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Conformational Changes Triggered by Mg^{2+} Mediate Transactivator Function†

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ABSTRACT: Transactivator protein C of bacteriophage mu is essential for the transition from middle to late gene expression during the phage life cycle. The unusual, multistep activation of mom promoter (P_{mom}) by C protein involves activator-mediated promoter unwinding to recruit RNA polymerase and subsequent enhanced promoter clearance of the enzyme. To achieve this, C binds its site overlapping the −35 region of the *mom* promoter with a very high affinity, in Mg^{2+}-dependent fashion. Mg^{2+}-mediated conformational transition in C is necessary for its DNA binding and transactivation. We have determined the residues in C which coordinate Mg^{2+}, to induce allosteric transition in the protein, required for the specific interaction with DNA. Residues E26 and D40 in the putative metal binding motif ($E_{26}X_{10}D_{37}X_2D_{40}$) present toward the N-terminus of the protein are found to be important for Mg^{2+} ion binding. Mutations in these residues lead to altered Mg^{2+}-induced conformation, compromised DNA binding, and reduced levels of transcription activation. Although Mg^{2+} is widely used in various DNA transaction reactions, this report provides the first insights on the importance of the metal ion-induced allosteric transitions in regulating transcription factor function.

Metal ions play very important roles in many of the biological processes in the cell. Of the various divalent cations present in the cell, Mg^{2+} is one of the most abundant and vital components in cellular biochemistry. Mg^{2+} interacts with nucleic acids and proteins, conferring them either structural stability or by endowing catalytic properties (*1*−*3*). Mg^{2+} is shown to be important in various DNA transaction processes like DNA replication, transcription, repair, recombination, restriction, and transposition. DNA and RNA polymerases (RNAP)[1] coordinate Mg^{2+} ions, where Mg^{2+} plays a catalytic role in the incorporation of dNTPs or NTPs, respectively, formation of phosphodiester bonds, and release of pyrophosphates (*4, 5*). In many of these enzymes including restriction endonucleases, Mg^{2+} also confers substrate specificity during catalysis (*6*). Transcription factors such as Gre proteins and their eukaryotic analogue TFIIS exert a role in rescuing the stalled elongation complex. They participate in RNA cleavage reaction by donating acidic residues to coordinate catalytic Mg^{2+} in RNAP active center (*7*).

We describe here yet another role for Mg^{2+}, distinct from its other elucidated functions in the cellular milieu. C protein is a middle gene product of phage mu and activates transcription by host RNAP from four late gene promoters P_{lys}, P_I, P_p, and P_{mom} (*8*). Transactivator C is a 16.5 kDa protein, and its dimer binds to the recognition sequence with a very high affinity only in the presence of Mg^{2+} (*9*). No other metal ion could effectively replace Mg-induced effect on the protein activity (*10*). CD spectral analysis of C protein in the presence of Mg^{2+} revealed an increase in the α-helical content of the protein, as compared to a more disordered structure in its absence (*11*). Mg^{2+}-mediated conformational change thus seemed to play an important role in DNA binding and the transactivating ability of C.

To establish the importance of conformational transition in C protein for its transactivation function, we have now sought to identify the metal binding residues and mutate them to determine their critical role. Several variant Mg^{2+} binding motifs have been identified as a result of the analysis of a large number of proteins. Some of the known Mg^{2+} binding motifs in diverse proteins are NADFDGD (*Escherichia coli* RNA polymerase) (*5*), GDD (poliovirus RNAP) (*12*), YGDTDS (DNA polymerase α) (*13, 14*), YXDD (reverse transcriptase), LXDD (telomerase) (*14*), EXDXELK (*E. coli* MutH) (*15*), DXEAK (restriction endonuclease *Eco*RI) (*16*−*18*), DXDIK (restriction endonuclease *Eco*RV) (*18, 19*), and DXELK (restriction endonuclease *Pvu*II) (*18, 20, 21*). Analysis of C protein revealed the presence of a $E_{26}X_{10}D_{37}X_2D_{40}$ motif toward the N-terminus. We demonstrate that residues E26 and D40 are the key determinants for Mg^{2+}-coordinated allosteric change in the protein, needed for DNA binding and transactivation.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Reagents. Bacterial strains and plasmids used in this study are listed in Table 1. Transcription templates comprising the wild-type *mom* promoter were

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; CD, circular dichroism; RNAP, RNA polymerase; HTH, helix−turn−helix; WT, wild type.

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Table 1: Strains and Plasmids

strains/plasmids	characteristics[a]	ref
E. coli MG1655	F^- λ^- *ilvG*$^-$ *rfb*$^-$ 50 *rph*$^-$ 1	laboratory stock
E. coli BL26(DE3)	F^- *ompT gal [dcm] [lon] hsdS*$_B$ (r_B^-, m_B^-) Δ*lac* (DE3) *nin5 lac*UV5- T7 gene *1*	laboratory stock
E. coli DH10B	Δ*(mrr-hsd rms-mcrBC) mcrA recA1*	laboratory stock
pVR7	Ap^r C under T7 promoter in pET11d	*10*
pUW4	220-bp *mom* promoter fragment cloned between *Eco*RI−*Bam*HI sites of pUC19	*10*
pCCV(−)	pACYC184 cloned with *mom-LacZ* fusion	*23*

[a] Antibiotic resistance to ampicillin and chloramphenicol are indicated by Ap^r and Cm^r.

1 MQHDLFEHDPAIRQLIGHID 20
21 NIPA*PE*$_{26}$*IFSRWPRS*VVD$_{37}$*IID*$_{40}$ 40
41 VLENELKRQNVSNPRELARK 60
61 QAVALSCFLGGRQFYIPCGD 80
81 TILTALRDDLLYCQFNGRNM 100
101 EELRRQVRLSQPQIYQIIAR 120
121 QRKLHTRRHQPDLFSPETPK 140

FIGURE 1: Primary amino acid sequence of C protein. The putative metal binding motif identified is shown in italics toward the N-terminus, and the key residues mutated in the metal binding motif are shown in bold and numbered. An overlapping coiled-coil motif is represented in the box. The stretch of central leucine zipper motif is shown in bold. The C-terminal helix−turn−helix motif (HTH), responsible for DNA binding, is underlined.

Table 2: Putative Metal Binding Mutants

plasmid	residue mutated	oligonucleotide used for mutagenesis
pVNC1	E26Q	5′ATCCCGGCCCCGCAGCTGGAATC3′
pVNC2	E26D	5′ATCCCGGCCCCGGACCTGGAATC3′
pVNC3	D37N	5′CCCGTTCGGTGGTTAATCTGATTG3′
pVNC4	D40N	5′ATCTGATTAATGTTCTGGAAAACG3′

prepared by releasing a 220-bp *Eco*RI and *Bam*HI fragment from plasmid pUW4 (*10*). NTPs and dNTPs were from Promega. The column materials used for the protein purification were from Amersham Biosciences. [γ-^{32}P]ATP and [α-^{32}P]UTP were purchased from Perkin-Elmer Life Sciences. The oligonucleotides were synthesized by Sigma-Aldrich. Restriction enzymes used were from New England Biolabs.

Generation of C Mutants. Plasmid pVR7 (*10*), consisting of C gene cloned under T7 promoter in pET11d vector, was used as a template for the generation of the mutants. Putative metal binding mutants in C protein were generated by site-directed mutagenesis using megaprimer inverse PCR method (*22*). Forward primer comprised the desired mutation, and T7 terminator primer was used as the reverse primer to generate megaprimer. Megaprimer was used in turn to amplify the entire plasmid (inverse PCR). The sequences of the primers (forward) used to incorporate mutations spanning residues E26, D37, and D40 in the putative metal binding motif and the mutants generated in this study are listed in Table 2.

In Vivo Transactivation Assays. The plasmids bearing either the wild-type or mutant C gene (under phage T7 promoter) (Table 2) were transformed into *E. coli* BL26(DE3) harboring *mom-lacZ* reporter construct pCCV(−) (*23*). β-Galactosidase assays were carried out as described by Miller (*24*).The data presented are an average of three independent measurements of activity.

Protein Purification. C protein and its mutants were purified from *E. coli* BL26(DE3) carrying plasmid pVR7 or pVNC1-4 as described before (*10*). RNAP was purified from *E. coli* K-12 MG1655 according to the method of Kumar and Chatterji (*25*).

DNA Binding. Electrophoretic mobility shifty assays (EMSA) were carried out with a 25-bp end-labeled ds DNA fragment containing C binding site (CBS, 5′AGATCGATTATGCCCCAATAACCAC3′), using the mutant and wild-type C protein. The assay was performed in $TMEG_{100}$ [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 100 mM NaCl]. Reaction mixtures containing labeled DNA were incubated with the protein on ice for 10 min, and the samples were analyzed on 6% native polyacrylamide gel (30:0.8) in 0.5× TBE buffer (45 mM Tris−borate and 1 mM EDTA) at 4 °C.

Surface Plasmon Resonance Spectrometry. The streptavidin matrix-coated sensor chip SA (Biacore Inc.) was preconditioned with three consecutive injections of 50 mM NaOH containing 1 M NaCl and further equilibrated with binding buffer [10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 150 mM NaCl, 5 mM β-ME] at a flow rate of 10 μL/min. A 24-mer 5′-biotinylated oligonucleotide comprising the C binding site (5′XGATCGATTATGCCCCAATAACCAC3′, where X stands for biotin moiety) was annealed with its complementary strand, and this in turn was immobilized on flow cell 2 of SA chip. Flow cell 1 was left unmodified to be used as a reference surface. All binding studies were performed in the binding buffer using Biacore program K*inject* (*26*). Increasing concentrations of the C protein and its mutants were passed onto the chip at a flow rate of 50 μL/min, and change in RU (response units) was monitored. The binding surface was regenerated each time by short 5 μL pulses of 0.05% SDS. Data analysis was performed using BIAevaluation software version 3.1 (BIAcore AB) using 1:1 Langmuir interaction with mass transfer model. All SPR experiments were carried out at room temperature (25 °C).

In Vitro Transcription. The reactions were carried out with 220-bp linear DNA templates comprising *mom* promoter (*10*) in transcription buffer [40 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM DTT, 100 mM KCl, 100 μg/mL BSA). $MgCl_2$ was supplemented to appropriate concentrations wherever required. Reactions were initiated by incubating 50 nM DNA template, 120 nM RNAP, and 300 nM C protein or its mutants on ice for 10 min and then shifted to 37 °C to facilitate the formation of open promoter complex. Multiple round transcriptions were initiated by the addition of 0.3 mM NTP mix and 2 μCi of [α^{32}P]UTP. After 30 min, reactions were terminated by the addition of stop dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene

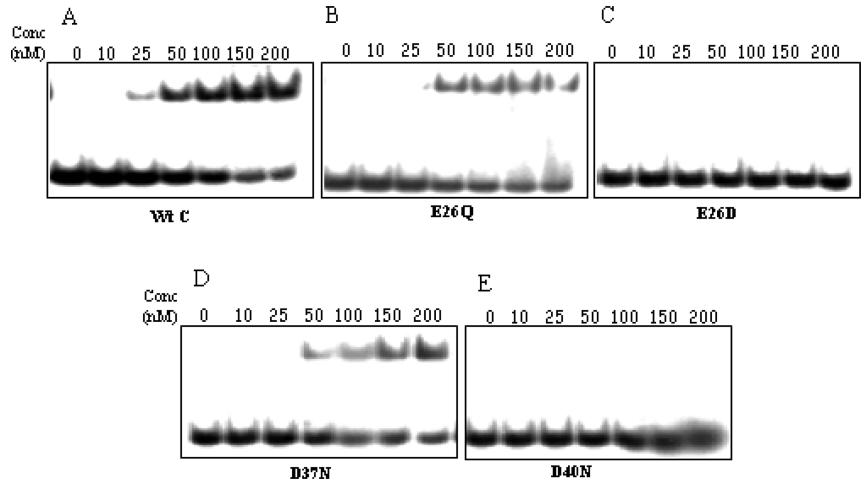


FIGURE 2: Mutants E26D and D40N show reduced DNA binding. A 25-bp duplex end-labeled oligonucleotide comprising the C binding site was incubated with C and mutant proteins and electrophoresed on a 6% native polyacrylamide gel as described in Experimental Procedures. DNA binding efficiencies of (A) C protein, (B) E26Q, (C) E26D, (D) D37N, and (E) D40N are represented in the figure.

cyanol), heat inactivated at 65 °C, and quenched on ice. The samples were analyzed on 8% polyacrylamide gel containing 8 M urea. The transcripts were quantitated by using Image Gauge software, and normalized values were plotted as a function of metal ion concentration.

ANS Fluorescence. Fluorescence emission spectra were recorded on a Jobin-Yvon fluorometer FluoroMax3, thermostated at 25 °C. EDTA-treated C protein or mutants E26D and D40N (1 μM concentration each) were incubated with different concentrations of metal ions (0−10 mM $MgCl_2$) for 15 min at 25 °C. ANS (8-anilinonaphthalene-1-sulfonic acid) dye was used as an extrinsic fluor. To 1 μM protein solution 100 μM ANS was added (made in Tris, pH 7.5, buffer) to obtain emission spectra. The protein samples were then subjected to excitation at 360 nm, and emission values were integrated between 400 and 600 nm. All the fluorescence emission spectra and fluorescence intensities were corrected for buffer, Mg^{2+}, and ANS intrinsic fluorescence.

Circular Dichroism Spectroscopy. The CD spectra were recorded at room temperature from 250 to 205 nm using a JASCO J-810 spectropolarimeter (Japan Spectroscopic Co., Inc., Tokyo, Japan) in a quartz cell of path length 0.2 cm. The spectra were collected at a scanning rate of 50 nm/min, and triplicate spectrum readings were collected per sample. Buffer runs [5 mM Tris-HCl (pH 8.0), 50 mM KCl] were carried out to determine baseline readings, and all samples were baseline corrected before calculations. EDTA-treated C protein and mutants E26D and D40N (0.2 μg/μL) were incubated with varying concentration of Mg^{2+} (0−10 mM) in order to equilibrate the protein sample. The ellipticity results were corrected for concentration of the protein (molar ellipticity) and expressed as mean residue ellipticity (θ)

$$\theta = \frac{\theta_{obs} \times 10^{-3} \times MW}{Cln \times 10^{-2}} \quad \text{deg dmol}^{-1}\,\text{cm}^2$$

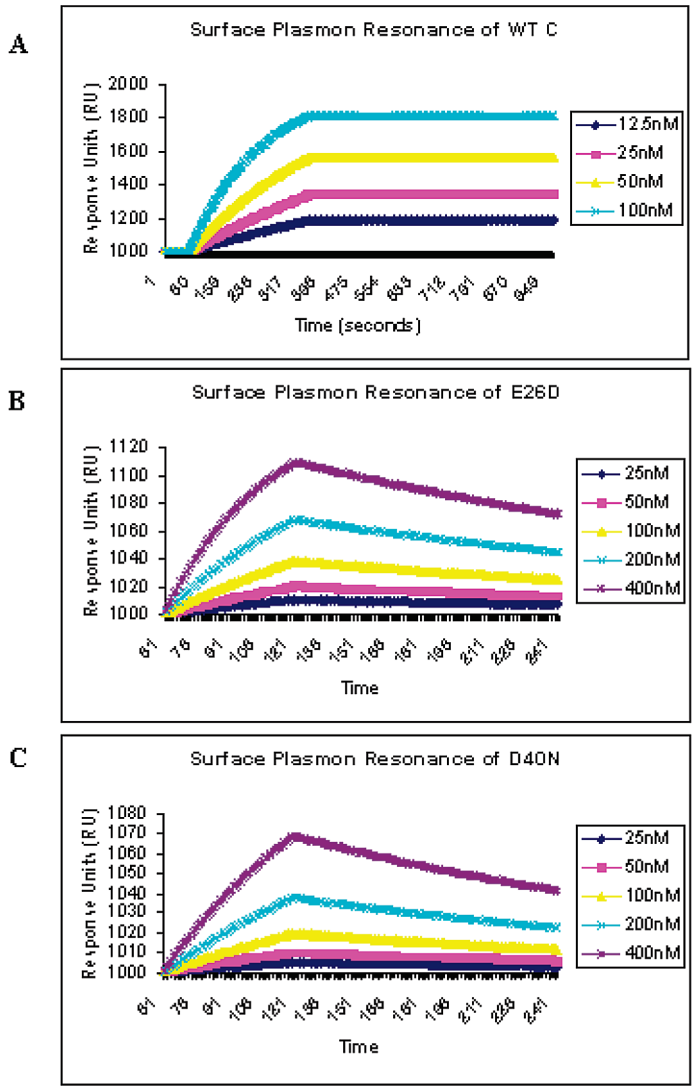
where θ_{obs} is the observed ellipticity, MW is molecular weight, C is concentration (in mg/mL), l is the path length of the cuvette (in centimeters), n refers to the number of residues, and deg is degrees. A mean amino acid residue molecular weight of 117.85 was used for calculation of molar ellipticity values of the proteins (*11, 27*).

RESULTS

Metal Binding Motif in C Protein. In the primary amino acid sequence of transactivator protein C shown in Figure 1, the protein contains a N-terminal coiled-coil motif, a central leucine zipper motif for dimerization, and a C-terminal HTH motif involved in DNA binding (*28, 29*). Further analysis of the sequence revealed the presence of a putative Mg^{2+} binding motif $E_{26}X_{10}D_{37}X_2D_{40}$ preceding and overlapping the N-terminal coiled-coil motif. To understand whether the residues in the putative motif are important for Mg^{2+} coordination, point mutations E26Q, E26D, D37N, and D40N were generated by site-directed mutagenesis, as described in the Experimental Procedures. C protein binds DNA and activates transcription in a dimeric form (*9*). The mutant proteins were purified and subjected to glutaraldehyde cross-linking to assess their dimerizing ability. The mutant proteins exhibited a concentration-dependent increase in dimerization similar to that of wild-type protein, indicating that the mutations did not confer any drastic structural perturbations in the protein affecting dimerization (Supporting Information Figure S1).

Analysis of DNA Binding and Transactivation Functions. The putative metal binding mutants generated were analyzed for their DNA binding ability. EMSA's were performed with C protein and its mutants using an end-labeled oligonucleotide consisting of the C binding sequence. Binding of mutants E26Q and D37N to the C recognition site is not greatly affected (Figure 2B,D). In contrast, mutants E26D and D40N were compromised for this activity (panels C and E of Figure 2, respectively). In order to obtain the quantitative data on the affinity of C and its mutants to the DNA, surface plasmon resonance spectroscopy experiments were performed. C protein bound to its site with high affinity, confirming our previous observations (*10, 30*) (Figure 3A). In contrast, mutants E26D and D40N had comparable association constants (K_a) to that of C, while the dissociation rates (K_d) were substantially faster (Figure 3B,C). As a result, the K_D values for the mutants were lower by several orders of magnitude (Figure 3D).

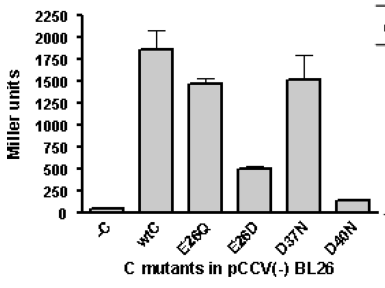
Next, we measured the *in vivo* transactivation ability of the C mutants at P_{mom} using a reporter construct pCCV(−)



D

Protein	K_a ($M^{-1}s^{-1}$)	K_d (s^{-1})	K_D (M)
C	5.58×10^4	1.91×10^{-6}	3.42×10^{-11}
E26D	4.34×10^4	3.46×10^{-3}	7.96×10^{-8}
D40N	1.69×10^4	4.22×10^{-3}	2.49×10^{-7}

FIGURE 3: Mutants E26D and D40N showed comparable association kinetics whereas higher dissociation rates when assayed by SPR spectroscopy. Biotinylated cognate C binding site was immobilized on a SA chip, and the proteins were passed over the DNA with restricted flow rates to measure the K_D, as described in Experimental Procedures. (A) Sensorgram of WT C. (B) Sensorgram of E26D. (C) Sensorgram of D40N. (D) Table comprising the association and dissociation values for the WT C and the mutants E26D and D40N.



C encoding plasmid	Miller units
-C	49 ± 10
Wild type C	1862 ± 380
E26Q	1470 ±118
E26D	513 ± 53.5
D37N	1524 ± 483
D40N	151 ± 15.2

FIGURE 4: Comparison of *in vivo* transactivation efficiency. Transactivation function of Mg^{2+} binding mutants of C was assessed by β-galactosidase assays. The experiments were carried out as described in Experimental Procedures. The values reported in Miller units are a mean of three independent measurements of the activity.

comprising P_{mom}-*lacZ* fusion (see Experimental Procedures). Mutants E26D and D40N were compromised in their ability to transactivate P_{mom}. The former mutant showed 4-fold reduction in β-galactosidase levels while in the latter the effect was even more pronounced (12-fold reduction) (Figure 4). Predictably, mutants E26Q and D37N, which showed comparable DNA binding to that of C, were competent in transactivation (Figure 4).

In parallel, as a further support for this observation, *in vitro* transcription assays were carried out evaluating the transactivation potential of the mutant activator proteins. Previous studies have established the absolute requirement of C for P_{mom} transcription. In the absence of C, no *in vitro* transcripts could be seen from P_{mom} when compared to the standard σ 70 promoters such as *lacUV5*. Initially, demeta-

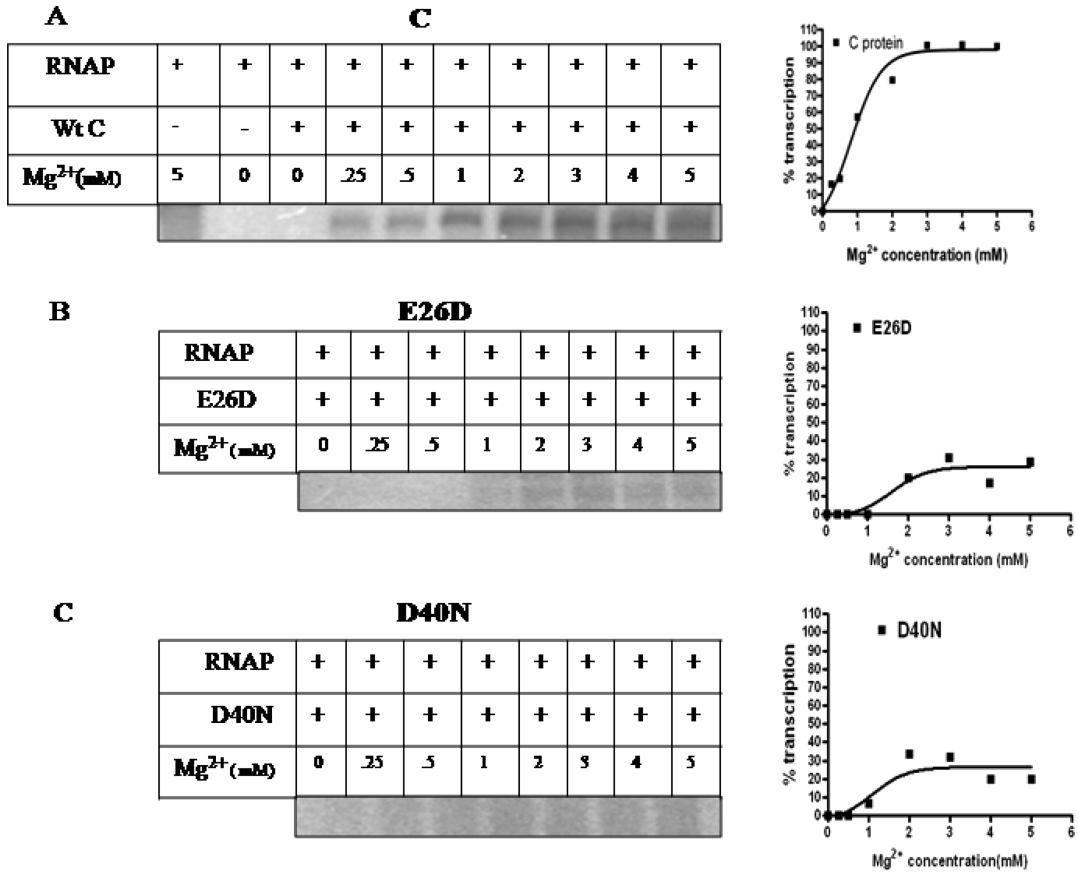


FIGURE 5: *In vitro* transcription assays. (A) Multiple round transcription assays were carried out on *mom* promoter construct in the presence of 300 nM C protein at varying concentrations of Mg^{2+} (0−5 mM). (B) Transactivation efficiency of E26D at increasing concentrations of Mg^{2+} (0−5 mM). (C) Transactivation efficiency of D40N at similar concentrations of Mg^{2+} (0−5 mM). Transcripts were quantitated using Multigauge software, and values were normalized by taking the transcript obtained with 5 mM Mg^{2+} in presence of WT C as 100%. Individual lanes have been subtracted for background. The corresponding graphs represent the normalized transcription percentages plotted as a function of Mg^{2+} concentration.

lated C protein was used along with increasing concentration of Mg^{2+} to measure transcription as a function of the metal ion concentration. Although 2−5 mM Mg^{2+} is used routinely in most transcription assays, 0.25 mM Mg^{2+} was found to be sufficient for initiating transcription at P_{mom}, and the transcript levels increased with the increasing concentration of the metal ion up to 2 mM. At 3 mM concentration of Mg^{2+}, complete saturation of transcription was observed (Figure 5A). In contrast, mutants E26D and D40N were compromised in transcription even at 5 mM Mg^{2+} concentration (Figure 5B,C). The results match the DNA binding and *in vivo* transactivation data obtained with these C mutants. From both *in vivo* and *in vitro* assays, D40N appears to be severely compromised in its transactivation potential. The lack of Mg^{2+} coordination in the mutants renders them inactive in transcription assays.

Metal Ion-Induced Secondary Structural Changes in C Protein. We performed circular dichroism analysis with C and its DNA binding defective mutants (E26D, D40N) as described in Experimental Procedures. Secondary structural changes occurring in C protein when incubated with Mg^{2+} is represented in Figure 6A. The spectra revealed an increase in α-helical content (with negative peaks at 208 and 222 nm, characteristic of α-helix) of the protein upon addition of Mg^{2+} (*11*). These conformational transitions in the protein upon the addition of the specific metal ion indicate the presence of the metal binding pocket. Coordination of Mg^{2+} to specific residues thus triggers the formation of the helical ordered structure required for its activity. In a similar analysis carried out with E26D and D40N, conformational transitions were not significant upon addition of the metal ion (Figure 6 B,C).

Mg^{2+}-Induced Tertiary Structural Changes in C Protein. ANS, a hydrophobic reporter dye, was used to analyze the tertiary structural changes in the protein. ANS, when subjected to excitation at 360 nm, exhibits emission maxima at 520 nm. It shows an enhanced fluorescence when bound to the hydrophobic surfaces of the proteins. Fluorescence spectra of C protein and the mutants E26D and D40N as a function of Mg^{2+} concentration were plotted. C protein displayed an increase in fluorescence intensity with the increase in concentration of Mg^{2+} (Figure 7A) revealing the conformational transition in the protein upon Mg^{2+} binding. In contrast, mutants E26D and D40N did not respond to the addition of the metal ion (Figure 7B,C) (refer to the scale on the *Y* axis). Both CD and fluorescence measurements thus reveal that Mg^{2+} binding is affected in these mutants. We conclude that the failure to achieve the allosteric changes by C protein mutants upon metal ion addition renders them inactive in the functional assays performed.

DISCUSSION

Mg^{2+}-dependent proteins are ubiquitous and participate in general metabolic pathways and nucleic acid biochemistry (*1, 2*). Mg^{2+} interacts with the phosphate backbone of DNA

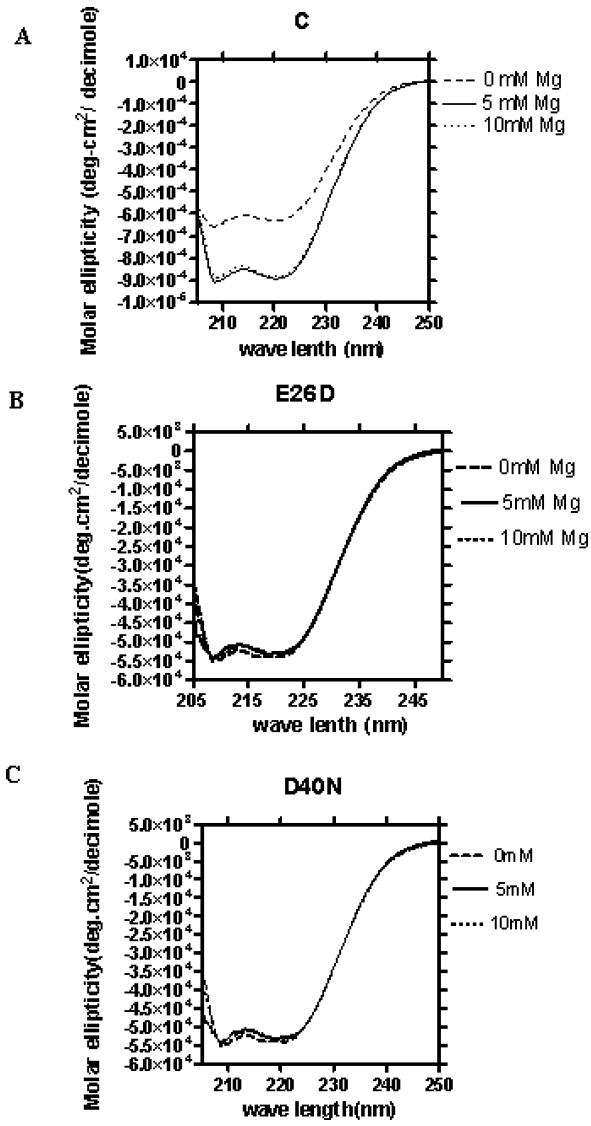


FIGURE 6: Mg^{2+}-induced secondary structural changes in C protein. Changes in secondary structure of the proteins were monitored at wavelengths ranging from 205 to 250 nm. (A) CD spectra of C protein. The molar ellipticity of CD spectra for demetalated C protein (dashed line) and Mg^{2+}-reconstituted C protein (5 mM, continuous line; 10 mM, dotted line). (B) and (C) represent the secondary structural changes observed in mutants E26D and D40N, respectively, upon addition of the metal ion.

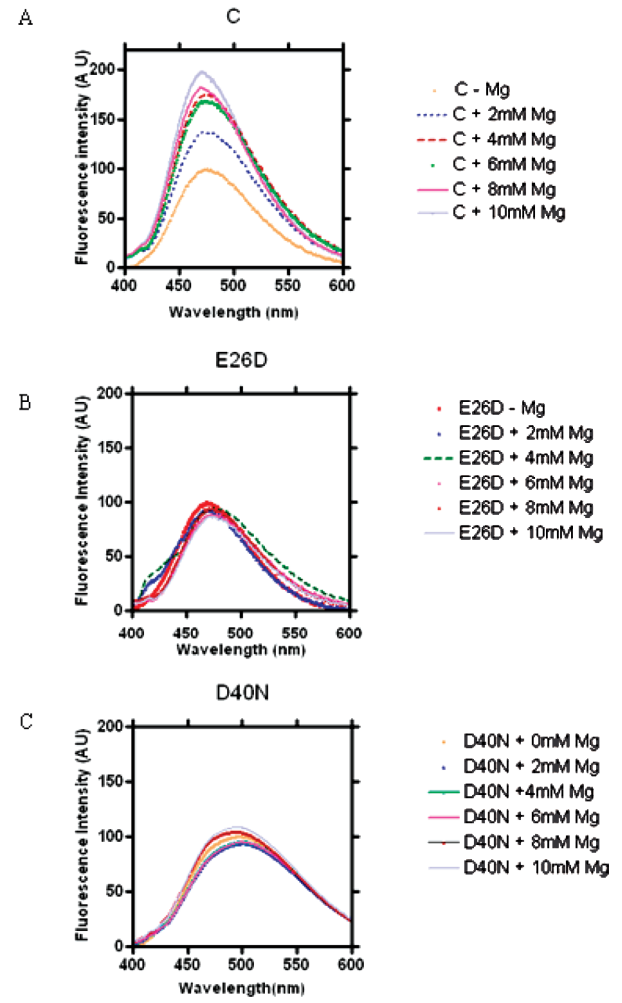


FIGURE 7: Tertiary structural changes in C protein. Fluorescence emission spectra were recorded as described in Experimental Procedures. ANS was used as the hydrophobic reporter dye. (A) Background corrected ANS fluorescence spectra of C protein (1 μM) with increasing amounts of $MgCl_2$ (0−10000 μM) in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl). (B) Mutant E26D and (C) mutant D40N ANS emission spectral pattern, respectively.

and enhances the structural stability by increasing its T_m. Mg^{2+} also plays a critical role in maintaining the structural and biochemical properties of tRNA and rRNA (*31*). Divalent metal ions, especially Mg^{2+}, help to fold RNA into a well-defined structure (ribozyme) and assist in catalysis by functioning either as a Lewis acid or a general acid when coordinated with H_2O (*32*). Recent reports also reveal a new role for Mg^{2+} in regulating the gene expression by modifying the structure of *mgtA* 5′UTR. This serves as the first example of a riboswitch that responds to a metal ion (*33*). We have now elucidated a new role for Mg^{2+} in transcription activation. We demonstrate the importance of Mg^{2+} coordination induced structural transitions in transcription activator protein C, essential for its function. Mg^{2+} is a common ingredient in almost every enzyme reaction involved in DNA transactions and transcription factor assays. However, in most cases barring its catalytic role in many enzymes, the exact function of the metal ion is not known. Requirement of Mg^{2+} coordination for conformation modulation in any regulatory protein involved in the transcription process is hitherto not described.

Among the residues targeted for mutagenesis in the putative metal binding motif ($E_{26}X_{10}D_{37}X_2D_{40}$) of C protein, only a few showed the strong phenotype. The most notable difference in the properties is observed in the case of mutants E26Q and E26D. The former mutant had DNA binding comparable to WT C while the latter lost its property of DNA binding possibly due to its inability to coordinate Mg^{2+}. Mg^{2+} is a hard ion with relatively high charge density and low polarizability. Its preferred coordination is with hard oxygen-containing ligands, viz., carboxylates, phosphates, hydroxyls, carbonyls, and water (*1*). In this particular case, it appears that side chain carbonyl oxygen (−C═O) of both Glu ($-COO^-$) in WT C protein and Gln ($-CONH_2$) in E26Q mutant could coordinate the metal ion. The loss of one methylene group ($-CH_2$) in the mutant E26D would decrease the side chain length by 1.54 Å, increasing the distance between the electron donor and the metal ion. This could probably be disrupting the coordination, thus affecting the activity of the mutant E26D.

A large number of DNA binding proteins bind DNA only in presence of metal ions, especially Mg^{2+}. In enzymes such as R.*Bam*HI and R.*Eco*RV, presence of Mg^{2+} increases their affinity to bind cognate DNA by 830000- and 100000-fold, respectively (*34, 35*). However, the extent of Mg^{2+}-mediated stimulation in DNA binding of C could not be estimated as no detectable binding is observed in the absence of the metal ion. From the data presented, it is evident that there is 1000-fold difference between the WT C protein and its mutants in DNA binding. C protein is thus the first example of a transcription factor that requires metal ion for conformational transition, needed for DNA binding and consequent transactivation.

Importance of the structural transitions in C protein for its DNA binding and subsequent downstream effects is evident by comparison of the fluorescence properties of the protein and its mutants. With the increase in Mg^{2+} concentration, C showed a corresponding increase in fluorescence intensity. In contrast, the mutants failed to respond to the addition of Mg^{2+} and do not show the gradual transition in the ANS fluorescence spectra. CD studies also revealed that helical ordered structure in C protein is achieved upon Mg^{2+} addition. The spectra obtained with the mutants indicate that the inability to coordinate metal ion leads to the lack of helical order achievement. Thus, both CD and fluorescence studies revealed the secondary and tertiary structural perturbations in the protein upon Mg^{2+} addition. Previously, we have demonstrated that the activator binds to DNA using HTH motif located toward the carboxy terminus of the protein (*28*) and that Mg^{2+} induces α-helicity into the protein (*11*). It appears that Mg^{2+} binding is probably required for attaining the proper HTH structure required for DNA binding. The mutants E26D and D40N do not respond to Mg^{2+}, and hence it is likely that HTH needed for DNA binding is not attained in them. Homology modeling studies of C protein based on the available structure of its closest homologue Mor protein (*36*), employing Frankensteins monster's approach (*37*), revealed that D40 residue along with other acidic residues (E45) forms a electronegative patch in the protein (data not shown). This region thus appears to be one of the most potential metal ion binding sites. From all of these studies, we infer that the appropriate coordination of Mg^{2+} in this region with residues E26 and D40 (and possibly others) and the consequent induced conformational changes are important for C to function as a transcription factor. Solution structure of C with Mg^{2+} and DNA should provide greater insights into this site-specific interaction process.

Conformation changes in the DNA and a large number of proteins which bind DNA in site-specific fashion result in formation of specific complexes. The "induced fit" originally proposed for enzyme−substrate complexes is successfully adapted by a large number of proteins during DNA recognition. Further, conformational flexibility concomitant to ligand binding has been well studied for several classes of regulatory proteins. For instance, alterations in the catabolite receptor protein CRP in the presence of the ligand cAMP has been studied in great detail (*38, 39*). However, metal ion-induced structural transition in transcription activator proteins is much less understood with the exception of the MerR family of transcription factors. Studies carried out to analyze the metal ion-mediated conformational changes reveal that MerR undergoes structural transitions in presence of Hg^{2+} (*40*). Comparison of MerR and C with respect to conformational transitions reveals a significant difference in their properties. MerR binds DNA in the absence of Hg^{2+} but exerts its effect on activation or repression of a gene or operon only upon the metal ion binding (*41, 42*). In contrast, C does not bind DNA on its own and requires Mg^{2+}-induced conformational changes to bind DNA. Thus, the effect of the metal ion-induced changes in these two proteins is very different. A detailed analysis with other transcription factors is likely to reveal the importance of Mg^{2+} and other divalent metal ion-mediated conformational changes in different steps of the transcription process.

A number of transcription factors appear to be intrinsically disordered and hence would not be able to function as transcriptional regulators in that state. A systematic analysis using Predictor of natural disorder regions (PONDR) was employed to identify the prevalence of intrinsic disorder in transcription factor data sets (*43*). The intrinsic disorderliness in the domains of these proteins would prevent their occupancy at the regulatory sites until orderliness is achieved by ligand binding. Thus it appears that disordered structure may be an important feature of some of these transcription factors in controlling gene expression.

In conclusion, we demonstrate the importance of binding of Mg^{2+} to a transcription factor for its conformational transition to bind DNA. In many cases where Mg^{2+} is required, the metal ion may have a similar role. In situations where Mg^{2+} would be limiting in the cell, the concentration-dependent conformational changes in the transcription factor may control the level of transactivation. Facilitating the availability of functional activator achieved by conformational transition triggered by a metal ion could be yet another general mode of control of gene expression.

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SUPPORTING INFORMATION AVAILABLE

Details of the experimental procedures and results of dimerization assay for metal binding mutants (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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