

Binding of MetJ Repressor to Specific and Nonspecific DNA and Effect of *S*-Adenosylmethionine on These Interactions[†]

Anne M. Augustus,[‡] Harvey Sage,[‡] and Leonard D. Spicer^{*‡§}

[‡]Departments of Biochemistry and [§]Radiology, Duke University, Durham, North Carolina 27710

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ABSTRACT: We have used analytical ultracentrifugation to characterize the binding of the methionine repressor protein, MetJ, to synthetic oligonucleotides containing zero to five specific recognition sites, called metboxes. For all lengths of DNA studied, MetJ binds more tightly to repeats of the consensus sequence than to naturally occurring metboxes, which exhibit a variable number of deviations from the consensus. Strong cooperative binding occurs only in the presence of two or more tandem metboxes, which facilitate protein–protein contacts between adjacent MetJ dimers, but weak affinity is detected even with DNA containing zero or one metbox. The affinity of MetJ for all of the DNA sequences studied is enhanced by the addition of SAM, the known cofactor for MetJ in the cell. This effect extends to oligos containing zero or one metbox, both of which bind two MetJ dimers. In the presence of a large excess concentration of metbox DNA, the effect of cooperativity is to favor populations of DNA oligos bound by two or more MetJ dimers rather than a stochastic redistribution of the repressor onto all available metboxes. These results illustrate the dynamic range of binding affinity and repressor assembly that MetJ can exhibit with DNA and the effect of the corepressor SAM on binding to both specific and nonspecific DNA.

Transcription regulators bind to specific DNA sequences in order to control expression of genes. Although they all have specificity, they also have flexibility in binding to various sequences since very rarely are the sequences completely identical. For instance, the binding matrices for over 70 *Escherichia coli* transcription factors in RegulonDB (1) show that there are no proteins which recognize a single sequence with complete stringency. Therefore, sequence variability is something transcription factors have to accommodate. When a DNA-binding protein can bind to multiple related sequences, it is common to represent these by finding one “consensus sequence” which captures the protein’s binding preferences. Variations of the binding sequence can affect the tightness of binding and therefore can modulate expression.

MetJ, the repressor which controls expression of the genes involved in methionine biosynthesis and transport in *E. coli* (2), recognizes many variable 8-bp¹ sites with the palindromic consensus sequence 5'-AGACGTCT-3' (3). Each of these is called a “metbox” and occurs in clusters of from two to five tandem sites in the operators of Met regulon genes. The different genes in this regulon are repressed to different extents, and this is likely related to the different metbox sequences (4, 5). In vitro analysis using SELEX (systematic evolution of ligands by exponential enrichment) has shown the consensus sequence to be MetJ’s preferred binding sequence (6), and operator mutations away from the consensus caused a decrease in MetJ binding (7–9). MetJ thus has a high intrinsic affinity for the consensus sequence, but as is the case with other transcription factors, this particular

sequence is rare, occurring only once in the operators for the Met regulon in the *E. coli* genome.

MetJ’s function in the cell is regulated by the binding of *S*-adenosylmethionine (SAM), a derivative of methionine (10). When methionine is abundant, SAM binds to MetJ which then represses the various genes of the Met regulon. MetJ does bind DNA in the absence of SAM but with a much lower affinity (11, 12). The mechanism of MetJ activation by SAM is unclear, although MetJ binding to DNA and SAM has been studied by various methods, including gel shifts, filter binding, and DNase footprinting. In this study we used analytical ultracentrifugation (AUC) to quantitatively characterize MetJ’s interactions with various DNA oligonucleotides in solution. AUC has the benefits that experiments are done in solution under equilibrium conditions and provide a direct measure of the interaction. AUC is capable of determining the size, shape, and stoichiometry of proteins or multicomponent complexes under physiological conditions (13, 14).

In the experiments reported here, we characterized the binding of MetJ to DNA oligos (containing zero to five metboxes with natural or consensus sequences) by determining the molecular weight changes which occur when protein and DNA are mixed. In order to best detect binding under conditions where DNA or SAM were present in large concentrations, we incorporated a rhodamine tag (which has an absorbance maximum at 545 nm) on the N-terminus of MetJ to follow the reaction in the visible range where neither DNA nor SAM has any appreciable absorbance.

MATERIALS AND METHODS

Preparation of Rhodamine-Labeled MetJ. For rhodamine labeling, residue Ala-1 was mutated to cysteine using the Quikchange mutagenesis kit (Stratagene). Mutant protein

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*Corresponding author. Tel: 919-684-4327. Fax: 919-684-8885. E-mail: spicer@biochem.duke.edu.

¹Abbreviations: AUC, analytical ultracentrifugation; bp, base pair; SAM, *S*-adenosylmethionine.

Table 1: DNA Constructs Used in This Study

name	bp	sequence (5' → 3') ^a	source
2con	20	gg-AGACGTCT-AGACGTCT-cc	two copies of consensus sequence
2nat	20	gg-AGACaTCc-AGACGTaT-cc	from the <i>metC</i> operator
3con	28	gg-AGACGTCT-AGACGTCT-AGACGTCT-cc	three copies of consensus sequence
3nat	28	gg-AGACGTCT-gGAAtGcCT-taACaTCc-cc	from the <i>metN</i> operator
4con	36	gg-AGACGTCT-AGACGTCT-AGACGTCT-AGACGTCT-cc	four copies of consensus sequence
4nat	36	gg-AGctaTCT-gGAAtGTCT-AaACGTaT-AagCGTaT-cc	from the <i>metA</i> operator
5con	44	gg-AGACGTCT-AGACGTCT-AGACGTCT-AGACGTCT-cc	five copies of consensus sequence
5nat	44	gg-cttCaTCT-ttACaTCT-gGACGTCT-AaACGgaT-AGAtGTgc-cc	from the <i>metF</i> operator
NS44	44	gg-AtggcTga-AtggagCg-gcgaaTaT-AtcaGcCc-AtACGctg-cc	first 40 bases of MetJ coding sequence
NS1con	44	gg-AtggcTga-AtggagCg-AGACGTCT-AtcaGcCc-AtACGctg-cc	NS44 with 1 consensus sequence

^aMatches to the consensus sequence are indicated by capital letters. Natural sequences are taken from the operators of genes recognized by MetJ. Each metbox within a given operator has a different sequence.

(MetJ-A1C) was expressed and purified using published protocols for wild-type MetJ (15). MetJ-A1C was labeled with maleimide-rhodamine (Sigma Chemicals) following manufacturer's instructions to generate MetJ-R. Although there is a cysteine in the wild-type sequence, it is not accessible to solvent. Labeled MetJ-R was separated from the free dye using an S100 gel filtration column (GE Healthcare). The degree of labeling was determined to be approximately one rhodamine molecule per MetJ dimer based on the absorbance at 280 and 551 nm. The remaining unlabeled cysteine was shown to be nonreactive by use of a DTNB assay (16). In addition, AUC experiments described below show no evidence for any higher order disulfide-linked multimers. MetJ exists almost exclusively as a dimer in solution, and all concentrations and stoichiometries reported here are in dimer equivalents. The rhodamine tag was probed by its fluorescence (in the gel shift assay) or absorbance (during centrifugation).

DNA Oligonucleotides. The various DNA oligos were purchased from IDT (Coralville, IA) and dissolved in TES buffer (20 mM Tris, pH = 8, 1 mM EDTA, 150 mM NaCl). Table 1 provides detailed characteristics for the DNAs used in these experiments. For nonpalindromic sequences, complementary strands were mixed at equimolar concentrations. All samples were then heat annealed to form double-stranded DNA.

Gel Shift Assay. Samples containing 50 nM each DNA were prepared in TES buffer with a 1.5-fold molar ratio of MetJ-R to metboxes. Binding was allowed to proceed at room temperature for 20 min. Aliquots were then applied to a 7.5% TGE (25 mM Tris, 192 mM glycine, and 2 mM EDTA) acrylamide gel. When SAM was present, it was incorporated in the gel at a concentration of 1 mM but not in the samples. Gels were run in TGE buffer and then stained with Vistra Green nucleic acid stain (GE Healthcare). Gels were imaged with a Typhoon 9410 phosphor-imager using a 520 BP 40 fluorescence filter for Vistra Green, and a 580 BP 30 fluorescence filter for MetJ-R.

Sedimentation Equilibrium. Studies were carried out with a fixed MetJ-R concentration of 10 μ M ($A_{545} \approx 0.25$) with various concentrations of DNA with and without SAM. Samples were prepared by dilution from stock solutions in TES buffer and then centrifuged at 8000–18000 rpm at 20 °C in six-channel center-piece cells in a Beckman Optima XL-A analytical ultracentrifuge. Unless stated otherwise, the SAM concentration used was 1 mM. Typically each run was done at three speeds appropriate for the size of the MetJ-R/DNA complex. A solvent density (ρ) of 1.0047 was calculated using SEDNTERP. The partial specific volume (\bar{v}) for MetJ-R (0.7242) was calculated from the known amino acid sequence by SEDNTERP. No correction was made for the

rhodamine tag (see Results). \bar{v} is assumed to be 0.55 for all of the DNAs (see Results). Buoyant MW (MW^b) values are reported rather than classical MW because mixtures of species with widely different \bar{v} values, such as protein and DNA, are difficult to interpret using MW. MW^b is also the property measured by sedimentation equilibrium. MW and MW^b are related by eq 1 (17).

$$MW^b = MW(1 - \bar{v}\rho) \quad (1)$$

Best fit MW^b was measured using the XL-A IDEAL 1 program by setting \bar{v} to zero. These were then compared to theoretical MW^b values for complexes of each DNA with varying (n) numbers of MetJ-R dimers according to eq 2.

$$MW_{\text{complex}}^b = MW_{\text{DNA}}^b + nMW_{\text{MetJ-R}}^b \quad (2)$$

Analysis of the equilibrium binding constants between MetJ-R and various DNA molecules was attempted using the SEDANALv345 program of W. F. Stafford and P. J. Sherwood (Boston Biomedical Research Institute). Global fittings of several different MetJ-R/DNA mixtures at two to three rotor speeds were done using HeteroAnalysis (J. L. Cole and J. W. Lary, University of Connecticut) with appropriate models.

Graphs of MW^b as a function of DNA or SAM concentration are shown with 5% error bars which was a typical value for repeated measurements.

RESULTS

Gel Shift Assay. In the absence of SAM (Figure 1A) most of the mixtures show a major unretarded green-staining band (free DNA) in the position reflecting oligo size. Both 4con and 5con (which have palindromic sequences) show a faster moving green-staining minor component which probably represents hairpin structures. 3con, 4con, 5con, and 5nat show a single major band as well as a broad retarded orange-red staining smear. This indicates binding by MetJ-R which fluoresces red. The intensity of MetJ-R staining was greater for the consensus DNAs than for the natural ones of the same metbox number. In the presence of 1 mM SAM (Figure 1B) there are major shifts in the positions and color of retarded bands. 2con and 2nat both show a new major band presumably representing two MetJ-R dimers bound per DNA. NS1con shows a major band and some faint slower bands shared by NS44. All of the others show ladder patterns with distinct bands increasing in number with increasing metbox number. Adding excess MetJ-R chases all of the bands in the ladder into the slowest moving (most highly bound) band (Figure 1C). In the case of 4con and 5con, the hairpin is also

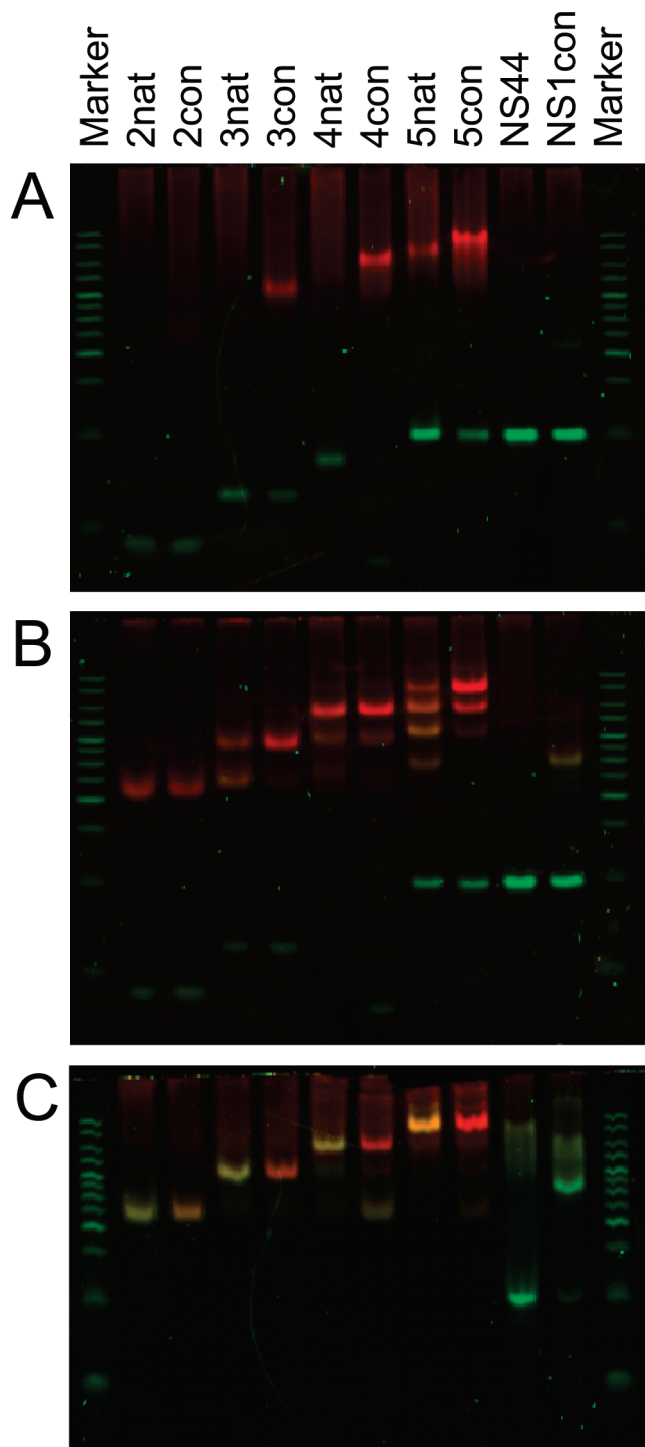


FIGURE 1: Gel shift showing MetJ-R binding to various DNA oligos in the absence (A) or presence (B) of 1 mM SAM and in the presence of SAM and excess MetJ (C). The DNA is stained with a dye that fluoresces green while MetJ-R fluoresces red. The molecular weight marker is Hyper Ladder V from Bionline.

shifted. Interestingly, the bands of the consensus DNAs tend to appear red while those of natural DNAs tend to appear orange. Examination of the single-color scans shows that this is due to a reduced Vistra Green signal intensity in the consensus sequence bands relative to the natural DNAs. We speculate that MetJ-R binds more tightly to consensus DNA sequences, blocking access of the DNA stain to the DNA and resulting in a decrease in the intensity of the signal.

Analytical Ultracentrifugation. Figure 2 shows representative sedimentation equilibrium curves plotted with the absorbance

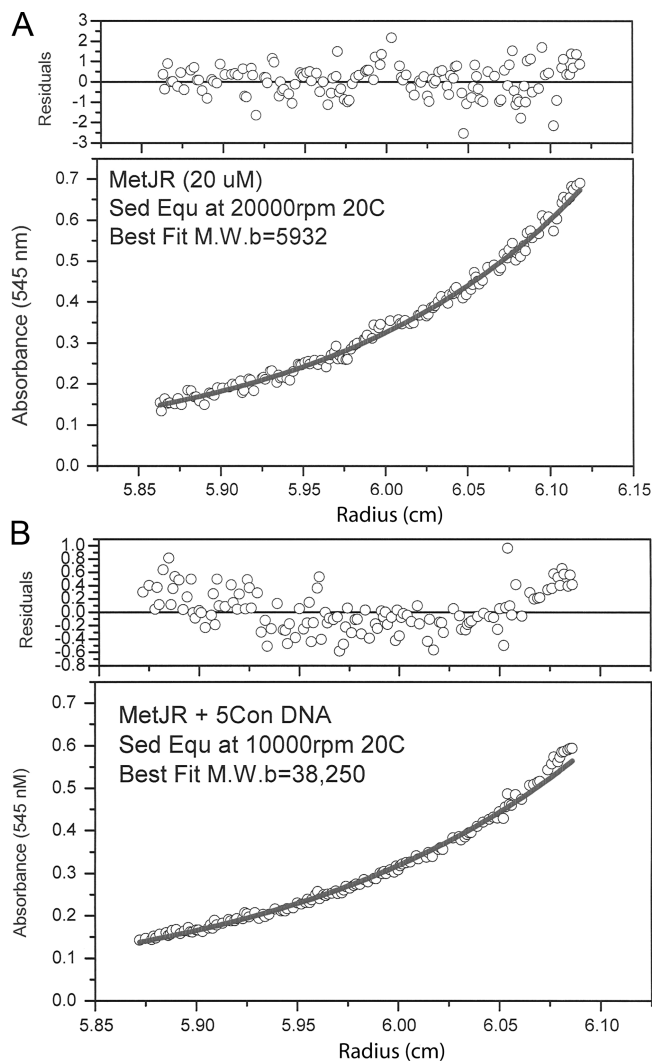


FIGURE 2: Sedimentation equilibrium study of MetJ-R alone (A) and in complex with 5con DNA (B). Residuals are in units of percent.

at 545 nm as a function of the radius. Figure 2A shows 20 μ M MetJ-R at 20000 rpm. The best fit MW^b for MetJ-R is ~ 6000 . In several experiments on both MetJ-R and unlabeled MetJ, the values for MW^b ranged from 5600 to 6200 which, from the calculated \bar{v} for MetJ (0.7242) and ρ_{20} of the solvent (1.0047), gives a value for the true MW of 20600–22800. This is somewhat lower than the expected MW based on the sequence (24019). This, plus the slight curvature of the residuals, suggests a small dissociation into the monomeric form occurring under these conditions. In the presence of 4 M urea, the MW^b falls to ~ 3400 , consistent with a noncovalent dissociation to monomers.

Figure 2B is a study of 10 μ M MetJ-R in complex with 2 μ M 5con DNA at 10000 rpm. The estimated MW^b is ~ 38000 . This value is consistent with an average of four to five MetJ-R dimers bound to each DNA molecule, assuming that MW^b_{DNA} is ~ 12000 and $MW^b_{MetJ-RNA}$ is ~ 6000 . The MW^b of 5nat DNA was measured at 18000 rpm at 20 $^{\circ}$ C using the A_{260} to follow sedimentation equilibrium and found to be 12038 and homogeneous (not shown). From its known classical MW (27038) eq 1 gives a \bar{v} of 0.55 in agreement with other published values for small double-stranded DNAs (18, 19) and was used to calculate the MW^b values used in eq 2 for all of the DNAs.

AUC Results with Oligos Containing Zero or One Met-box. Several studies indicate that MetJ can bind to DNA with no

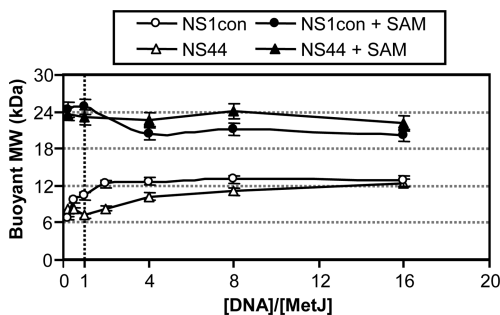
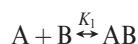


FIGURE 3: MW^b values for MetJ-R complexes with DNA containing zero (NS44) or one metbox (NS1con) at various MetJ:DNA ratios. MW^b for both DNAs is ~ 12040 . The theoretical MW^b for a 1:1 MetJ:DNA complex is ~ 18200 and for a 2:1 complex is ~ 24400 .

obvious metbox sequences (20–22). To investigate this and to compare such binding to a single consensus metbox, a pair of 44-bp DNAs (the same size as 5con and 5nat) was designed containing no metbox (NS44) and a centrally located consensus metbox (NS1con). Figure 3 shows the best fit MW^b values as a function of MetJ-R:DNA ratio for both DNAs with and without SAM. In the absence of SAM, binding is weak and noncooperative for both DNAs. Although binding is slightly higher for the NS1con DNA, both DNAs approach a similar MW^b of 12000 at high DNA concentration. The theoretical MW^b for a 1:1 complex is 18000. The most likely situation is that there is a mixture of free MetJ-R and 1:1 complexes in this concentration range. Using the simple model



HeteroAnalysis gives values for K_1 of 7.19×10^3 and 4.45×10^4 M^{-1} for NS44 and NS1con, respectively (Table 2). This represents a $\Delta\Delta G^\circ$ of -1.25 kcal/mol in going from a nonspecific to the consensus sequence. SAM dramatically increases binding to both DNAs, showing cooperativity approaching a 2:1 stoichiometry. Using the model



HeteroAnalysis yielded values for K_1 and K_2 of 4.85×10^3 and 1.62×10^7 for NS44 and 1.55×10^5 and 2.21×10^7 for NS1con, respectively (Table 2). SAM appears to affect binding to both DNAs to a similar extent.

Since MetJ-R apparently prefers to bind DNA as a dimer of dimers, even when only one metbox is present, we investigated the direct formation of MetJ-R tetramers in solution. Equilibrium ultracentrifugation, however, showed that the molecular weight of MetJ-R was invariant over a range of SAM concentrations from 0 to 20 mM (1000-fold excess), indicating that MetJ-R neither associates nor dissociates in the presence of SAM.

AUC Results with Oligos Containing Two Metboxes. The smallest oligos examined contained two consecutive metboxes and were capable of binding two MetJ-R dimers. The 2con construct contained two copies of MetJ's preferred consensus sequence, while the 2nat construct contained a natural 16-bp recognition sequence from the *metC* operator in *E. coli*. Figure 4 shows the results of MetJ-R binding to these oligos in the presence or absence of SAM and with varying amounts of DNA.

In the absence of SAM, MW^b for MetJ-R/2con is $\sim 15,000$ at metbox:MetJ-R ratios of 1:1 or higher while that for MetJ-R/2nat was $\sim 10,500$. This compares with the theoretical MW^b values of $\sim 11,600$ for the 1:1 complex and $\sim 17,800$ for the 2:1

Table 2: Binding Constants for MetJ-R/DNA Interactions^a

DNA	-SAM		+SAM	
	K_1	K_2	K_1	K_2
NS44	7.19×10^3		4.85×10^3	1.62×10^7
NS1con	4.45×10^4		1.55×10^5	2.21×10^7
2nat	7.83×10^4	1.71×10^6	$K_a > 5 \times 10^7$	

^a K_1 , K_2 , and K_a are in units of M^{-1} .

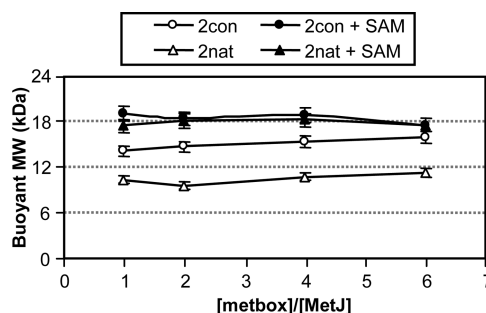


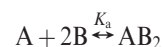
FIGURE 4: MW^b values for MetJ-R complexes with DNA containing two metboxes (2con and 2nat). MW^b for both DNAs is ~ 5440 . The theoretical MW^b for a 1:1 MetJ:DNA complex is ~ 11600 and for a 2:1 complex is ~ 17800 .

complex. SAM raised the MW^b of both to 17,000–18,000 suggesting an almost exclusive population of 2 dimers of MetJ bound per DNA molecule. In all of these systems MW^b is relatively invariant over a wide range of metbox:MetJ-R ratios. This behavior is also seen with NS44 and NS1con in the presence of SAM above. This strongly suggests that such binding is highly cooperative since little re-equilibration occurs when DNA is added above the 1:1 metbox:MetJ-R ratio.

Attempts to apply the



and



models to the binding of MetJ-R to two-metbox DNA had limited success. Using the former two-step model, values for K_1 and K_2 for 2nat in the absence of SAM were found to be 7.83×10^4 M^{-1} and 4.71×10^6 M^{-1} (Table 2). For 2nat in the presence of SAM and for 2con under both conditions the models were rejected as being out of the useful range for the concentrations of MetJ-R and DNA used in this study. This suggests that the K_a values were greater than 5×10^7 M^{-1} .

AUC Results with Oligos Containing Five Metboxes. The largest naturally occurring MetJ-binding sites in *E. coli* contain five metboxes, so oligos containing five tandem metboxes were used to determine conditions where the largest number of MetJ-R dimers bind a single DNA. Figure 5 shows the results of MetJ-R binding to DNA containing either five copies of the consensus sequence (5con) or the naturally occurring sequence from the *metF* operator (5nat). The dashed lines outline the range of MWs expected if all the MetJ-R is bound to DNA (i.e., there is no free MetJ-R). The upper line represents the theoretical MW^b for the 5:1 MetJ-R:DNA complex (42600) while the lower one represents the case where MetJ-R dimers evenly redistribute onto all available DNA molecules with a minimum of one dimer per DNA.

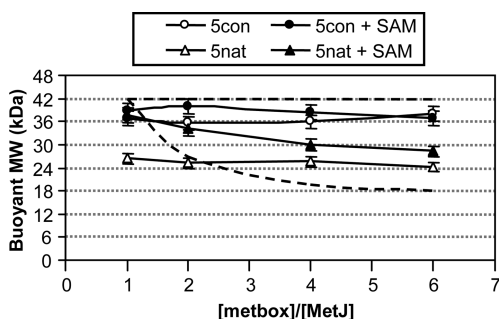


FIGURE 5: MW^b values for MetJ-R complexes with DNA containing five metboxes (5con and 5nat). MW^b for both DNAs is ~ 12040 . The theoretical MW^b for a 1:1 MetJ:DNA complex is ~ 18200 , for a 2:1 complex is ~ 24400 , for a 3:1 complex is ~ 30600 , for a 4:1 complex is ~ 36700 , and for a 5:1 complex is ~ 42900 . The upper dashed line represents the 5:1 complex, while the lower dashed line indicates the reequilibration curve expected for a noncooperative process.

Both in the presence and in the absence of SAM, 5con bound four to five MetJ-R dimers per DNA with strong cooperativity and little or no reequilibration. This behavior was similar to that for 2con and 2nat above and indicated that there was saturation of all metbox sites on that population of DNA molecules which bound MetJ-R even in the presence of a considerably larger number of DNA molecules containing little or no bound MetJ-R. On the other hand, binding to 5nat in the absence of SAM was significantly less than with 5con. In the presence of SAM, binding at the 1:1 metbox:MetJ-R stoichiometry was comparable to 5con, but as more DNA was added, reequilibration occurred, and the MW^b approached those seen for 5nat in the absence of SAM. Attempts to fit AUC data to the model



were rejected routinely for all of the DNAs containing three, four, or five metboxes as being out of the range for experimental conditions. When the root-mean-square average error of the data exceeds certain limits relative to the observed MW change with concentration, the data are rejected.

In order to clarify the effect of SAM on binding, a titration was performed, holding the concentration of MetJ-R and DNA constant (Figure 6). For the 5con DNA, there is no significant change in MW^b at any SAM concentration, whereas for 5nat there was an increase in MW^b as the concentration of SAM increased. At SAM concentrations at or above the stoichiometric ratio, the MW^b of 5nat approached that of 5con.

AUC Results for Other Oligos. Studies were primarily done on two-metbox and five-metbox DNA because these represent the shortest and longest naturally occurring MetJ binding sites. We also looked at three-metbox and four-metbox oligos but only with stoichiometric amounts of DNA. Figure 7 shows the results for all oligos in the presence or absence of SAM at a 1:1 metbox:MetJ-R ratio. (For NS44, an equimolar ratio of DNA was used.) Results are reported as the number of MetJ-R dimers bound to each DNA (using eq 2) in order to compare the effect of SAM independent of the MW^b of the DNA or the complexes.

DNA containing three or more repeats of the consensus sequences showed near-maximal binding even in the absence of SAM. For natural sequences in the absence of SAM, there was always less binding than with the consensus repeats. However, in the presence of SAM maximal binding was reached, and there was little difference between natural and consensus sequences.

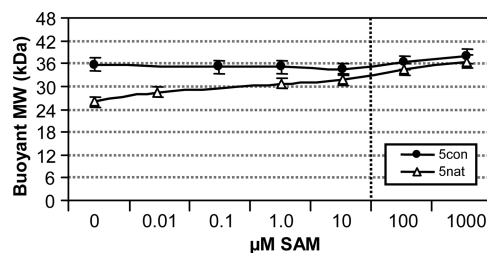


FIGURE 6: MW^b values for complexes containing MetJ-R and DNA with five metboxes as a function of SAM concentration. The DNAs used were 5nat (Δ , $MW^b = 12038$) and 5con (\bullet , $MW^b = 12040$). The DNA:MetJ was always at a 1:1 [metbox]:[MetJ-R] ratio. Stoichiometric SAM is $20 \mu\text{M}$, indicated by the vertical dashed line between 10 and $100 \mu\text{M}$ SAM.

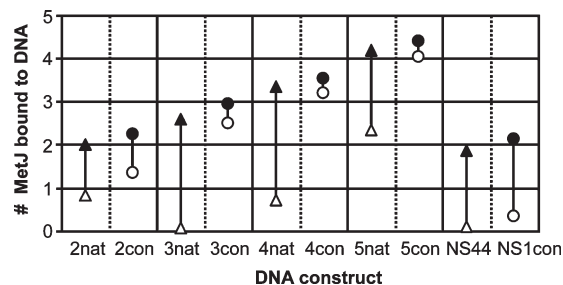


FIGURE 7: Average number (n) of MetJ-R dimers bound to stoichiometric concentrations of various DNAs in the presence (filled symbols) or absence (open symbols) of 1 mM SAM. The value of n was determined from the data using eq 2.

DISCUSSION

SAM Enhances MetJ Binding to All DNA Sequences. The interaction of MetJ-R with metbox sequences is complex, and SAM contributes to this complexity. In every case, DNAs containing multiple consensus sequences bound MetJ-R more tightly than their same-length counterparts containing natural sequences. In general, SAM increases binding and decreases reequilibration of MetJ-R dimers between DNA molecules. This is evident from a comparison of gel shift assays in the presence and absence of an excess of SAM. When SAM is present, distinct bands are seen representing various MetJ-R/DNA complexes. In its absence the bands merge into a smear, suggesting dissociation of the complexes as they migrate through the gel. These patterns are similar to those predicted for protein–nucleic acid complexes and observed for other systems (23).

SAM is not required for MetJ-R to bind to DNA even for those oligos without a target sequence. Binding to NS44 is very weak and involves a single MetJ-R dimer with a K_1 in the 10^4 M^{-1} range. The single consensus sequence in NS1con increases binding by less than 10-fold, and the natural metboxes should lie between these limits. In contrast, the addition of SAM causes two dimers of MetJ-R to bind to both NS44 and NS1con with high cooperativity and a K_2 value in the 10^7 range, an increase of ~ 1000 -fold. As before, the DNA with the single consensus metbox binds MetJ-R only slightly more tightly.

Two dimers of MetJ-R binding to DNA with zero or one metbox are not due to the formation of a tetramer in free solution induced by SAM, nor is it obvious where on NS44 (which is the same size as 5con and 5nat DNA) they bind. The slight increase in the binding of NS1con in the presence of SAM (compared to NS44) suggests that the MetJ-R dimers lie at adjacent sites.

With two-metbox DNA in the absence of SAM, tetramer binding occurs, but it is relatively weak, and the consensus

sequence shows significantly tighter binding. The K_a values for binding of one and two MetJ-R dimers to 2nat are similar to those seen for NS44 and NS1con in the presence of SAM. Attempts to measure these values for 2con were not successful because K_2 was too high to measure with the concentrations of MetJ-R and DNA used in these studies, probably greater than $5 \times 10^7 \text{ M}^{-1}$. SAM shifts MetJ-R binding of both 2con and 2nat to strongly cooperative tetramer binding. Hence the presence of two adjacent metboxes contributes significantly to cooperative binding, and SAM increases such binding.

With the five-metbox DNAs, 5con shows near-saturation of all the metboxes in the presence or absence of SAM, again indicating strongly cooperative binding. The presence of a minor amount of hairpin DNA for 5con (and 4con) does not have a significant effect on the measured MW^b , which is close to its maximal value. In the absence of SAM, 5nat shows significantly reduced binding as do all of the natural metbox DNAs. In the presence of SAM, in contrast, MetJ-R binds 5nat at near-saturation when the metbox:MetJ-R ratio is 1:1 but decreases significantly as excess 5nat is added, approaching MW^b values seen in the absence of SAM, which indicates extensive reequilibration. For 5nat DNA, each metbox has a different sequence, with different matches to the consensus and therefore different affinities for MetJ. At equilibrium, it is reasonable that MetJ-R would dissociate from a weaker metbox and bind to a stronger metbox on a different DNA molecule, as long as it can bind as a tetramer. The effect of SAM is strongest at concentrations above a SAM:MetJ-R ratio of 2:1, suggesting that SAM binds in a weak noncooperative manner to each subunit of MetJ, in agreement with published studies (21, 24). Attempts to measure binding constants by HeteroAnalysis for all of the other DNAs with or without SAM failed because the K_a values were too high for the concentration of MetJ-R and DNAs used here. This is a consequence of the absorbance optics of the XL-A. Some of these could be measured in principle using fluorescence optics (25) or using radiolabeled MetJ and/or DNA with the method of Minton (26).

A more detailed analysis of MetJ, SAM, and DNA binding is beyond the scope of this report, but it should be pointed out that an earlier, elegant theoretical approach to ligand binding in matrices containing n linear binding sites has been developed by McGhee and von Hippel (27). Their model applies to both noncooperative and cooperative binding but would require significant extension to accommodate the biologically important corepressor SAM as a third independent binding element in most of the complexes reported here. In addition, for all natural contiguous metbox sequences from *E. coli*, the binding constants at each metbox will vary with the specific 8-bp sequence, requiring reformulation of the simplifying assumption of an intrinsic K in the reported model (27). Nevertheless, such a model would be a valuable tool to complement experiments in complex regulatory systems like the methionine repressor in *E. coli*.

SAM Activation of MetJ. Under *in vitro* conditions, MetJ can bind DNA in the absence of SAM, but this situation is unlikely to occur *in vivo* because SAM is an essential metabolite that is required for cell growth and can never be completely depleted in the cell (28). In the living cell, the concentration of SAM is thought to be in the range of 1–100 μM (29), which is usually in excess over MetJ. Therefore, the shift to active repression occurs not when the SAM concentration goes from 0 to 1 mM (as in most of our experiments) but more likely when it goes from low to high micromolar, a range which is still

responsive to SAM as seen in Figure 6 with the 5nat sequence. The purpose of SAM, then, is not just to enable MetJ to bind DNA but to regulate its binding and make it sensitive to the concentration of SAM in the cell.

The mechanism for SAM activation of MetJ is unknown. It has been suggested that SAM works by a long-range electrostatic interaction (30, 31) or possibly by changing protein dynamics (32), but neither hypothesis has been proven conclusively. The crystal structure of MetJ bound to two-metbox DNA shows no major changes in conformation (relative to the structure of MetJ alone or MetJ bound to SAM) that would account for activation by SAM (33), but NMR experiments in our laboratory suggest that SAM has a profound effect on MetJ in solution, as shown by radical changes in the HSQC fingerprint spectra after the addition of SAM (unpublished data). In addition, neutron scattering experiments also suggest a conformational change in the complex upon binding (15). One possibility that we considered was that SAM affected MetJ's higher order oligomerization status. Our results here do not provide evidence for this, but there is still the possibility that a small amount of MetJ tetramer is formed, which has little effect on the measured molecular weight.

Cooperativity Is Important for MetJ Binding. Although we found no evidence for tetramer formation in solution, tetramer formation on DNA is required for repression. MetJ's affinity for a single metbox is weak (21), and higher order assemblies of at least two dimers are required for stable binding (7, 20). The importance of cooperative binding is clearly demonstrated by our AUC results where the interaction of MetJ with DNA which contains a single metbox (NS1con) is not significantly stronger than its interaction with DNA containing no metboxes (NS44). In the presence of SAM, they both tend to bind two MetJ dimers. We do not know whether these dimers are on adjacent sites and interact with each other, but if they are not, then we would expect only one MetJ dimer per DNA under conditions of large DNA excess, and that is not observed. Alternatively, the binding of one MetJ dimer may affect the conformation of the DNA, enhancing the binding of a second dimer to a nonadjacent site on the same oligo, but this type of long-range effect seems unlikely since multiple metboxes in nature are always directly adjacent to each other, without extra bases between them. Other evidence for the impact of cooperativity comes from the fact that at high DNA:protein ratios there is far less redistribution of MetJ on the DNA than we would expect based on a random distribution.

Nonspecific Binding Is an Early Step on the Pathway to Repression. The similar behavior of NS1con and NS44 indicates not only the importance of cooperativity but the importance of nonspecific DNA interactions as well. Even though MetJ binding to nonspecific DNA is weak, as is the binding of a single MetJ dimer to a metbox, when there is a large abundance of DNA, as there is in the cell, the equilibrium may very well favor such weak binding. Our previous NMR experiments suggest that in the cell MetJ is always associated nonspecifically with DNA (22). This association only leads to active repression during times of methionine abundance, however, indicating the important role played by SAM in regulation. This weak nonspecific binding has also been reported using filter-binding assays (20) and calorimetry (21).

One model for repression, therefore, incorporates an initial state with MetJ loosely associated with genomic DNA. Considering the near-identical behavior of NS1con and NS44 in solution, the binding of MetJ to the first site shows no measurable

preference for the consensus sequence. This is also supported by calorimetry data showing the binding energy of MetJ to random DNA (-5.7 kcal/mol) is comparable to that of MetJ to a single metbox (-5.8 kcal/mol) (21). When SAM concentrations increase and the corepressor binds to MetJ, the protein's affinity for DNA is increased. Despite this nonspecific increase in DNA affinity, MetJ will only tightly bind DNA if it can do so as a complex of two or more dimers at adjacent metboxes which contain favorable sequences. Thus stable binding only comes from the sum of the energies of two dimers binding plus a cooperativity factor (-1.3 kcal/mol) (21). This requirement for two adjacent metboxes is beneficial because it allows for greater specificity of binding (requiring a 16-bp recognition sequence rather than just 8) without having to use a larger protein and without having to be too strict about deviations from the consensus.

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