the region is identified, the ability of the sequence to adopt specific structures can be analyzed using available bioinformatics tools. This is important as the ChIP primers are usually 200 bp long and may have multiple conformations. Therefore, using bioinformatics tools to identify the structures would help shorten the length of the oligonucleotide to one structure.

Finally, if the protein of interest is an ATP-dependent chromatin remodeling protein, the ability of the oligonucleotides to act as an effector must be checked using ATPase assays. In both CD spectroscopy and ATPase assays, care should be taken to ensure that saturating concentrations of ligands are used in the reaction. If possible, the dissociation constant (K_d) for the protein-ligand interaction should be calculated before proceeding with CD spectroscopy. Numerous methods are available for calculating the binding parameter. Using ATPase assays, the Michaelis-Menten constant (K_M) can be calculated by titrating increasing concentrations of DNA. The K_M, in many cases, can be approximated to the binding constant. If the protein is fluorescent, binding constants can be calculated using fluorescence spectroscopy. If neither of these techniques is feasible, the electrophoresis mobility shift assay (EMSA) can be used.

The main problem with CD spectroscopy arises when the cuvettes are not cleaned or when the reagents are impure. If the baseline is too high, it is advisable to clean the cuvettes. Numerous cuvette cleaning solutions are available. Placing the cuvettes in a dilute acid solution for 16–24 h also helps clean the cuvette. It is advisable to purchase reagents that are >95% pure and to use double-distilled and autoclaved water. Baseline drift is another potential problem. If doing a long-term experiment, it is advisable to periodically check the baseline. The peaks of DNA when studying protein–DNA interactions may not exactly correspond to the peaks/bands obtained with DNA alone. Protein peaks are usually observed in the Far UV range between 190 and 230 nm. Therefore, peaks below 250 nm might have interference from the protein peak and might not provide reliable information. DNA can adopt a variety of non-B conformations depending upon the sequence. The longer the DNA sequence, the higher the chance of multiple conformations co-existing within the DNA oligonucleotide. This can make analysis difficult. Hence, it is advisable to use shorter oligonucleotides corresponding to the potential structures predicted by the bioinformatic tools.

The other major drawback of CD spectroscopy is that it does not allow for atomic-level structure analysis, and the obtained spectrum is insufficient to identify the only viable structure. For example, both X-ray crystallography and protein NMR spectroscopy provide atomic resolution data, whereas CD spectroscopy provides less detailed structural information. However, CD spectroscopy is a rapid approach that does not necessitate vast quantities of proteins or considerable data processing. As a result, CD may be used to investigate a wide range of solvent variables such as temperature, pH, salinity, and the presence of various cofactors. It can also be used to monitor structural changes (due to complex formation, folding/unfolding, denaturation due to temperature, denaturants, and changes in amino acid sequence/mutation) in dynamic systems. By connecting it to the stop-flow apparatus, it can also be used to study the kinetics of protein/DNA-ligand interactions.

Well-characterized DNA conformers include A/B/Z DNA, triplex, hairpin, and G-quadruplexes. All these forms of DNA are associated with an open DNA conformation, i.e., unwound DNA that serves as the sink for negative supercoiling. Transcription is associated with negative supercoiling as the formation of an open complex is a prerequisite for the movement of RNA polymerase. Therefore, transcription of most genes involves increased negative supercoiling in the promoter region. Studies have shown that nucleosome unfolding leads to A-DNA conformation, which, however, is unstable[39]. One possibility is that the protein of interest, e.g., SMARCAL1, binds to such structures and stabilizes them, thus facilitating transcription, as seen in the case of *DROSHA* promoters. The guanine quadruplex is based on guanine tetrads bound by Hoogsteen hydrogen bonds. *In silico* analysis has confirmed that G4-forming sequences are notably enriched proximal to gene promoters and at transcription start sites. These G4 sequences can both activate and repress transcription.

In the case of the *MYC* promoter[40], the formation of G-quadruplex acts as a repressor, while in the case of human vascular endothelial growth factor (*VEGF*), the G-quadruplex structure functions as a docking site for transcription factors[41], thus activating the expression of this gene. In the case of SMARCAL1, the G-quadruplex structure was observed when ADAAD interacted with *DGCR8* pair 7 promoter sequences. As the occupancy of SMARCAL1 and RNAPII increased on this primer pair, it is hypothesized that the formation of G-quadruplex,

in this case, correlates with transcription activation of this gene. DNA can also be positively supercoiled, and the progress of RNA polymerase is known to generate positive supercoiling in front of it. This transcription-generated (+) supercoiling can disrupt or eliminate road-block proteins, destabilizing nucleosome structures to make the DNA more accessible to RNA polymerase. The X-DNA is a conformation of DNA that acts as sinks for positive supercoiling. The striking feature of an X-DNA is it can form in a sequence-specific manner on the promoter of a gene. In the case of SMARCAL1, ADAAD induced A-X and B-X transitions in an ATP-dependent manner in the *DICER* and *DGCR8* promoters, respectively. Combined with *in vivo* data where increased SMARCAL1 and RNAPII occupancy was found on these promoters in the presence of doxorubicin-induced DNA damage, it can be hypothesized that X-DNA formation facilitates transcription by removing barriers/blocks. Triple DNA helices do not have a characteristic spectrum. The CD spectra of the DNA sequences present in the *MYC* promoter and the synthetic stem-loop DNA showed two positive peaks—one at 258 nm with a shoulder at 269 nm and a larger peak at 210 nm in the DNA. This type of spectrum can be obtained in the case of triplexes[37]. Triplexes are difficult to unwind and, therefore, are known to block transcription[42]. Hence, it is hypothesized that the formation of this structure in the c-*MYC* promoter by SMARCAL1 leads to repression of transcription.

It should be noted that ATP also binds to ATP-dependent chromatin remodeling proteins. The conserved arginine present in the motif VI of the helicase domain of these proteins interacts via electrostatic interactions with the γ-phosphate of the protein[43]. In the case of ADAAD, the K_d of protein–ATP interaction is $(1.5 \pm 0.1) \times 10^{-6}$ M[38]. The binding of ATP induces a conformational change in the protein such that the affinity of the DNA increases. The binding of DNA also induces a conformation change in the protein leading to an increased 10-fold affinity for ATP[31]. For example, in the case of ADAAD, bands/peaks are observed at -212 nm and -222 nm. ATP also gives bands at 197 nm, +210 nm, -222 nm, -247 nm, and -270 nm. These must be subtracted from the spectra of DNA + ADAAD + ATP to obtain the "net" conformation of the DNA in the presence of the ligands.

Thus, this paper shows the convenience of CD spectroscopy for the study of the conformational changes occurring in the DNA in the presence of ATP-dependent chromatin remodeling proteins. Correlating the changes in the DNA conformation with ChIP data can provide the investigators with information regarding how the DNA conformers activate/repress transcription.

ACKNOWLEDGEMENT:
The authors would like to thank Advanced Instrumentation Research Facility, JNU, for the CD spectrophotometer. V.J. and A.D. were supported by a fellowship from CSIR.

DISCLOSURES:
The authors have no conflict of interest to declare.

REFERENCES:

1. Woody, R.W. [4] Circular dichroism. *Methods in Enzymology*. **246**, 34–71 (1995).
2. Kelly, S., Price, N. The Use of Circular Dichroism in the Investigation of Protein Structure and Function. *Current Protein & Peptide Science*. **1** (4), 349–384 (2000).

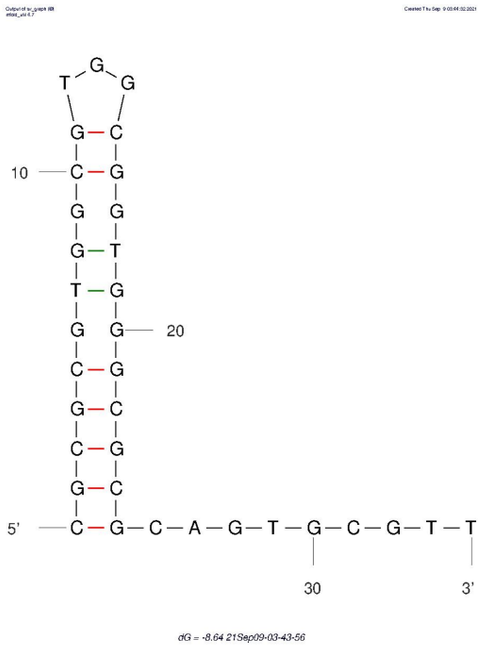
3. Rodger, A., Marshall, D. Beginners guide to circular dichroism. *The Biochemist*. **43** (2), 58–64 (2021).
4. Greenfield, N. J. Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols*. **1** (6), 2876–2890 (2006).
5. Kypr, J., Kejnovská, I., Bednářová, K., Vorlíčková, M. Circular Dichroism Spectroscopy of Nucleic Acids. *Comprehensive Chiroptical Spectroscopy*. 575–586 (2012).
6. Kypr, J., Kejnovska, I., Renciuk, D., Vorlickova, M. Circular dichroism and conformational polymorphism of DNA. *Nucleic Acids Research*. **37** (6), 1713–1725 (2009).
7. Miyahara, T., Nakatsuji, H., Sugiyama, H. Helical Structure and Circular Dichroism Spectra of DNA: A Theoretical Study. *The Journal of Physical Chemistry A*. **117** (1), 42–55 (2013).
8. Khomyakova, E. B. Parallel intramolecular DNA triple helix with G and T bases in the third strand stabilized by Zn^{2+} ions. *Nucleic Acids Research*. **28** (18), 3511–3516 (2000).
9. Kypr, J. et al. The unusual X-form DNA in oligodeoxynucleotides: dependence of stability on the base sequence and length. *Journal of Biomolecular Structure & Dynamics*. **13** (6), 999–1006 (1996).
10. Zohoorian-Abootorabi, T., Sanee, H., Iranfar, H., Saberi, M. R., Chamani, J. Separate and simultaneous binding effects through a non-cooperative behavior between cyclophosphamide hydrochloride and fluoxymesterone upon interaction with human serum albumin: multi-spectroscopic and molecular modeling approaches. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*. **88**, 177–191 (2012).
11. Sharifi-Rad, A., Mehrzad, J., Darroudi, M., Saberi, M. R., Chamani, J. Oil-in-water nanoemulsions comprising Berberine in olive oil: biological activities, binding mechanisms to human serum albumin or holo-transferrin and QMMD simulations. *Journal of Biomolecular Structure & Dynamics*. **39** (3), 1029–1043 (2021).
12. Mokaberi, P., Babayan-Mashhadi, F., Amiri Tehrani Zadeh, Z., Saberi, M. R., Chamani, J. Analysis of the interaction behavior between Nano-Curcumin and two human serum proteins: combining spectroscopy and molecular stimulation to understand protein-protein interaction. *Journal of Biomolecular Structure & Dynamics*. **39** (9), 3358–3377 (2021).
13. Danesh, N. et al. Determining the binding site and binding affinity of estradiol to human serum albumin and holo-transferrin: fluorescence spectroscopic, isothermal titration calorimetry and molecular modeling approaches. *Journal of Biomolecular Structure & Dynamics*. **36** (7), 1747–1763 (2018).
14. Sadeghzadeh, F. et al. Characterizing the binding of angiotensin converting enzyme I inhibitory peptide to human hemoglobin: influence of electromagnetic fields. *Protein and Peptide Letters*. **27** (10), 1007–1021 (2020).
15. Chamani, J. et al. Cooperative alpha-helix formation of beta-lactoglobulin induced by sodium n-alkyl sulfates. *Journal of Colloid and Interface Science*. **293** (1), 52–60 (2006).
16. Dareini, M. et al. A novel view of the separate and simultaneous binding effects of docetaxel and anastrozole with calf thymus DNA: Experimental and in silico approaches. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*. **228**, 117528 (2020).
17. Dehghani Sani, F. et al. Changes in binding affinity between ofloxacin and calf thymus DNA in the presence of histone H1: Spectroscopic and molecular modeling investigations. *Journal of Luminescence*. **203**, 599–608 (2018).
18. Hargreaves, D. C., Crabtree, G. R. ATP-dependent chromatin remodeling: genetics, genomics and mechanisms. *Cell Research*. **21** (3), 396–420 (2011).

19. Morettini, S., Podhraski, V., Lusser, A. ATP-dependent chromatin remodeling enzymes and their various roles in cell cycle control. *Frontiers in Bioscience: A Journal and Virtual Library*. **13**, 5522–5532 (2008).
20. Flaus, A., Martin, D. M. A., Barton, G. J., Owen-Hughes, T. Identification of multiple distinct Snf2 subfamilies with conserved structural motifs. *Nucleic Acids Research*. **34** (10), 2887–2905 (2006).
21. Flaus, A., Owen-Hughes, T. Mechanisms for ATP-dependent chromatin remodelling: the means to the end. *The FEBS Journal*. **278** (19), 3579–3595 (2011).
22. Mizuguchi, G. et al. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science (New York, N.Y.)*. **303** (5656), 343–348 (2004).
23. Elizondo, L. I. et al. Schimke immuno-osseous dysplasia: SMARCAL1 loss-of-function and phenotypic correlation. *Journal of Medical Genetics*. **46** (1), 49–59 (2009).
24. Baradaran-Heravi, A. et al. SMARCAL1 deficiency predisposes to non-Hodgkin lymphoma and hypersensitivity to genotoxic agents in vivo. *American Journal of Medical Genetics. Part A*. **158A** (9), 2204–2213 (2012).
25. Bansal, R. et al. SMARCAL1, the annealing helicase and the transcriptional co-regulator. *IUBMB life*. **72** (10), 2080–2096 (2020).
26. Yusufzai, T., Kadonaga, J. T. HARP is an ATP-driven annealing helicase. *Science (New York, N.Y.)*. **322** (5902), 748–750 (2008).
27. Yusufzai, T., Kong, X., Yokomori, K., Kadonaga, J. T. The annealing helicase HARP is recruited to DNA repair sites via an interaction with RPA. *Genes & Development*. **23** (20), 2400–2404 (2009).
28. Patne, K. et al. BRG1 and SMARCAL1 transcriptionally co-regulate DROSHA, DGCR8 and DICER in response to doxorubicin-induced DNA damage. *Biochimica et Biophysica Acta*. **1860** (9), 936–951 (2017).
29. Sharma, T., Bansal, R., Haokip, D. T., Goel, I., Muthuswami, R. SMARCAL1 negatively regulates c-Myc transcription by altering the conformation of the promoter region. *Scientific Reports*. **5**, 17910 (2015).
30. Muthuswami, R., Truman, P. A., Mesner, L. D., Hockensmith, J. W. A eukaryotic SWI2/SNF2 domain, an exquisite detector of double-stranded to single-stranded DNA transition elements. *The Journal of Biological Chemistry*. **275** (11), 7648–7655 (2000).
31. Nongkhlaw, M., Dutta, P., Hockensmith, J. W., Komath, S. S., Muthuswami, R. Elucidating the mechanism of DNA-dependent ATP hydrolysis mediated by DNA-dependent ATPase A, a member of the SWI2/SNF2 protein family. *Nucleic Acids Research*. **37** (10), 3332–3341 (2009).
32. Kikin, O., D'Antonio, L., Bagga, P. S. QGRS Mapper: a web-based server for predicting G-quadruplexes in nucleotide sequences. *Nucleic Acids Research*. **34** (Web Server), W676–W682 (2006).
33. Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research*. **31** (13), 3406–3415 (2003).
34. Gupta, M. et al. Ligand-induced conformation changes drive ATP hydrolysis and function in SMARCAL1. *The FEBS Journal*. **282** (19), 3841–3859 (2015).
35. Dutta, P. et al. Global epigenetic changes induced by SWI2/SNF2 inhibitors characterize neomycin-resistant mammalian cells. *PloS One*. **7** (11), e49822 (2012).
36. Muthuswami, R. et al. Phosphoaminoglycosides inhibit SWI2/SNF2 family DNA-dependent molecular motor domains. *Biochemistry*. **39** (15), 4358–4365 (2000).

37. Gondeau, C. Circular dichroism and UV melting studies on formation of an intramolecular triplex containing parallel T*A:T and G*G:C triplets: netropsin complexation with the triplex. *Nucleic Acids Research*. **26** (21), 4996–5003 (1998).
38. Nongkhlaw, M., Gupta, M., Komath, S. S., Muthuswami, R. Motifs Q and I are required for ATP hydrolysis but not for ATP binding in SWI2/SNF2 proteins. *Biochemistry*. **51** (18), 3711–3722 (2012).
39. Luchnik, A. N. DNA conformational transitions induced by supercoiling control transcription in chromatin. *Gene Regulation and Systems Biology*. **8**, 89–96 (2014).
40. Siddiqui-Jain, A., Grand, C. L., Bearss, D. J., Hurley, L. H. Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proceedings of the National Academy of Sciences of the United States of America*. **99** (18), 11593–11598 (2002).
41. Uribe, D. J., Guo, K., Shin, Y.-J., Sun, D. Heterogeneous nuclear ribonucleoprotein K and nucleolin as transcriptional activators of the vascular endothelial growth factor promoter through interaction with secondary DNA structures. *Biochemistry*. **50** (18), 3796–3806 (2011).
42. Young, S. L., Krawczyk, S. H., Matteucci, M. D., Toole, J. J. Triple helix formation inhibits transcription elongation in vitro. *Proceedings of the National Academy of Sciences of the United States of America*. **88** (22), 10023–10026 (1991).
43. Dürr, H., Flaus, A., Owen-Hughes, T., Hopfner, K.-P. Snf2 family ATPases and DExx box helicases: differences and unifying concepts from high-resolution crystal structures. *Nucleic Acids Research*. **34** (15), 4160–4167 (2006).

Figure 1

A.



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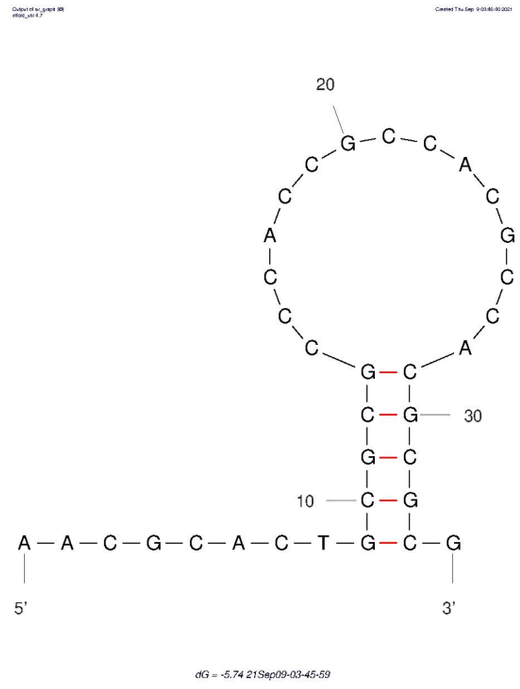

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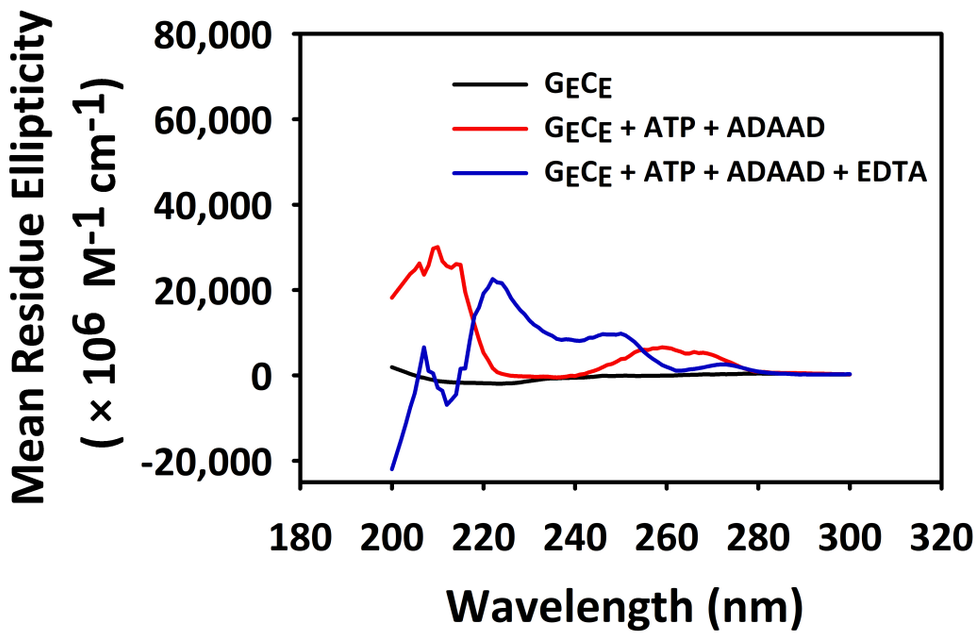

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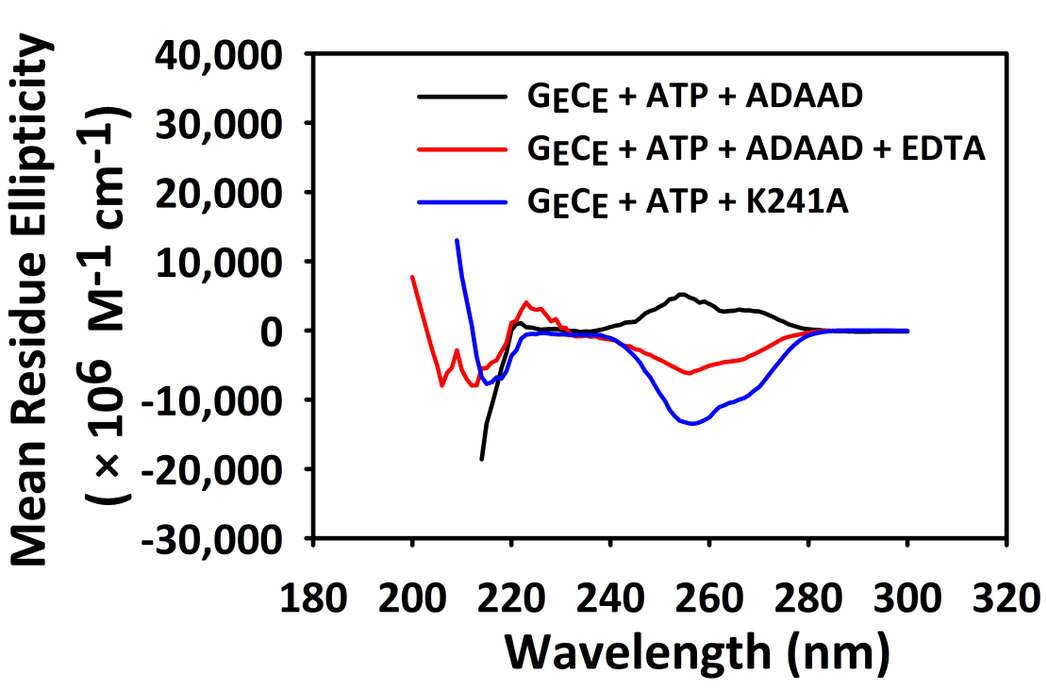

Figure 2

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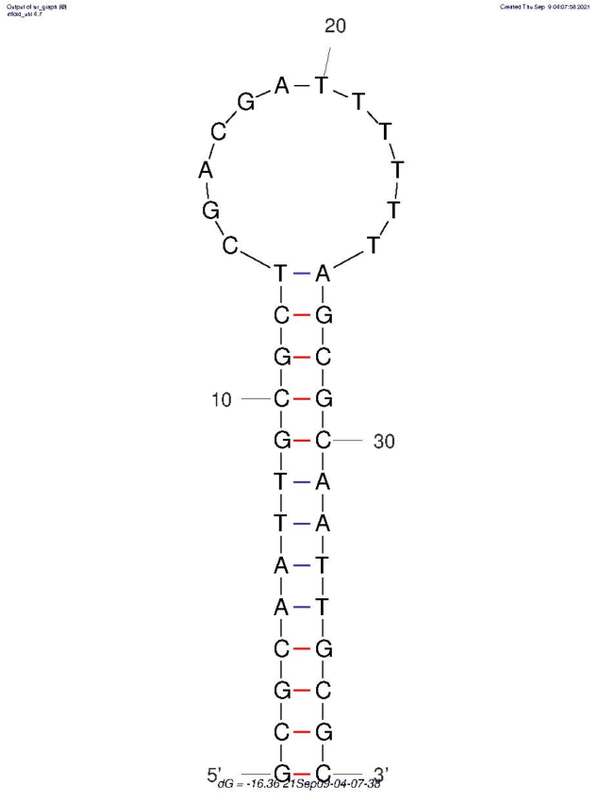

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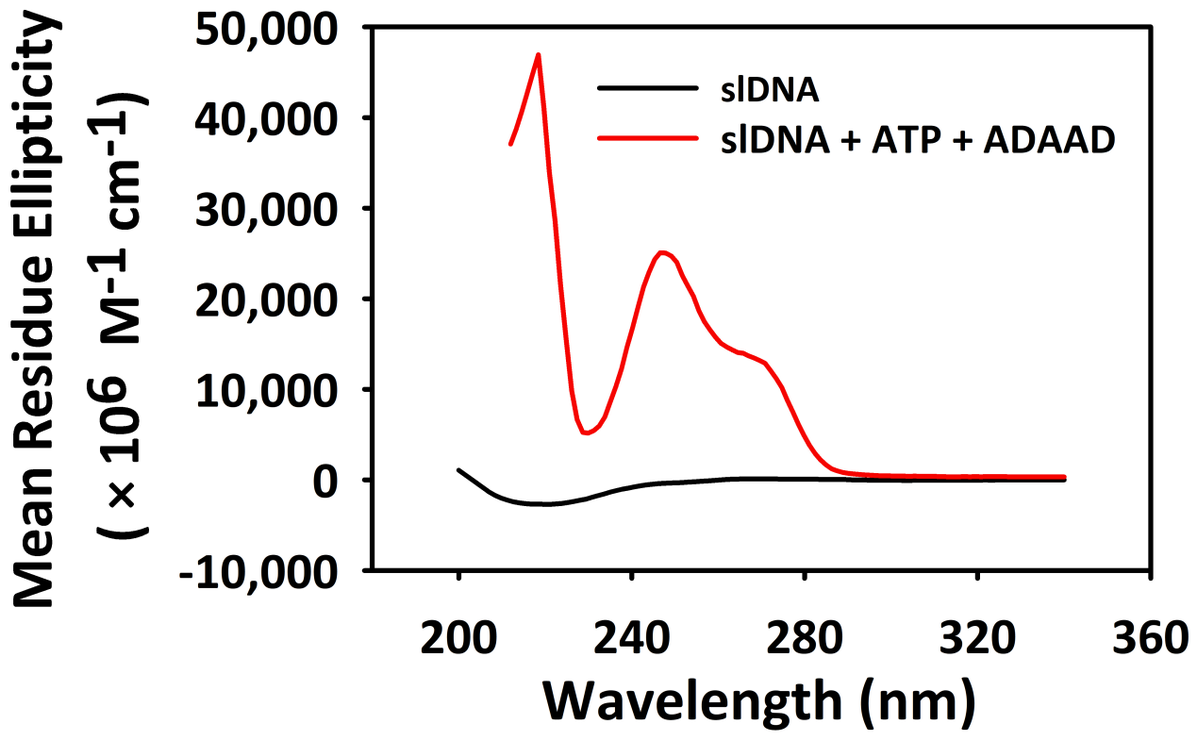
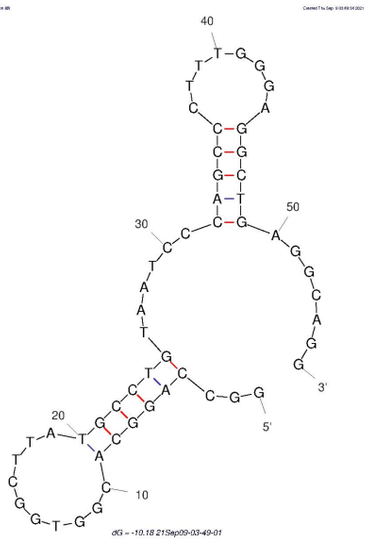


Figure 3

A.



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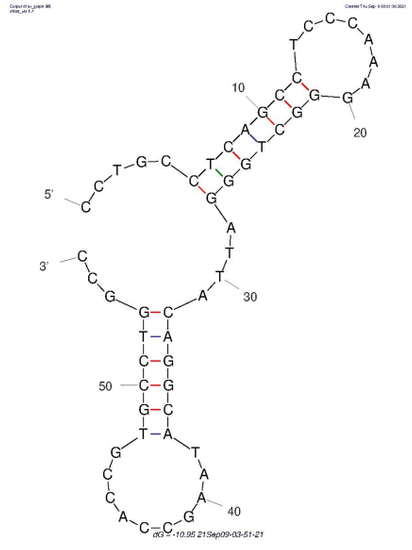

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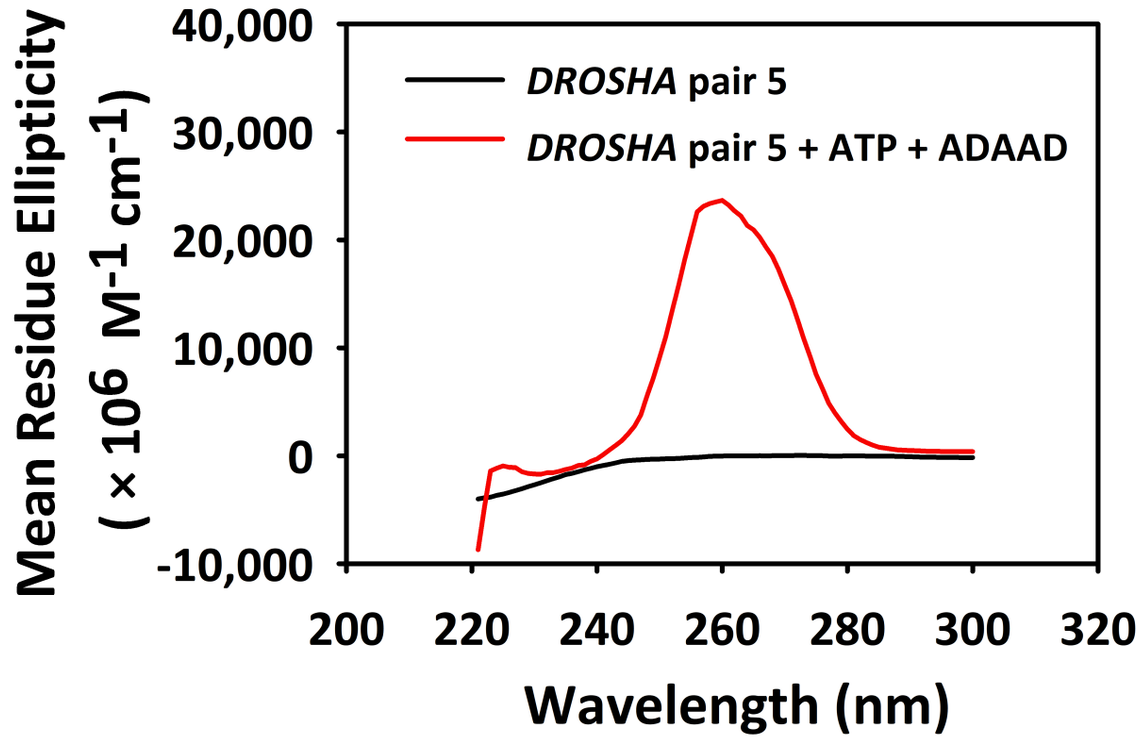
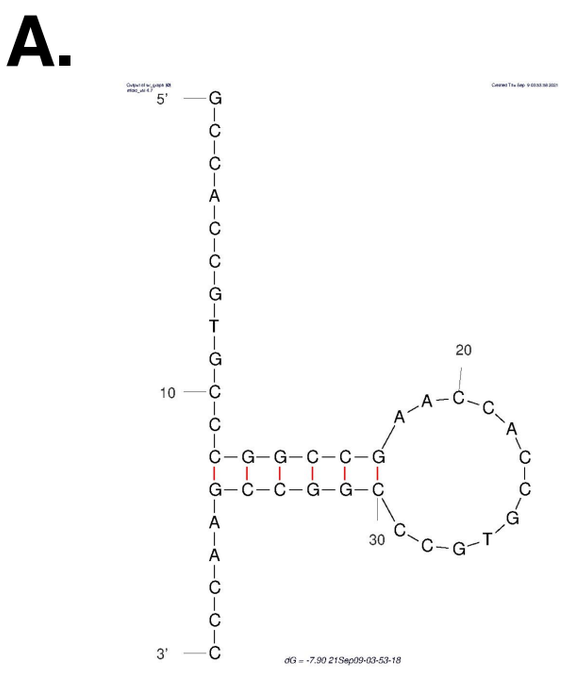
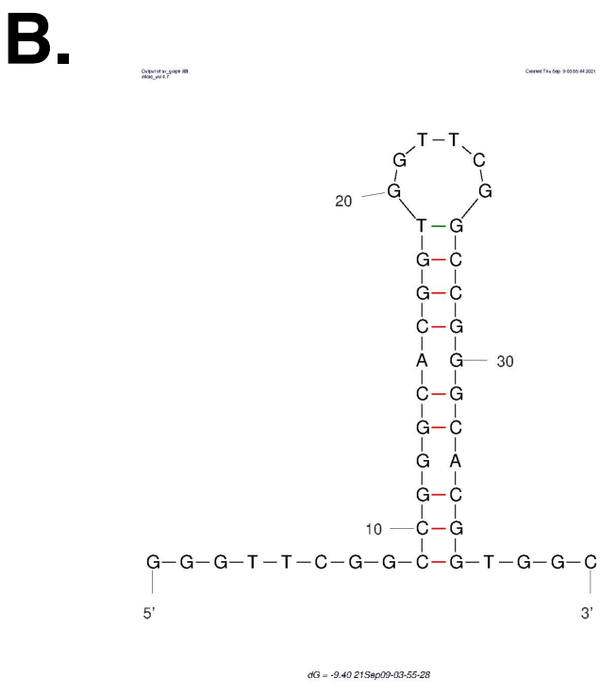
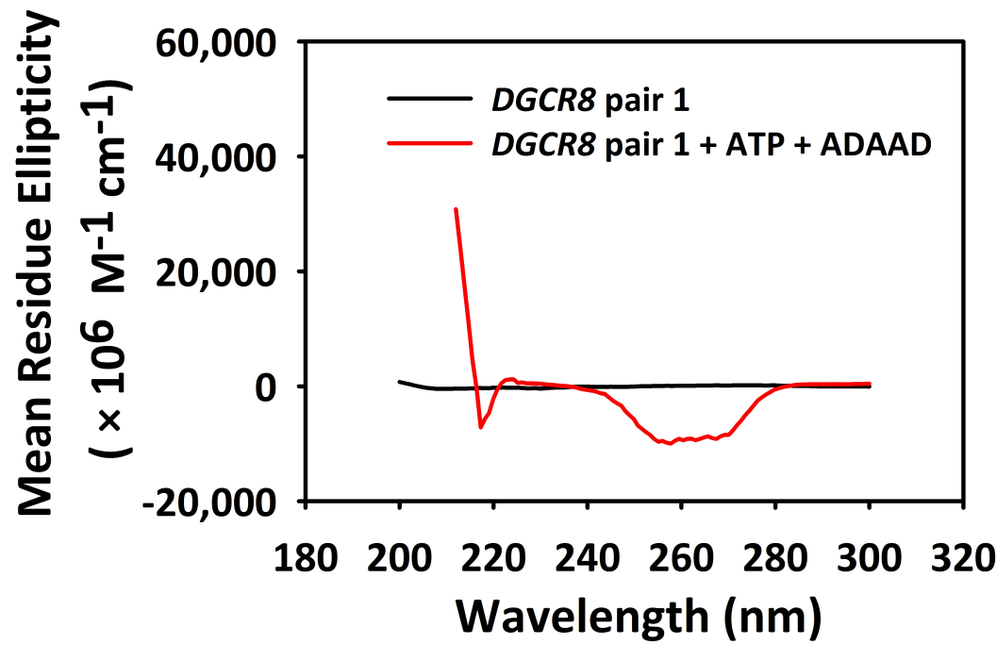
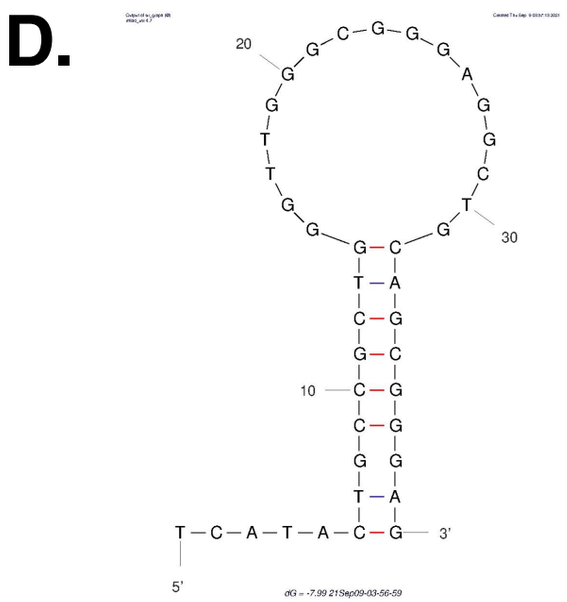


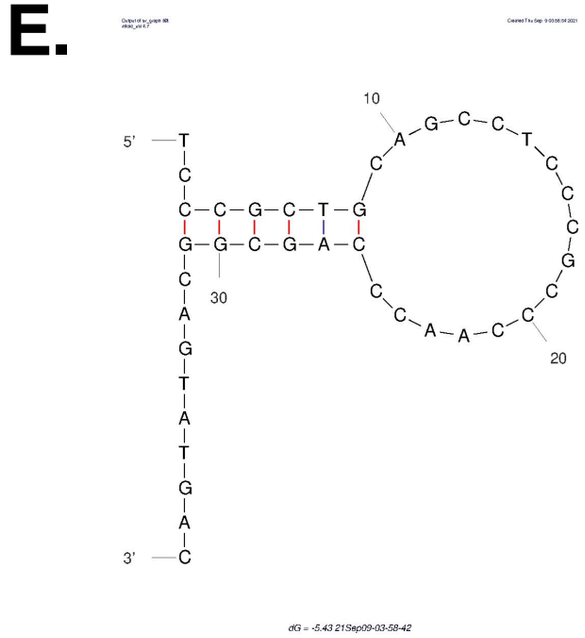
Figure 4











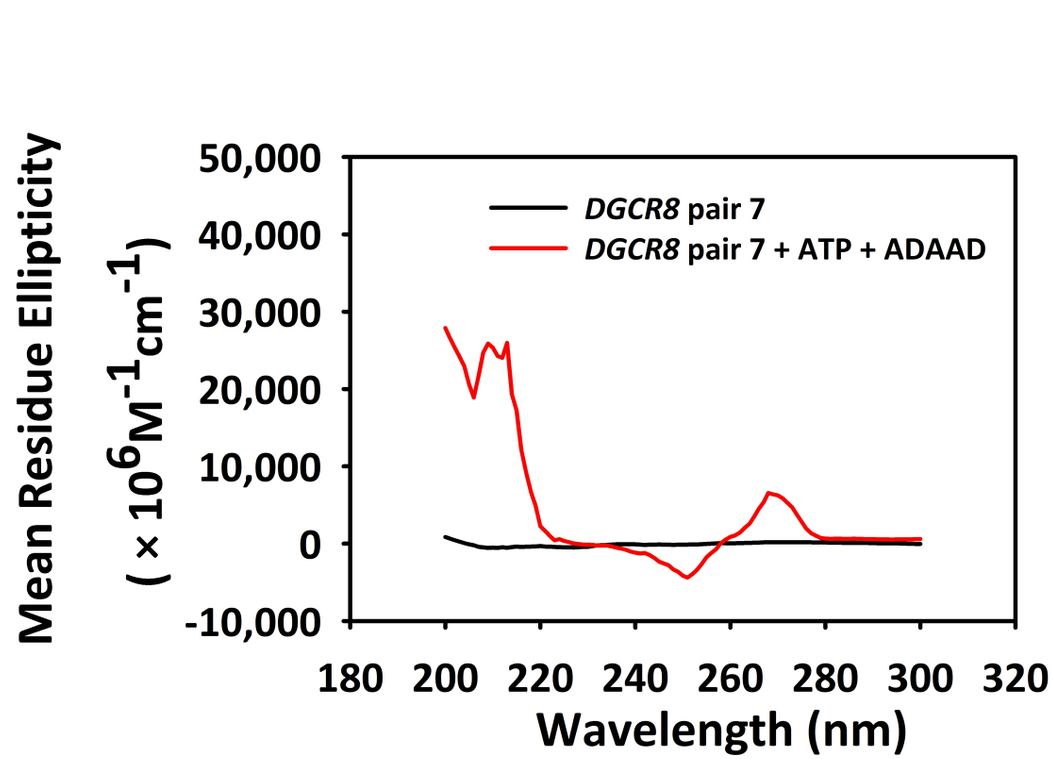
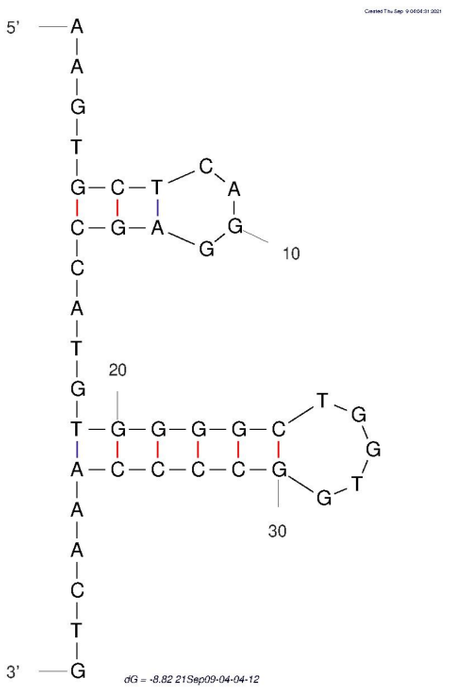


Figure 5

A.



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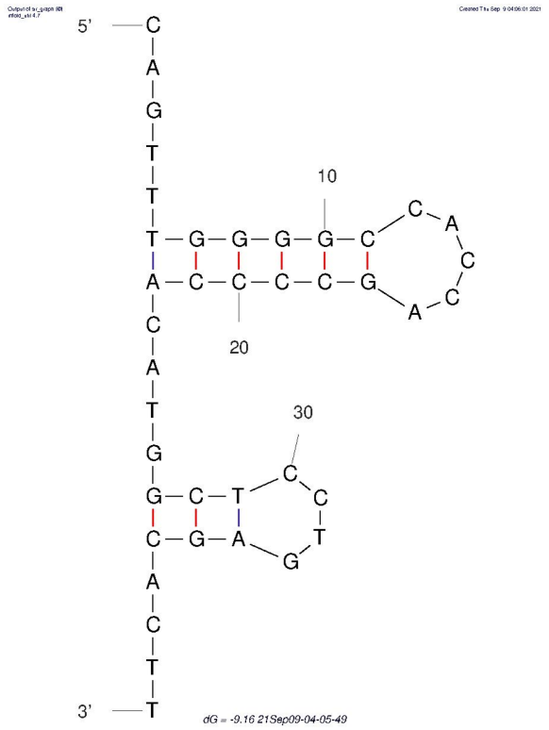

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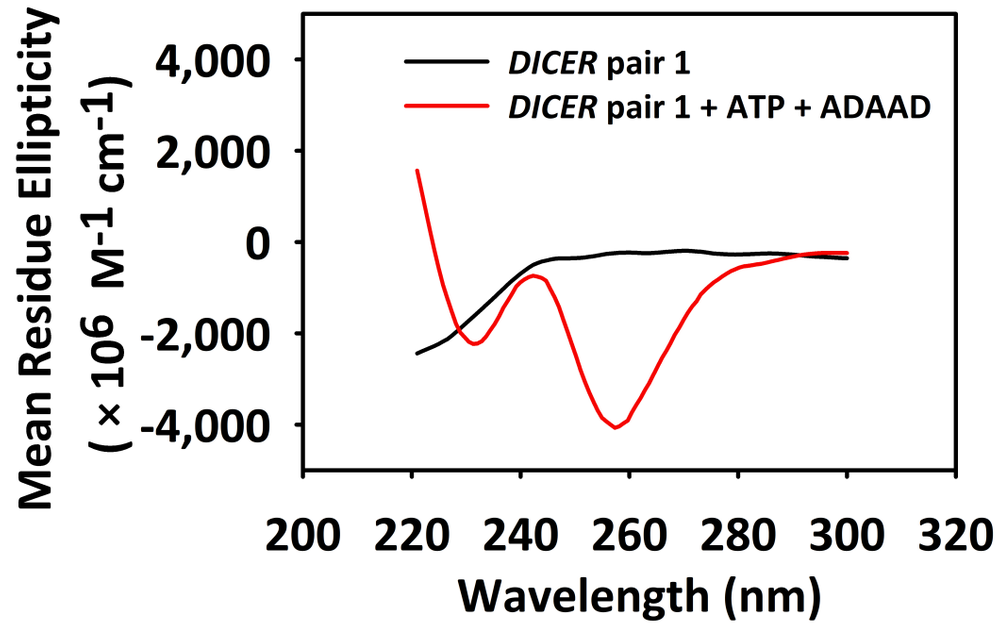

5x REG buffer

Component	**Working concentration**
Lactate dehydrogenase (LDH)	50 units/mL
Magnesium acetate ($Mg(OAc)_2$)	30 mM
Phosphoenolpyruvate (PEP)	6.8 mg/mL
Pottasium acetate (KOAc)	300 mM
Pyruvate kinase (PK)	50 units/mL
Tris acetate (Tris-OAc)	125 mM
β-mercaptoethanol (β-ME)	25 mM

Table 2

Table 2

Oligonucleotides	Forward sequence	ΔG (m-Fold) Kcal/mol	G-score (QGRS mapper)	Reverse sequence	ΔG kcal/mol (Mfold prediction)
slDNA	GCGCAATTGCGCTCGACGATTTTTTAGCGCAATTGCGC	-16.36	-	-	-
MYC G_EC_E	CGCGCGTGGCGTGGCGGTGGGCGCGCAGTGCGTT	-8.64	19	AACGCACTGCGCGCCCACCGCCACGCCACGCGCG	-5.74
DROSHA Pair 5	GGCCAGGCACGGTGGCTTATGCCTGTAATCCCAGCCCTTTGGGAGGCTGAGGCAGG	-10.18	19, 19	CCTGCCTCAGCCTCCCAAAGGGCTGGGATTACAGGCATAAGCCACCGTGCCTGGC	-10.95
DGCR8 Pair 1	GCCACCGTGCCCGGCCGAACCACCGTGCCCGGCCGAACCC	-7.9	-	GGGTTCGGCCGGGCACGGTGGTTCGGCCGGGCACGGTGGC	-9.4
DGCR8 Pair 7	TCATACTGCCGCTGGGTTGGGCGGGAGGCTGCAGCGGGAG	-7.99	33	TCCCGCTGCAGCCTCCCGCCCAACCCAGCGGCAGTATGAC	-5.43
DICER Pair 1	AAGTGCTCAGGAGCCATGTGGGGCTGGTGGCCCCAAACTG	-8.82	19	CAGTTTGGGGCCACCAGCCCCACATGGCTCCTGAGCACTT	-9.16

G-score (QGRS mapper)
-
-
-
21
-
-

Table 3

Oligonucleotides	CD Spectra Peaks (After incubating with ATP and ADAAD)	Form of DNA	Role in Transcription
slDNA	+215, +250, +272	Triplex	Repression
MYC G_EC_E	+210, +258, +269	Triplex	Repression
DROSHA Pair 5	-210, +260	A-DNA	Initiation/Activation
DGCR8 Pair 1	+210,-260	B-X Transition	Positive supercoiling/Activation
DGCR8 Pair 7	+210, -250, +270	G-quadruplex	Activation
DICER Pair 1	+210, -230, -257	A-X Transition	Positive supercoiling/Activation

RESPONSE TO REVIEWERS' COMMENTS

We would like to thank the reviewers for their comments and suggestions to improve the manuscript.

Reviewer #1:

Minor:
line 78 - Which conditions? Substitute "these" for "the"

Our Response: As suggested we have done the substitution.

lines 84 and 303 - CD spectroscopy may be an "easy and inexpensive method" provided you have access to the adequate "not so inexpensive" equipment and software.

Our Response: We have made the changes in the lines 84 and 303 as suggested.

line 134 - Substitute "must be" for "was"

Our Response: We have made the substitution in the revised manuscript.

line 325 - Specify EMSA in full.
``
Our Response: We have now specified EMSA in full in the revised manuscript.

Reviewer #2:
1- The introduction section needs more literature survey for better literature review. Some relevant references to this research should be cited for literature survey review as follow: a) Journal of Biomolecular Structure and Dynamics, 2021, 39 (3), 1029-1043. b) Journal of Biomolecular Structure and Dynamics, 2021, 39 (9), 3358-3377. c) Journal of Biomolecular Structure and Dynamics, 2018, 36 (7), 1747-1763. d) Journal of Colloid and Interface Science, 2006, 293 (1), 52-60. e) Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2012, 88, 177-191. f) Journal of Luminescence, 2018, 203, 599-608. g) Protein and peptide letters, 2020, 27 (10), 1007-1021. h) Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2020, 228, 117528.

Our Response: We thank the reviewer for pointing us to these references. We have now included them in the Introduction section.

> Changes in CD spectra can also reveal details about ligand–protein interactions, and therefore, have been added to the arsenal of molecular methods for detecting drug-protein interactions [1–5]. CD spectra has also been used to monitor the changes in the secondary structure of proteins during folding process [6]. Similarly, CD spectra can also be used for probing ligand-DNA interactions [7, 8]

2- In Fig. 1C, what is the EDTA role in the GECE-ATP-ADAAD complex formation? Which one of GECE, ATP and ADAAD, accept effect in the presence of EDTA? Please discuss in the text of manuscript. –

Our Response: ATP forms a coordination complex with Mg^{+2} and thus, this cation is essential for ATP hydrolysis EDTA chelates Mg^{+2} and thus, inhibits the ATP hydrolysis. We have discussed this in the manuscript.

ATP forms a coordination complex with Mg^{+2} and this cation is essential for ATP hydrolysis. Addition of EDTA chelates Mg^{+2} leading to inhibition of ATP hydrolysis [9]. Therefore, to understand whether ATP hydrolysis by ADAAD was important for conformational change, EDTA was added to the reaction mix. Addition of EDTA to the reaction abrogates this conformation.

3- In Fig. 1D, ATP and ADAAD have CD spectrum in the same regions (similar wavelength? How did the authors separate the spectra and discuss about the complex spectrum? The legend of Fig. 1D should be added.

Our Response: CD spectra for buffer alone, buffer + ATP alone, buffer + ADAAD alone recorded. Then CD spectra for buffer + DNA is recorded, followed by buffer + DNA +ATP and finally, Buffer + DNA +ATP +ADAAD is recorded. To calculate the conformation change induced in DNA due to ADAAD and ATP, the CD spectra of buffer + ATP +ADAAD is subtracted from buffer + DNA+ATP+ ADAAD. We apologize for the oversight.
Figure 1D legend has been added in the revised manuscript.

4- In Fig. 2B, what is the slDNA role in the ellipticity values of ATP-ADAAD complex from the viewpoint of energetic content of complex and type of interaction behavior between ATP and ADAAD? Two relevant references to this research should be cited for better understanding as follow: a) Journal of Biomolecular Structure and Dynamics, 2019, 37 (9), 2265-2282. b) Journal of Colloid and Interface Science, 2006, 297 (2), 561-569.

Our Response: We have recorded the CD spectra of the protein in the presence of ATP and DNA. We do find that the secondary structure of the protein is altered in presence of the ligands. We have published the data on the alterations in the protein structure[9–11].
However, as the focus was on DNA structure, we have not emphasized much on the protein structure other than to point out that the protein CD spectra should be subtracted out from the DNA+ protein spectra.

5- If the experiments were done at situation in vivo, does the binding cause between ATP and ADAAD? Please explain in the text of manuscript with using binding constant values.

Our Response: Yes, in vivo, ADAAD (or rather the full-length SMARCAL1) binds to DNA and ATP. This interaction is essential for transcription regulation as well as for DNA damage response. There are no reports calculating the binding constants *in vivo*. *In vitro*, the dissociation constant for the interaction between ATP and ADAAD in the absence of DNA is $(1.5 \pm 0.1) \times 10^{-6}$ M. In the presence of DNA, the affinity for ATP increases by 10-fold. We have now explained it in the manuscript.

It should be noted that ATP also binds to the ATP-dependent chromatin remodeling proteins. The conserved arginine present in the motif VI of the helicase domain of these proteins interacts via electrostatic interactions with the γ-phosphate of the protein. In case of ADAAD, the K_d of protein-ATP interaction is $(1.5 \pm 0.1) \times 10^{-6}$ M. Binding of ATP induces a conformational change in the protein such that the affinity of the DNA increases. Binding of DNA also induces a conformation change in the protein leading increased 10-fold affinity for ATP. For example, in case of ADAAD, bands/peaks are observed at -212 nm and -222 nm. ATP also gives bands at + 197 nm, +210 nm, -222 nm, -247 nm and -270 nm. These must be subtracted from the spectra of DNA + ADAAD + ATP to obtain the "net" conformation of the DNA in the presence of the ligands.

6- In Fig. 3C, the authors should determine the type of chirality's of ATP-ADAAD complex and discuss about this.

Our Response: We have performed CD spectra of ATP-ADAAD. We do not see any peak reversal leading us to conclude that the chirality of the protein does not alter when in complex with ATP.

7- The authors should discuss about the type of interaction forces between ATP and ADAAD from the viewpoint of conformational changes of them.

Our Response: ADAAD belongs to the SF2 superfamily of helicases. The SF2 superfamily contains conserved helicase motifs- Q, motif I (GKT box), motif II (DEAD box), motif III (SAT box), motif IV, motif V, and motif VI. Studies with helicases as well as crystal structures of ATP-dependent chromatin remodeling proteins like Rad54, have shown that Q, motif I, motif II, and motif III are present in RecA-like domain I while motif III, motif IV, and motif V are present in RecA-like domain 2. The motifs Q and I are required for ATP hydrolysis. The γ-phosphate of ATP binds with the conserved arginine of the motif VI via electrostatic interaction.
We have provided a short paragraph in the discussion:
It should be noted that ATP also binds to the ATP-dependent chromatin remodeling proteins. The conserved arginine present in the motif VI of the helicase domain of these proteins interacts via electrostatic interactions with the γ-phosphate of the protein. In case of ADAAD, the K_d of protein-ATP interaction is $(1.5 \pm 0.1) \times 10^{-6}$ M. Binding of ATP induces a conformational change in the protein such that the affinity of the DNA increases. Binding of DNA also induces a conformation change in the protein leading increased 10-fold affinity for ATP. For example, in case of ADAAD, bands/peaks are observed at -212 nm and -222 nm. ATP also gives bands at 197 nm, +210 nm, -222 nm, -247 nm and -270 nm. These must be subtracted from the spectra of DNA + ADAAD + ATP to obtain the "net" conformation of the DNA in the presence of the ligands.

References:

1. Zohoorian-Abootorabi, T., Sanee, H., Iranfar, H., Saberi, M.R., Chamani, J. Separate and simultaneous binding effects through a non-cooperative behavior between cyclophosphamide hydrochloride and fluoxymesterone upon interaction with human serum albumin: multi-spectroscopic and molecular modeling approaches. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*. **88**, 177–191, doi: 10.1016/j.saa.2011.12.026 (2012).
2. Sharifi-Rad, A., Mehrzad, J., Darroudi, M., Saberi, M.R., Chamani, J. Oil-in-water nanoemulsions comprising Berberine in olive oil: biological activities, binding mechanisms to human serum albumin or holo-transferrin and QMMD simulations. *Journal of Biomolecular Structure & Dynamics*. **39** (3), 1029–1043, doi: 10.1080/07391102.2020.1724568 (2021).
3. Mokaberi, P., Babayan-Mashhadi, F., Amiri Tehrani Zadeh, Z., Saberi, M.R., Chamani, J. Analysis of the interaction behavior between Nano-Curcumin and two human serum proteins: combining spectroscopy and molecular stimulation to understand protein-protein interaction.

Journal of Biomolecular Structure & Dynamics. **39** (9), 3358–3377, doi: 10.1080/07391102.2020.1766570 (2021).
4. Danesh, N., Navaee Sedighi, Z., Beigoli, S., Sharifi-Rad, A., Saberi, M.R., Chamani, J. Determining the binding site and binding affinity of estradiol to human serum albumin and holo-transferrin: fluorescence spectroscopic, isothermal titration calorimetry and molecular modeling approaches. *Journal of Biomolecular Structure & Dynamics*. **36** (7), 1747–1763, doi: 10.1080/07391102.2017.1333460 (2018).
5. Sadeghzadeh, F. *et al.* Characterizing the Binding of Angiotensin Converting Enzyme I Inhibitory Peptide to Human Hemoglobin: Influence of Electromagnetic Fields. *Protein and Peptide Letters*. **27** (10), 1007–1021, doi: 10.2174/1871530320666200425203636 (2020).
6. Chamani, J. *et al.* Cooperative alpha-helix formation of beta-lactoglobulin induced by sodium n-alkyl sulfates. *Journal of Colloid and Interface Science*. **293** (1), 52–60, doi: 10.1016/j.jcis.2005.06.015 (2006).
7. Dareini, M. *et al.* A novel view of the separate and simultaneous binding effects of docetaxel and anastrozole with calf thymus DNA: Experimental and in silico approaches. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*. **228**, 117528, doi: 10.1016/j.saa.2019.117528 (2020).
8. Dehghani Sani, F., Shakibapour, N., Beigoli, S., Sadeghian, H., Hosainzadeh, M., Chamani, J. Changes in binding affinity between ofloxacin and calf thymus DNA in the presence of histone H1: Spectroscopic and molecular modeling investigations. *Journal of Luminescence*. **203**, 599–608, doi: 10.1016/j.jlumin.2018.06.083 (2018).
9. Nongkhlaw, M., Gupta, M., Komath, S.S., Muthuswami, R. Motifs Q and I are required for ATP hydrolysis but not for ATP binding in SWI2/SNF2 proteins. *Biochemistry*. **51** (18), 3711–3722, doi: 10.1021/bi2014757 (2012).
10. Nongkhlaw, M., Dutta, P., Hockensmith, J.W., Komath, S.S., Muthuswami, R. Elucidating the mechanism of DNA-dependent ATP hydrolysis mediated by DNA-dependent ATPase A, a member of the SWI2/SNF2 protein family. *Nucleic Acids Research*. **37** (10), 3332–3341, doi: 10.1093/nar/gkp178 (2009).
11. Bansal, R., Arya, V., Sethy, R., Rakesh, R., Muthuswami, R. RecA-like domain 2 of DNA-dependent ATPase A domain, a SWI2/SNF2 protein, mediates conformational integrity and ATP hydrolysis. *Bioscience Reports*. **38** (3), doi: 10.1042/BSR20180568 (2018).

Reviewer #3:

A. Major points

1. Although it is stated that protein CD spectrum might interfere with the DNA spectra recorded here, provision is not made for the presentation of at least one CD spectrum of the ADAAD protein, at least in the UV range 180-250nm, where proteins also exhibit CD peaks. Ideally, the potential users should be guided to subtract the spectrum of the protein from the spectrum of the oligonucleotide plus protein mixture, so that they can base their interpretations on DNA conformation on the "net" oligonucleotide spectrum. This would be the rigorous process to follow. But, even if they do not propose this correction, the authors should at least show and suggest recording of a protein CD spectrum so that the users can judge by themselves which of the peaks recorded are exclusively due to the DNA and which ones might also result from the protein itself, so that they do not misassign their peaks and misinterpret their data on DNA conformation.

Our Response: ADAAD, bands/peaks are observed at -212 nm and -222 nm. ATP also gives bands at 197 nm, +210 nm, -222 nm, -247 nm and -270 nm. These must be subtracted from the spectra of DNA + ADAAD + ATP to obtain the “net” conformation of the DNA in the presence of the ligands.

Therefore, CD spectra for buffer alone, buffer + ATP alone, buffer + ADAAD alone, and buffer + ADAAD + ATP is recorded. Then CD spectra for buffer + DNA is recorded, followed by buffer + DNA +ATP and finally, Buffer + DNA +ATP +ADAAD is recorded. To calculate the conformation change induced in DNA due to ADAAD and ATP, the CD spectra of buffer + ATP +ADAAD is subtracted from buffer + DNA+ATP+ ADAAD. This explanation is included in protocol step 7.7.

2. In several cases (lines 278, 291, Figures 3C, 5C) there is a mention of a negative or positive peak at 210nm, although inspection of the relevant figures reveals the absence of any signal in this region.

Our Response: The CD spectra reading at 210 nm is very high in these cases. If it is plotted, then the other peaks would not be visible. Therefore, for plotting purposes, the 210 nm peak was removed.

B. Minor points
Line 124 Give some examples of buffers and materials that should not be used in CD.

Our Response: In the revised manuscript we have indicated that buffers with chlorides and citrates should be avoided.

1.1 Buffers for CD spectroscopy must not contain any materials that are optically active and should be as transparent as possible. Buffers with chlorides and citrates must be avoided.

Line 177 Instead of "Setting up the baselines" with sth like "Preparing mixtures to record baseline spectra"

Our Response: We have revised the manuscript as per your suggestion.

Line 186 "Preparing reaction mixtures to record CD spectra" should have been a more appropriate heading

Our Response: We have now changed this heading to "Setting up the experiments to record CD spectra".

Line 249 and Table 1 It is advisable to include in Table 1 the scores of the prediction methods QGRS and Mfold so that one can get an idea of how strong is the predicted result.

Our Response: We have now included the scores of the QGRS and Mfold in Table 1.

Figures 1C and 1D The spectra shown for GECE+ATP+ADAAD are not identical in the two figures, as they should have been. This oversight should be corrected.

Our Response: Figure 1D data was generated using purified GST-tagged ADAAD. After purification, the GST tag was removed using PreScission Protease. In Figure 1C and all other figures, 6X His-tagged ADAAD purified using Ni^{+2}-NTA affinity chromatography was used. Therefore, the CD spectra of ADAAD and 6X-His ADAAD are not identical. We have now clarified this point in the revised manuscript.

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