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MeCP2 Binding Cooperativity Inhibits DNA Modification-Specific Recognition

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Abstract

Methyl-CpG binding protein 2 (MeCP2) is a multifunctional protein that guides neuronal development through its binding to DNA, recognition of sites of methyl-CpG (mCpG) DNA modification, and interaction with other regulatory proteins. Our study explores the relationship between mCpG and hydroxymethyl-CpG (hmCpG) recognition mediated by its mCpG binding domain (MBD) and binding cooperativity mediated by its C-terminal polypeptide. Previous study of the isolated MBD of MeCP2 documented an unusual mechanism by which ion uptake is required for discrimination of mCpG and hmCpG from CpG. MeCP2 binding cooperativity suppresses discrimination of modified DNA and is highly sensitive to both the total ion concentration and the type of counterions. Higher than physiological total ion concentrations completely suppress MeCP2 binding cooperativity, indicating a dominant electrostatic component to the interaction. Substitution of SO_4^{2-} for Cl^- at physiological total ion concentrations also suppresses MeCP2 binding cooperativity. This effect is of particular note as the intracellular Cl^- concentration changes during neuronal development. A related effect is that the protein-stabilizing solutes, TMAO and glutamate, reduce MeCP2 (but not isolated MBD) binding affinity by 2 orders of magnitude without affecting the apparent binding cooperativity. These observations suggest that polypeptide flexibility facilitates DNA binding by MeCP2. Consistent with this view, nuclear magnetic resonance (NMR) analyses show that ions have discrete effects on the structure of MeCP2, both MBD and the C-terminal domains. Notably, anion substitution results in changes in the NMR chemical shifts of residues, including some whose mutation causes the autism spectrum disorder Rett syndrome. Binding cooperativity makes MeCP2 an effective competitor with histone H1 for accessible DNA sites. The relationship between MeCP2 binding specificity and cooperativity is discussed in the context of chromatin binding, neuronal function, and neuronal development.

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Notes

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Graphical Abstract

Epigenetic marking of the human genome programs tissuespecific gene expression and, thereby, cell differentiation and development. Until recently, the only known epigenetic mark was 5-methylcytosine (5mC) that is placed by DNA methyltransferases (DNMTs), preferentially at CpG dinucleotides.^{1,2} Modification of CpG by 5mC (mCpG) is associated with gene repression and generally regarded as a stable, highly heritable determinant. DNA methylation patterns undergo genome-wide reprogramming during the establishment of primordial germ cells and after fertilization.³ The molecular mechanisms of epigenetic reprogramming are actively being explored.⁴ The enzyme TET1 oxidizes 5mC to 5-hydroxymethylcytosine (5hmC),⁵ a modification first detected in mammalian DNA.⁶ While first viewed as an intermediate in 5mC removal, 5hmC is now also viewed as a discrete epigenetic modification that adds another layer of complexity to epigenetic regulation.⁵

Three major protein families, MBD, Kaiso and Kaiso-like proteins, and SRA domain proteins, discriminate between mCpG and hmCpG chromatin modification.⁷ The 486-residue MeCP2 is a member of the MBD protein family that is highly expressed in neuronal tissue.⁸ Alternative splicing generates two MeCP2 N-terminal isoforms, the longer of which, MeCP2e1, is enriched with acidic and hydrophobic residues. Isoform MeCP2e2 is shorter but is considered functionally equivalent to MeCP2e1.⁹ MeCP2 is composed of five domains:¹⁰ the aforementioned “N-terminal” domain (NTD, residues 1–78) and MBD (residues 79–162), as well as the “intervening” (ID, residues 163–206), “transcriptional repression” (TRD, residues 207–310), and “C-terminal” (CTD, residues 311–486) domains.

The importance of MeCP2 to cellular regulation and development is highlighted by the extent to which its dysfunction causes human developmental disorders such as Rett syndrome, the most common genetic cause of severe intellectual disability in females.¹¹ The

majority of Rett syndrome cases are caused by mutation in the X-linked MECP2 gene.¹² Many point and deletion MECP2 mutations have been identified in Rett syndrome patients and, less frequently, in other forms of intellectual disability, including autism, schizophrenia, mental retardation, and Angelman syndrome.¹³ The disability-causing mutations in MeCP2 affect either MBD function, C-terminal domain function, or both.^{14–17}

MeCP2 is a biologically complex protein. It is multifunctional, activating or repressing target genes,^{15,16} modulating chromatin structure,^{20,21} and controlling RNA splicing that together direct brain development.^{1,8,17,18} Because MeCP2 binds to chromatin, it competes with linker histones for the DNA between nucleosomes.¹⁹ MeCP2 function can be modulated by its binding within different chromatin types composed of particular histone variants and/or modifications.²⁰ Chromatin interactions by MeCP2 independent of specific binding to mCpG are reported to be transient and weak.²¹ Because of its high level of expression in neuronal tissues,⁸ both mCpG-specific and nonspecific (electrostatic) interactions contribute to MeCP2's biological function.^{10,16} The intrinsically disordered C-terminal polypeptide is reported to restructure upon DNA binding and thus may allosterically influence the interaction of MeCP2 with its regulatory partners.^{22–25}

Different domains mediate specific MeCP2 functions. MBD mediates recognition of sites of mCpG and hmCpG modification. Our thermodynamic studies of the isolated MBD from MeCP2 revealed an unusual dependency of mCpG and hmCpG binding specificity on the ionic milieu.²⁶ AT-Hook motifs within the TRD contribute to nonspecific binding and chromatin restructuring.^{10,21,27–29} Disruption of a highly conserved AT-hook alters chromatin compaction, causing neurological dysfunction.²⁸ The effects of MeCP2 mutations outside MBD are dose-dependent; a single copy of wild-type MeCP2 can restore normal development.³⁰

This paper extends our exploration of the effect of ions on DNA binding by MBD to full-length MeCP2.²⁶ Our motivation is twofold. First, changing solution conditions is a well-established tool with which to probe the energetics of macromolecular interactions.²⁶ Second, changes in the neuronal ionic milieu during function and development and changes of tens of millimolar in sodium and chloride ions have been measured during neuronal differentiation.^{31–37} MeCP2 functions in competition with linker histones,^{38,39} whose interaction with DNA is mostly electrostatic.²⁷ Thus, ionic changes have the potential to regulate the partitioning of MeCP2 between specific and nonspecific sites on chromatin and exchange of MeCP2 with linker histones.

We find that the DNA binding cooperativity and the mCpG and hmCpG specificity of MeCP2 are modulated by both the concentration and type of ions present in solution. Surprisingly, we observe that MeCP2 binding cooperativity inhibits the protein's specificity for modified nucleotides. Only when cooperativity is suppressed do we observe full discrimination by MeCP2 of mCpG and hmCpG. Ions also alter the structure of MeCP2. Ordering of the C-terminal polypeptide by osmolytes inhibits binding affinity but not the apparent cooperativity. Cooperativity makes MeCP2 an effective competitor with histone H1 for DNA sites. We discuss the hypothesis of how the ionic changes that accompany neuronal differentiation could separately regulate methylation-specific, nonspecific, and cooperative

components of MeCP2 DNA binding and thus modulate the molecular dysfunction evoked by disability-causing mutations.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification.

The DNA binding domain of human MeCP2 (MBD, residues 76–167; Uniprot entry P51608) was expressed and purified as previously described.²⁶ The human MeCP2 (Uniprot entry P51608) sequence was inserted into the pET30 expression vector and expressed as previously described for MBD.²⁶ Molar extinction coefficients ($\epsilon_{M,280\text{ nm}}$) of 10810 and 12090 were used to determine MBD and MeCP2 concentrations, respectively. The cells containing expressed protein are typically packed and frozen at -80°C . Cell pellets are thawed on ice and suspended at 5 mL/g in 50 mM NaPO_4 (pH 7.9), 10% ethylene glycol, 1 M NaCl, 5 mM β -mercaptoethanol (BME), 10 mM imidazole (buffer A), and complete protease inhibitor cocktail.²⁶ Lysis is accomplished with 4×30 s microtip sonication pulses (setting 6, FB705, Fisher Scientific) in a container jacketed by a NaCl-saturated ice/water mixture with 2 min allowed between each sonication pulse. The uniformly viscous and clump free sonicated sample is then centrifuged at $13000g$ and 4°C for 20 min. DNA is precipitated by dropwise addition during stirring of 2.5 mL of 10% Protamine Sulfate to 20 mL of the retained supernatant, which continued for 10 min following addition of the last drop. The precipitate is removed by centrifugation at $13000g$ and 4°C for 15 min.

Our purification of wild-type MeCP2 takes advantage of the seven consecutive histidine residues within the C-terminal polypeptide that effectively bind a nickel purification column. At 4°C , a nickel column bed volume of 3 mL (Econo-Pac Disposable column, Bio-Rad) is equilibrated with buffer A by passage of 10 bed volumes. The sample is loaded, and the column is washed with 5 bed volumes of buffer A. Next, 5 bed volumes each of 50 mM NaPO_4 (pH 7.9), 10% ethylene glycol, 200 mM NaCl, 5 mM BME, and 20 mM imidazole (buffer B) and 50 mM NaPO_4 (pH 7.9), 10% ethylene glycol, 200 mM NaCl, 5 mM BME, and 60 mM imidazole (buffer C) are passed through the column. MeCP2 is eluted from the column with 5 bed volumes of 50 mM NaPO_4 (pH 7.9), 10% ethylene glycol, 200 mM NaCl, 5 mM BME, and 250 mM imidazole (buffer D). MeCP2 containing flow-through is dialyzed overnight at 4°C against 20 mM HEPES (pH 7.5), 10% glycerol, 100 mM NaCl, and 0.5 mM DTT, typically concentrated with Amicon Ultra centrifugal filters (10 kDa cutoff), and stored in small aliquots at -80°C .

Isotope Labeling of MBD and MeCP2.

The cell growth conditions used for isotope labeling are the same as those used for natural abundance MeCP2 except that M9 with the corresponding isotope(s) is used as the medium. An overnight culture is inoculated at a 1:100 ratio into 1 L of YT medium with 0.4% sorbitol and antibiotics. When the cell density reaches 0.8 at 600 nm, the cells are gently pelleted. The pellet is suspended in 100 mL of isotope labeling medium for each liter of culture and shaken overnight. The cells in the overnight culture(s) are pelleted and suspended in 1 L of labeling medium for each 100 mL culture. Sorbitol is necessary for ^{15}N but not ^{13}C labeling. We shake the cultures for 30 min let the cells relax and then add isopropyl β -D-1-

thiogalactopyranoside to a final concentration of 1 mM to induce protein expression. Induction is allowed to proceed for 4 h, after which the cells are collected and purified as described above for natural abundance protein.

Reagents and DNA.

The reagents and DNA used herein have been previously described.²⁶ The 20 bp oligonucleotide 5'-TCTGGAACGGAATTCTTCTA-3', with C being modified or not modified, was used in our study. This sequence is taken from promoter III of the mouse BDNF gene and is the DNA present in the MBD cocrystal structure.⁴⁰

Fluorescence, Absorption, and Light Scattering Measurements.

Binding experiments were performed in “standard buffer” containing 25 mM Tris-HCl, 6% glycerol, 100 μ g/mL BSA, 0.1 mM EDTA, 0.1 mM TCEP at pH 7.6 and 20 °C, and additional salts whose type and concentration are stated in the text or figure legends. Protein concentration determinations were made spectroscopically using a Nano-Drop 2000 UV-vis spectrophotometer. Fluorescence measurements were taken with a Jobin Yvon (Edison, NJ) Fluoromax-3 or Fluoromax-4 spectrofluorometer. The intensity of the Raman scattering band of water was used as the internal standard of fluorometer sensitivity. To control for protein aggregation, elastic light scattering (ELS) was recorded using the spectrofluorometer to control for protein aggregation; the scattered 350 nm light was collected at an angle of 90° to the incident illumination.

Analytical Ultracentrifuge.

Sedimentation equilibrium and velocity experiments were performed using the absorption optics of a Beckman XL-I analytical ultracentrifuge as described previously.²⁶

Equilibrium Binding.

MeCP2 binding isotherms were calculated and analyzed as previously described.²⁶ The DNA concentration is 5 nM. Briefly, fractional saturation ($\bar{Y}$) is calculated as

$$\bar{Y} = \frac{A_{\text{obs}} - A_{\text{min}}}{A_{\text{max}} - A_{\text{min}}} \quad (1)$$

where A_{obs} , A_{min} , and A_{max} are the observed, minimal, and maximal values of the relative fluorescence anisotropy (A_r), respectively. $A_r = (A_m - A_{\text{DNA}})/A_{\text{DNA}}$, where A_m and A_{DNA} are the measured anisotropies of the complex and the labeled DNA, respectively. Equilibrium dissociation constant k_d and Hill coefficient n_H are calculated from

$$(\bar{Y})^2 - \left(1 + \frac{k_d}{O_{\text{tot}}} + \frac{P_{\text{tot}}^{n_H}}{O_{\text{tot}}} \right) \times \bar{Y} + \frac{P_{\text{tot}}^{n_H}}{O_{\text{tot}}} = 0 \quad (2)$$

where P_{tot} and O_{tot} are the total protein and DNA concentrations, respectively. Equation 2 reduces to the single-site (Langmuir) binding model when n_H is unity and $P_{\text{tot}} \sim P_{\text{free}}$ ($k_d \gg O_{\text{tot}}$).

Nuclear Magnetic Resonance.

Isotopically enriched MBD was purified and concentrated to 1 mM in 150 mM NaCl, 10 mM sodium phosphate, and 5 mM BME (pH 7.4). ^{15}N -enriched full-length MeCP2 was purified and concentrated to 0.3–0.5 mM in 10 mM potassium phosphate and 5 mM BME (pH 7.4) with additional salt (e.g., KCl or K_2SO_4) added as described in the text. All NMR data were acquired at 25 °C on 600 MHz cryoprobe-equipped Bruker or Varian instruments. HSQC-based pulse sequences were used on MBD samples. TROSY-based pulse sequences were used for the much larger MeCP2. The deposited MBD backbone chemical shift assignments⁴¹ were transferred to our MBD construct²⁶ and buffer conditions using three-dimensional HNCA, HN- (CO)CA, HNCOC, and HNCACB triple-resonance data sets. All NMR data sets were processed with nmrPipe⁴² and analyzed using CCPN Analysis 2.4.⁴³ Referencing was done with respect to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

RESULTS

MeCP2 Binding Specificity and Cooperativity.

Key characteristics of a protein–DNA interaction that drive its biological function are its affinity and specificity for its target site, whether the protein binds as a monomer or oligomer and, if the latter, whether there is discernible cooperativity to its assembly on the DNA. Our studies of the isolated MBD of MeCP2 demonstrated that substantial cation uptake is required for mCpG binding specificity, binding of a second monomer follows at sufficiently high protein concentrations, and assembly is resolutely noncooperative.²⁶ Like MBD,²⁶ MeCP2 is monomeric in solution,⁴⁴ behavior confirmed by analytical ultracentrifugation under our solution conditions (data not shown). We initiated our study of MeCP2 by asking if the thermodynamics of binding of MeCP2 to DNA differ from that of the isolated MBD.

MBD and MeCP2 share a salt requirement for specific binding to mCpG and hmCpG. However, modification-specific binding by MeCP2 requires salt concentrations higher than used for isolated MBD.²⁶ At 150 mM KCl, MeCP2 binds identically to mCpG and CpG DNA (Figure 1, blue filled and empty circles, and Table 1). These isotherms also reflect the MeCP2 binding cooperativity previously reported for assembly of the protein to long fragments of DNA and chromatin.⁴⁵

Increasing the salt concentration to 250 mM completely suppresses binding cooperativity and reveals 30-fold specificity for mCpG (Figure 1, black filled and empty circles, and Table 1). Comparison of the blue and black isotherms of Figure 1 highlights the observation that suppression of MeCP2 binding cooperativity allows for mCpG binding specificity to emerge.

A different relationship is observed for hmCpG binding. At 150 mM KCl, binding to of MeCP2 both symmetrically and asymmetrically modified (hmCpG/mCpG) DNA is not

discernibly cooperative (Figure 1A, blue solid and half-solid squares, and Table 1). This behavior results in the surprising outcome that despite the loss of cooperativity, binding of MeCP2 to either symmetrically or asymmetrically hmCpG-bearing templates is similar relative to that for mCpG and CpG at physiological salt concentrations.

Specificity for hmCpG relative to CpG again becomes evident when the salt concentration is increased to 250 mM KCl, although the affinity of MeCP2 for this modification is lower than that for mCpG (in Figure 1, compare black ■ with black ●; Table 1). The additivity of binding of MeCP2 to hemimodified hmCpG previously observed for MBD²⁶ is also evident at high salt concentrations where binding cooperativity is suppressed (Figure 1, black ■ and black ▣). Isolated MBD and MeCP2 have comparable binding affinities and specificities under common solution conditions when MeCP2 binding cooperativity is suppressed (in Figure 1, black data symbols compared with those previously published for MBD²⁶). The results shown in Figure 1 and Table 1 demonstrate energetic coupling (depending on the ionic milieu and nucleotide modification) between modification-specific and cooperative binding.

Nonspecific Binding Cooperativity Is Specific to Anion Type.

The inhibition of MeCP2 binding cooperativity by high salt concentrations (Figure 1) indicates an electrostatic component to the interaction. Although 250 mM is higher than the physiological concentration, ion concentrations can vary widely in both the cytoplasm and the nucleus.⁴⁶ We thus asked whether ions whose concentrations change with neuronal development (e.g., Na⁺ and Cl⁻)^{31–37} might modulate MeCP2 binding and cooperativity at physiological concentrations. Substituting the anion SO₄²⁻ for Cl⁻ while keeping the total K⁺ concentration constant at 150 mM eliminates cooperativity to CpG binding but has virtually no effect on mCpG binding (Figure 2, blue symbols and curves, and Table 1). The result of this selective suppression of cooperativity is that a small degree of mCpG binding specificity emerges at higher protein concentrations. Essentially, the total cation concentration supersedes the effect of anion type. At 250 mM K⁺, anion type has no effect on MeCP2 binding (in Figures 1 and 2, compare black ● and black ○).

We next explored the effect of cation type on MeCP2 binding. Substituting NaCl for KCl at 150 mM very slightly and equivalently decreases the binding affinity of MeCP2 for mCpG and CpG. (Figure 3A and Table 1). In contrast, there is unusual synergy when both the cation and anion type are changed. With Na⁺ as the countercation, binding of MeCP2 to mCpG is enhanced and the cooperativity of binding CpG is eliminated when Cl⁻ is replaced by SO₄²⁻ (Figure 3B and Table 1). The net result of these changes is almost 10-fold mCpG binding specificity in solution at physiological ionic strength that is rich in Na⁺ and poor in Cl⁻. Lastly, we explored binding in mixed anion solutions and found that the Cl⁻ concentration has a graded effect on the cooperativity of binding of MeCP2 to CpG (Figure 4 and its inset). Thus, Cl⁻ flux at a constant total ionic strength has the potential to alter the distribution of the MeCP2 between modified sites and genomic DNA. As discussed below, changes in binding cooperativity could hypothetically regulate the competition for chromatin between MeCP2 and the highly cooperative linker histones.

Divalent Cations.

Divalent cations can influence protein–DNA interactions through a variety of mechanisms, including specific and nonspecific interactions with the nucleic acid, protein, or both macromolecules. The separate analysis of monovalent and divalent cations allows the unique activities of each to be discerned. The addition of 5 mM Mg^{2+} at a physiological monovalent cation concentration has no effect on mCpG binding by MeCP2 but depresses CpG binding by a modest 3-fold (Figure 5A and Table 1). As observed with monovalent cations alone (Figure 1), discrimination of mCpG from CpG results from depression of the level of binding of MeCP2 to CpG at higher ionic strengths rather than enhancing the binding affinity of the modified nucleotide. Interestingly, MgCl_2 depresses CpG binding without diminishing the apparent binding cooperativity.

Because there are seven consecutive histidine residues (residues 366–373) within the C-terminal polypeptide of MeCP2, we also explored whether Zn^{2+} might influence DNA binding. Presumably, Zn^{2+} coordinates to residues 366–373 as we effectively used this internal His tag to purify the protein. Zn^{2+} has an effect that is distinct from that of Mg^{2+} . Unlike Mg^{2+} , the addition of 2 mM ZnCl_2 does not differentially affect mCpG or CpG binding by MeCP2. Rather, Zn^{2+} diminishes the binding affinity of MeCP2 for both mCpG and CpG equivalently (Figure 5B and Table 1). These data show that the divalent cations, Mg^{2+} and Zn^{2+} , have mechanistically distinct effects on MeCP2 specificity for modified DNA. Mg^{2+} appears to inhibit nonspecific binding through canonical cation screening of the DNA, while Zn^{2+} apparently binds to a specific C-terminal polypeptide.

Osmolyte Stabilization of the C-Terminal Polypeptide.

The C-terminal polypeptide of MeCP2 is reported to play multiple functional roles, including binding of DNA and cofactors. Because the C-terminal polypeptide of MeCP2 is intrinsically disordered, we reasoned that solutes that alter the structure or flexibility of the polypeptide might alter DNA binding affinity, specificity, or cooperativity. Indeed, binding of Zn^{2+} to residues 366–373 would alter both the structure and flexibility of a portion of the C-terminal polypeptide that may be the cause of the reduced DNA binding affinity presented above (Figure 5B).

The osmolyte trimethylamine N-oxide (TMAO) belongs to the family of “counteracting” osmolytes that naturally accumulate in marine organisms and affect both protein stability and function.^{47,48} DNA binding by MeCP2 is profoundly influenced by 1 M TMAO. At a physiological total salt concentration, the presence of 1 M TMAO decreases the binding affinity of MeCP2 for both mCpG and CpG by 2 orders of magnitude while they remain highly cooperative (Figure 6, black lines and symbols). It is interesting that specificity of MeCP2 for mCpG emerges in the presence of the osmolyte TMAO.

To explore the chemical generality of the effect of the osmolyte on MeCP2 DNA binding, we next substituted glutamate for chloride. Glutamate has been shown to stabilize protein secondary structure.⁴⁹ The binding affinity of MeCP2 for DNA also decreases upon this exchange of anions. As with TMAO, modest specificity for mCpG emerges upon this exchange of anions (Figure 6, magenta lines and symbols). Interestingly, this anion

exchange does not result in the loss of binding cooperativity observed when Cl^- is replaced with SO_4^{2-} (Figure 4), highlighting the complexity of the impact of ions on MeCP2 function.

The effects of Zn^{2+} , TMAO, and glutamate together suggest that disorder and/or flexibility of the C-terminal polypeptide is important to the process that results in the higher binding affinity of MeCP2 relative to that of MBD; C-terminal polypeptide flexibility is reduced upon the preferential interaction of the osmolytes. We confirmed that the effect of these osmolytes resides in the C-terminal domain by demonstrating that neither the binding affinity nor modification specificity changes when the same experiments are conducted with isolated MBD (data not shown).

We additionally explored whether TMAO has any effect on the MBD within intact MeCP2 by monitoring the thermal melting of MeCP2 and isolated MBD using intrinsic tryptophan fluorescence. Because the two tryptophan residues of MeCP2 are located within MBD, this assay reports on the domain's folding within the full-length protein. Remarkably, these reactions are almost fully reversible. For MeCP2, T_m values of 54.3 ± 2.4 and 60.5 ± 0.7 °C are measured in 150 mM buffer without and with 1 M TMAO, respectively. The same experiment conducted with isolated MBD yields T_m values of 55.7 ± 1.7 and 59.8 ± 3.0 °C, respectively. The comparable values measured for MeCP2 and MBD show that the stability of MBD is essentially independent of the C-terminal polypeptide and only moderately stabilized by osmolyte. The latter effect is that expected of folded proteins.⁵⁰ From these results, we conclude that the effect of osmolyte on DNA binding by MeCP2 is exclusive to the C-terminal polypeptide.

Ionic Effects on MeCP2 Structure.

We turned to NMR to help distinguish ionic effects on MeCP2 itself from their condensation around duplex DNA. Because the structure of the MBD had been determined by NMR,⁴¹ we anticipated that the spectrum of the structured MBD would also be discernible within the limited dispersion signals from the unstructured C-terminal polypeptide. In the overlay of the $^1\text{H}^{15}\text{N}$ spectrum of MBD with that from MeCP2, most of the backbone amide cross peaks from MBD are indeed observed in MeCP2 (Figure 7A). Most of the cross peaks from isolated MBD superimpose nearly perfectly with those of the domain within MeCP2. Noticeable changes are observed only for the residues at the end of MBD (residues 157–160) that link the domain to the C-terminal domain of MeCP2.

Following some reassignment of MBD signals to adjust for construct and buffer differences from the original NMR studies, we probed for ion-dependent effects on assigned MBD residues and unassigned MeCP2 residues (Figure 7B). The effect of total ionic strength on chemical shift intensity is limited over 50–250 mM K^+ regardless of whether the anion is SO_4^{2-} or Cl^- . Slight intensity changes and shifts are observed for residues 107–111, the outer edge of the β -hairpin facing away from the DNA in the protein–DNA complex, and small but slightly larger chemical shift changes for linker residues 156–160. No changes are obvious for the non-MBD resonances. An even weaker effect is observed via comparison of NaCl and KCl; there are no obvious NMR spectral changes between these two cations at 150 mM.

In contrast to the results described above, substitution of SO_4^{2-} for Cl^- at a K^+ concentration of either 150 or 250 mM has more pronounced effects on the NMR chemical shifts (Figure 7C). Given the observed impact of cation type on apparent binding cooperativity (Figures 1–4), it is surprising that the chemical shift effects are predominantly in the MBD rather than the C-terminal polypeptide (Figure 7B and its inset). The largest changes are in the loop that stabilizes the methyl binding pocket and constitutes the linker between MBD and the C-terminal polypeptide (Figure 7C and its inset). The most pronounced chemical shift change is for Thr160.

Comparison of MeCP2, MBD, and Histone H1 Binding Affinity.

Linker histones, particularly histone H1, are structurally and functionally similar with MeCP2.³⁸ Both proteins possess an N-terminal globular domain and disordered C-terminal polypeptide that changes its structure in response to the surrounding environment.^{51,52} Histone H1 and MeCP2 bind mainly to linker DNA in chromatin; the exchange of histone H1 and MeCP2 has been shown *in vivo* and *in vitro* to be necessary to maintain chromatin structure and function.^{45,53} Because DNA binding by MeCP2 is highly sensitive to solution conditions, we considered it essential to evaluate the relative affinity of these proteins for DNA under identical solution conditions. Figure 8 plots isotherms determined for MeCP2 (Figure 1) and MBD²⁶ under our quasi-physiological 150 mM KCl reference condition. The higher affinity of cooperative binding of MeCP2 to mCpG bearing DNA relative to noncooperative MBD binding is clearly evident (Figure 8, dashed vs dotted lines).

As expected, human histone H1 binds cooperatively and identically to our standard 20 bp duplex bearing mCpG or CpG and a random sequence of equal length under these solution conditions (Figure 8, triangles). The cooperativity and lack of specificity is characteristic of histone H1.⁵⁴ Remarkably, the K_d measured for histone H1 is coincident with that of MBD. Because MBD binds without cooperativity, the isolated domain would be at a competitive disadvantage with histone H1 for binding to DNA within chromatin. In contrast, the 2 order of magnitude higher affinity and high cooperativity of MeCP2 binding provide the full-length protein with the ability to readily displace linker histones from DNA. While competition between MeCP2 and linker histones is clearly more complicated in the context of chromatin, these results support the hypothesis that the 2 order of magnitude energetic boost provided by the cooperative component of MeCP2 DNA binding would promote its displacement of histone H1 in the context of chromatin.

DISCUSSION

MeCP2 is a member of the MBD family of proteins whose biological function is to bind to sites of methylation in the genome.^{55,56} MeCP2-mediated epigenetic regulation starts with recognition of mCpG by its DNA binding domain, MBD,⁵⁵ and continues with multiple processes involving the other domains of the protein.¹⁰ Among the “simple roots” that determine MeCP2 function is its recognition of modified DNA.⁵⁷ However, complexity resides within this basic function as studies indicate unique roles for recognition of discrete DNA modifications in MeCP2 mediation of development and cellular metabolism.^{58,59} In addition, DNA binding cooperativity and the ability to alter chromatin structure are another

mechanism by which MeCP2 regulates development and cellular metabolism.⁶⁰ Understanding the biological activity of these mechanisms requires understanding their physical nature. Our study demonstrates that DNA binding specificity and cooperativity can be effectively decoupled by ion type and concentration, allowing discrete study of each process within the context of the full-length protein. MeCP2 binding cooperativity is sensitive to both the nature of the bound DNA and the concentration and type of ions. The latter observation suggests the hypothesis that changes in the neuronal environment may regulate both the stability and localization of the protein on chromatin. The changes in the ionic environment that accompany the maturation of neurons could contribute to developmental regulation of MeCP2 function.^{31–37}

Our published study of isolated MBD revealed that cation uptake is required for modification-specific binding.²⁶ Extending our analysis to full-length MeCP2 revealed the unusual property that binding cooperativity obscures specific recognition of modified DNA at physiological salt concentrations (Figure 1). This behavior is contrary to typical cooperative sequence-specific protein–DNA interactions in which cooperativity enhances binding affinity as well as specificity. MeCP2 binding cooperativity is substantial, resulting in 2 order of magnitude tighter binding compared to that of isolated MBD (Figure 8).

An important subtlety to MeCP2 function is the apparent linkage between modification recognition and binding cooperativity. Cooperativity is lower for MeCP2 binding to either symmetrically or asymmetrically hydroxymethyl-modified DNA (Figure 1). Because binding to modified DNA contributes to MeCP2's biological function,^{57,61} a multistep process in which cooperativity facilitates DNA binding to permit successful competition with linker histones for chromatin DNA (Figure 8) that is then suppressed to allow modification discrimination (Figure 1, black lines and symbols) plausibly rationalizes the protein's physiological function.

Cooperativity appears to be substantially if not exclusively electrostatic in nature as an increase is completely suppressed when the cation concentration increases from the physiological concentration by 100 mM (Figure 1). Despite the apparent dominance of electrostatics in MeCP2 binding cooperativity, the influence of salts on MeCP2 binding is not simple. Mg^{2+} has no effect on the cooperativity of binding to either methylated or unmethylated DNA. Interestingly, only modest specificity for mCpG by MeCP2 is observed in the presence of Mg^{2+} at physiological salt concentrations (Figure 5A). We had previously observed no effect of Mg^{2+} on DNA binding by the isolated MBD.²⁶ This observation argues against preferential interaction of Mg^{2+} with the DNA duplex underlying the effect of the divalent cation on MeCP2 binding cooperativity. Conversely, the fact that the other divalent cation tested, Zn^{2+} , does not influence modification-specific binding suggests that Mg^{2+} does have a discrete influence on this process (Figure 5B). The mechanism underlying the synergy between Na^+ and Cl^- with regard to the energetics of MeCP2 binding cooperativity and specificity remains puzzling and a topic for future study.

Our studies of the effect of TMAO, glutamate, and Zn^{2+} (Figures 5 and 6) point to conformational flexibility of the C-terminal polypeptide as being important for the higher affinity of binding of DNA to MeCP2 relative to that to MBD (Figure 8). While a more

detailed understanding of the mechanistic role of the flexibility of the C-terminal polypeptide awaits future study, it is plausible that the binding of coregulatory factors to MeCP2 would also inhibit cooperativity and thus enhance mCpG binding specificity. Thus, one can envision a multistep process in MeCP2-mediated regulation in which high-affinity/low-specificity cooperative binding brings MeCP2 onto the DNA that is followed by mCpG-specific assembly of regulatory complexes. It should also be noted that the contribution of cooperativity to DNA binding by MeCP2 might confound the interpretation of the *in vivo* binding dynamics of proteins bearing dysfunction-causing mutations. Preliminary analyses show that the cooperativity also obscures the modification specificity of MeCP2 bearing Rett syndrome mutations (S. Khrapunov and M. Brenowitz, unpublished results).

In contrast, the suppression of MeCP2 binding cooperativity by substitution of SO_4^{2-} for Cl^- (Figure 4) has a unique structural signature. We show by anion type that the largest chemical shift changes occur within MBD rather than the C-terminal polypeptide (Figure 7). The fact that the largest chloride-induced shifts are within MBD suggests that the structure and/or dynamics of MBD influences the binding cooperativity mediated by the C-terminal polypeptide. Such structural crosstalk between MBD and the C-terminal polypeptide has been shown by hydrogen/deuterium exchange studies of MeCP2.⁶² The fact that there is crosstalk between MBD and the C-terminal polypeptide is further supported by the lower cooperativity of binding of MeCP2 to hmCpG compared to that for binding to CpG (Figure 1). It is also noteworthy that the largest anion-induced chemical shifts are located within the MBD loop that links it to the C-terminal domains (Figure 7C) and also contains the site of the most prevalent Rett syndrome mutation, T158M.⁶³ These observations suggest that mutations within MBD affect MeCP2 binding cooperativity as well as domain stability and mCpG binding specificity (ref 63 and unpublished results of S. Khrapunov and M. Brenowitz).

A surprising observation is that in a solution containing 250 mM KCl, conditions under which binding cooperativity is completely suppressed (Figure 1), binding of MeCP2 to mCpG-bearing DNA is 500-fold tighter than that of isolated MBD.²⁶ Because high salt inhibits nonspecific electrostatic interactions, electrostatic interactions identified in the C-terminal polypeptide^{28,44,64,65} are unlikely to be the source of the increased stability of MeCP2–DNA complexes relative to that of isolated MBD. Whether the additional binding energy is the result of an unidentified nonelectrostatic interaction or stabilization of MBD is unclear. While our data rule out global stabilization of MBD by the C-terminal polypeptide (the thermal stability of MBD and MeCP2 is the same in the absence and presence of a stabilizing osmolyte), it is possible an additional N- or C-terminal polypeptide orders MBD in a local and heretofore unknown way. Lastly, we note that the structural basis of binding enhancement by the A-tract sequence adjacent to mCpG (present in our target sequence) remains unknown.⁶⁵

The apparent additivity of each strand's modification to the binding strength of MeCP2 suggests that hemihydroxymethyl modification potentially represents a unique signal for MeCP2 binding *in vivo*. The affinity of mCpG/hmCpG DNA for MeCP2 is intermediate to that of the parent symmetrically modified targets (Figure 1). We observed similar behavior by isolated MBD.²⁶ The additivity of modification recognition is somewhat surprising, as

DNA sequence-specific recognition is often a highly concerted process. For example, a base pair change on one site of a palindromic binding site can completely eliminate sequence-specific binding. We note that contradictory pictures of binding of MeCP2 to the hydroxymethyl modification have been reported in the literature.^{58,59,63} Results such as those summarized in Figure 1 suggest that conditions and context play an important role in modification recognition.

Our understanding of the recognition of modification specificity comes from the cocrystal structure of MBD bound to mCpG-bearing DNA.⁶⁶ The high reported hydration level within the methyl binding pocket is consistent with the ready accommodation of the hydroxymethyl modification. We have noted in our study of MBD the plausibility that cations rather than water are required for modification recognition.²⁶ However, if water-mediated binding of a modification is the dominant contact between protein and DNA, it is unclear why hydroxymethyl is bound with an affinity lower than that for methyl. This observation argues for a contribution of determinants such as modification-induced DNA structure to modification recognition. The latter view is also consistent with observed strand additivity of modification recognition. Further studies are required to more clearly describe the discrimination of the discrete modifications bound by MeCP2. An issue complementary to modification recognition that we have not addressed in this paper is sequence recognition and potential synergy between these two modes of interaction of MeCP2 with DNA. Complicating our understanding the contribution of DNA sequence to MeCP2 site recognition is emerging evidence that the protein is sensitive to local changes in conformation that can be induced by modification as well as sequence.^{65,67,68}

The diverse sensitivity of MeCP2 to the solution environment reflects an unusual synergy in the effect of ion type and ion concentration on a protein–DNA interaction. Of particular note is the fact that the ionic milieu of neuronal tissues changes during development, notably by an efflux of Na⁺ and Cl[−].³⁷ Our results suggest the hypothesis that changes in the concentrations of these ions may directly alter MeCP2 DNA binding activity *in vivo*.^{31–37} From an energetic point of view, normal neuronal development and function requires a balance of mCpG-specific, CpG, nonspecific, and cooperative binding processes that are regulated by the ionic environment within neurons. Because classic Rett syndrome manifests during development,¹² it is possible that the disorder's onset may be linked to changes in the neuronal ionic milieu that in turn alter the interaction of MeCP2 with chromatin, making manifest a molecular dysfunction caused by mutation.

What might trigger a switch between the high-affinity/low-specificity and lower-affinity/high-specificity states that result in localization of MeCP2 to discrete regulatory sites? As noted above, changes in the neuronal ionic milieu can modulate binding cooperativity and modification specificity. Other possibilities are post-translational modifications that alter the conformational dynamics of the C-terminal polypeptide and the binding of coregulatory factors. In general, our results show that MeCP2 structure and thermodynamics of MeCP2–DNA complex formation are dynamic depending on the conditions of the surrounding environment. The intrinsically disordered structure of the C-terminal polypeptide can be transformed depending on the particular conditions of the surrounding

environment. These results open an avenue to investigate the mechanisms of the numerous MeCP2 single-point mutations leading to many human diseases such as Rett syndrome.

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ABBREVIATIONS

mCpG	5-methylcytosine-guanine dinucleotide
hmCpG	5- hydroxymethylcytosine-guanine dinucleotide
MeCP2	methyl-CpG binding protein 2
MBD	mCpG binding domain

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Figure 1.

Isotherms determined by fluorescence anisotropy (ex_{490} ; em_{520}) for the binding of MeCP2 to the fluorescein-labeled duplex 5'-TCTGGAACGGAATTCTTCTA-3', with C being symmetrically methylated (●), unmethylated (○), hydroxymethylated (■), or hemimodified [methylated/hydroxymethylated (◻)], in "standard buffer" containing 150 (blue) to 250 (black) mM KCl. The isotherms were determined in "standard buffer" that contains 25 mM Tris-HCl, 6% glycerol, 100 $\mu\text{g}/\text{mL}$ BSA, 0.1 mM EDTA, and 0.1 mM TCEP (pH 7.6) to which was added either 150 or 250 mM KCl. The lines depict the fits of each data set to the Hill equation (Table 1). The fits to ○ and ◻ are denoted with blue dashed lines for the sake of clarity.

Figure 2.

Isotherms determined as described in the legend of Figure 1 except that the standard buffer contained either 75 (blue) or 125 (black) mM K₂SO₄ with C symmetrically methylated (●) or unmethylated (○). The symbol designations are the same as in Figure 1. The isotherms were determined in standard buffer to which was added either 75 or 125 mM K₂SO₄. The lines depict the fits of each data set to the Hill equation (Table 1).

Figure 3.

(A) Isotherms determined as described in the legend of Figure 1 except that the standard buffer contains either 150 mM KCl (● and ○) or NaCl (blue filled and empty circles). Isotherms for MeCP2 binding mCpG are denoted by black and blue filled circles and those for CpG by black and blue empty circles. (B) Isotherms determined as described in the legend of Figure 1 except that the standard buffer contains 150 mM NaCl (● and ○) or 75 mM Na₂SO₄ (blue filled and empty diamonds). Isotherms for MeCP2 binding mCpG are denoted by blue filled diamonds and black filled circles and those for CpG by blue empty diamonds and black empty circles. The isotherms were determined in standard buffer to which was added the indicated salt. The lines depict the fits of each data set to the Hill equation (Table 1).

Figure 4.

Isotherms determined for binding of MeCP2 to CpG as described in the legend of Figure 1 except that the standard buffer contains 75 mM Na₂SO₄ (⊗), 80 mM NaCl, 35 mM Na₂SO₄ (⊙), or 150 mM NaCl (○) with a constant Na⁺ concentration of 150 mM. The lines depict the fits of each data set to the Hill equation (Table 1). The inset summarizes the change in binding cooperativity as reflected in the Hill coefficient, n_H , for isotherms obtained in buffer containing the indicated concentration of NaCl with the balance to 150 mM cation made up with Na₂SO₄.

Figure 5.

Influence of divalent cations on the binding of MeCP2 with DNA. Isotherms determined as described in the legend of Figure 1, except that the standard buffer contains 150 mM KCl (● and ○) and 150 mM KCl with (A) 5 mM MgCl₂ (◆ and ◇) or (B) 2 mM ZnCl₂ (◆ and ◇). Filled symbols are for symmetric mCpG and empty symbols for symmetric CpG. The isotherms were determined in standard buffer to which was added the indicated divalent salt. The lines depict the fits of each data set to the Hill equation (Table 1).

Figure 6.

Influence of trimethylamine N-oxide (TMAO) on the binding of MeCP2 with DNA.

Isotherms determined as described in the legend of Figure 1, except that the standard buffer contains 150 mM KCl in the absence (● and ○) and presence (■ and □) of 1 M TMAO or with 150 mM potassium glutamate substituted for KCl (magenta filled and empty triangles). Filled symbols depict data for MeCP2 binding to symmetric mCpG and empty symbols data for symmetric CpG bearing DNA. The lines depict the fits of each data set to the Hill equation (Table 1).

Figure 7.

(A) Overlay of 2D MBD and MeCP2 600 MHz NMR spectra. ^{15}N HSQC spectrum of MBD (blue) and ^{15}N TROSY-HSQC spectrum of MeCP2 (red) in 150 mM NaCl (pH 7.4). The ^{15}N HSQC spectrum of MBD was shifted by 45 Hz in both dimensions to coincide with the TROSY spectrum of MeCP2. (B) Overlay of TROSY-HSQC spectra of MeCP2 in 250 mM KCl (red) and 125 mM K_2SO_4 (blue). Several peaks from residues within MBD that differ between 250 mM KCl and 125 mM K_2SO_4 are highlighted in the insets. (C) Chemical shift changes in the MBD of full-length MeCP2 comparing 250 mM KCl and 125 mM K_2SO_4 . Residues were color-coded according to average chemical shift differences between MeCP2 in 250 mM KCl and 125 mM K_2SO_4 in ^{15}N TROSY-HSQC spectra. The colors are green (<0.02 ppm), yellow (between 0.02 and 0.07 ppm), red (>0.07 ppm), and dark gray (undetermined).

Figure 8.

Isotherms comparing DNA binding by histone H1 (—), MeCP2 (---), and the isolated MBD of MeCP2 (····) to mCpG bearing DNA in standard buffer containing 150 mM KCl conducted as described in the legend of Figure 1. Human histone H1 was bound to DNA containing symmetric mCpG (▲), symmetric CpG (△), and random DNA sequence (half-filled triangles). The data points for the MeCP2 and MBD isotherms presented in Figure 1 and ref 26 have been omitted for the sake of clarity. The solid line depicts the global fits of the data to the Hill equation. The DNA concentration is 5 nM.

Summary of the Dissociation Constants (K_d) and Cooperativities (n_H , Hill coefficient) Determined for MeCP2 in Each of the Figures

Table 1.

	Sample	K_d (nM)	n_H
Fig. 1 (KCl)	m/m (●) 150 mM (blue)	5.2 ± 0.4	2.5 ± 0.5
	m/m (●) 250 mM	36.5 ± 1.4	1.1 ± 0.1
	-/- (○) 150 mM (blue)	6.3 ± 0.3	2.7 ± 0.2
	-/- (○) 250 mM	1,192.0 ± 915.0	0.9 ± 0.3
Fig. 2 (K ₂ SO ₄)	hm/m (■) 150 mM (blue)	7.8 ± 4.7	1.1 ± 0.7
	hm/m (■) 250 mM	112.0 ± 17.0	0.9 ± 0.1
	hm/hm (■) 150 mM (blue)	6.6 ± 2.0	1.1 ± 0.3
	hm/hm (■) 250 mM	336.0 ± 70.0	1.1 ± 0.2
	m/m (●) 75 mM (blue)	5.3 ± 0.4	3.4 ± 1.0
	m/m (●) 125 mM	8.6 ± 0.8	1.1 ± 0.1
Fig. 3A (150 mM)	-/- (○) 75 mM (blue)	5.4 ± 1.0	1.1 ± 0.2
	-/- (○) 125 mM	195.0 ± 37.0	1.1 ± 0.1
	m/m (●) NaCl (blue)	10.7 ± 0.2	2.5 ± 0.3
	m/m (●) KCl	5.2 ± 0.4	2.5 ± 0.5
Fig. 3B (150mM)	-/- (○) NaCl (blue)	14.4 ± 1.1	3.8 ± 0.8
	-/- (○) KCl	6.3 ± 0.3	2.7 ± 0.2
	m/m (◆) Na ₂ SO ₄ (blue)	5.8 ± 0.4	2.5 ± 0.5
	m/m (◆) NaCl	10.7 ± 0.2	2.5 ± 0.3
	-/- (◇) Na ₂ SO ₄ (blue)	21.5 ± 2.5	0.9 ± 0.1
	-/- (◇) NaCl	14.4 ± 1.1	3.8 ± 0.8
Fig. 4 (-/-)	75 mM Na ₂ SO ₄ (⊗)	21.5 ± 2.5	0.9 ± 0.1
	80 mM NaCl & 35 mM Na ₂ SO ₄ (⊙)	28.3 ± 0.6	1.9 ± 0.1
	150 mM NaCl (○)	10.5 ± 0.6	2.8 ± 0.6
Fig. 5A (150 mM KCl)	m/m (●)	5.2 ± 0.4	2.5 ± 0.5
	m/m 5 mM MgCl ₂ (◆)	5.9 ± 0.5	2.3 ± 0.5
	-/- (○)	6.3 ± 0.3	2.7 ± 0.2
	-/- 5 mM MgCl ₂ (◇)	19.1 ± 2.0	3.5 ± 0.7
Fig. 5B (150 mM KCl)	m/m (●)	5.2 ± 0.4	2.5 ± 0.5

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Sample	K_d (nM)	n_H
m/m 2 mM ZnCl ₂ (◆)	18.0 ± 1.0	1.6 ± 0.1
-/- (○)	6.3 ± 0.3	2.7 ± 0.2
-/- 2 mM ZnCl ₂ (◆)	18.0 ± 0.7	1.7 ± 0.1
m/m (●)	5.2 ± 0.4	2.5 ± 0.5
m/m (■), 1 M TMAO	80.3 ± 2.5	3.0 ± 0.2
m/m (▼), Glutamate	28.3 ± 1.1	3.1 ± 0.2
-/- (○)	6.3 ± 0.3	2.7 ± 0.2
-/- (□), 1 M TMAO	154.9 ± 3.7	4.3 ± 0.3
-/- (▼), Glutamate	56.5 ± 1.8	3.3 ± 0.4

Fig. 6(150 mM KCl)