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# Calorimetric studies of the energetics of protein–DNA interactions in the *E. coli* methionine repressor (MetJ) system

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**Abstract**

Calorimetric measurements of binding of a specific DNA fragment and *S*-adenosyl methionine (SAM) co-repressor molecules to the *E. coli* methionine repressor (MetJ) show significant differences in the energetics of binary and ternary protein–DNA complexes. Formation of the MetJ:SAM:DNA ternary complex is significantly more exothermic ($\Delta H \simeq -99$ kJ·mol$^{-1}$) than either MetJ:DNA or MetJ:SAM binary complexes alone ($\Delta H \simeq -10$ kJ·mol$^{-1}$ each). The protein is also significantly more stable to unfolding ($\Delta T_m \simeq 5.4$°C) when bound to DNA. These observations suggest that binding of SAM to the protein–DNA complex leads to a significant reduction in dynamic flexibility of the ternary complex, with considerable entropy–enthalpy compensation, not necessarily involving any overall conformational change.

*Key words:* Protein–DNA interaction; Calorimetry; Energetics; Dynamics; Methionine repressor

## 1. Introduction

The *E. coli* methionine repressor, MetJ, is a member of the ribbon-helix-helix (RHH) family of sequence-specific DNA binding proteins, which includes the phage P22 repressors Arc and Mnt, and the Tra Y proteins of F′ and related episomes [1–3]. Operator sequence recognition is primarily achieved by hydrogen bonding from side chains located on the $\beta$-ribbon to the edges of the base pairs in the operator [4–6]. The repressor exists in solution as a non-covalent dimer of subunit $M_r$ 11,996, but forms extended arrays when bound to operator sites. Array formation is co-operative with respect to protein concentration [6,7]. Operator binding is dependant on a small co-repressor molecule, *S*-adenosyl methionine (SAM), two molecules of which bind non-cooperatively to each repressor dimer, enhancing the interaction with DNA approximately 10-fold, depending on conditions [8]. The SAM binding sites are distant from the sequence-specific DNA binding regions on the opposite face of the molecule [5], and SAM binding does not appear to alter repressor conformation significantly. Consequently it has been thought that the co-repressor effect might arise purely out of the long range electrostatic effects of the positive charge carried by the tertiary sulphur atom in SAM [6,9]. High resolution crystal structures are available for both the apo-repressor and the co-repressor-bound holo-repressor [10]. A structure has also been obtained for the minimal stable repressor–operator complex, which contains two repressor dimers bound to a fragment encompassing a 16 bp operator sequence [5]. Natural operators contain multiple tandem repeats of an 8 bp recognition sequence, the met box, with consensus dAGACGTCT [7]. Operators can have up to five such met box repeats (*metF* and *metB*) the minimum being two met boxes (*metC*), although the minimum effective operator in vivo probably contains four repeats ([11]; Wild, Stockley et al., unpublished).

Ultimately the transcriptional flux through the *met* operons is determined by the kinetics of assembly/disassembly and the stability of the repressor–operator complexes relative to the passage by RNA polymerase, and the energetics of such processes are of considerable general interest [12]. The interactions of MetJ with synthetic variants of the operators have been investigated by both gel retardation and nitrocellulose filter binding experiments, which have allowed estimates of the equilibrium constant and free energy of the interaction to be made [4,6,7]. However, the indirect nature of these assays means that these values are necessarily only estimates. We report here on direct calorimetric determination of the energetics of these interactions.

## 2. Materials and methods

### 2.1. Protein

Wild-type methionine repressor protein was over-expressed in *E. coli* and purified to homogeneity, as judged on polyacrylamide gels [13], as previously described [8], with minor modifications involving chromatographic separations on DEAE–Sepharose and S–Sepharose. Purified protein was stored as an ammonium sulphate precipitate at 4°C until use. Samples for calorimetry were suspended in 25 mM potassium phosphate buffer at pH 7.0, containing 0.1 M KCl, extensively dialysed against the same buffer at 4°C, and centrifuged to remove traces of undissolved material. Buffers for DSC experiments also contained 1 mM DTT (dithiothreitol) to guarantee full reversibility of the MetJ thermal unfolding transition [14]. Samples of the final dialysis buffer were used for calorimeter equilibration and DSC reference baseline corrections. Protein concentrations were determined using $\varepsilon_{280}$ =

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15,340 $M^{-1} \cdot cm^{-1}$ for the MetJ monomer ($A_{280}$ = 1.28 for 1 mg/ml) on the basis of amino acid composition [15].

*2.2. DNA*

A synthetic 16 bp oligonucleotide (HPLC-purified and freeze-dried) of sequence dAGACGTCTAGACGTCT, comprising two consensus met boxes [7], was obtained from OSWEL (Edinburgh). Samples for calorimetry were dissolved in the appropriate dialysis buffer, centrifuged, their and concentration determined from UV absorbance assuming 1 $OD_{260}$ ≡ 31.5 μg ($\varepsilon_{260}$ = 154,860 $M^{-1} \cdot cm^{-1}$) of single-stranded oligonucleotide [16]. Control experiments for non-specific DNA effects were done using degraded herring sperm oligonucleotides (Sigma).

*2.3. Co-repressor*

*S*-Adenosyl methionine (SAM, *p*-toluenesulphonate salt) from Sigma was used without further purification, and concentrations were verified by UV absorbance using $\varepsilon_{260}$ = 15,400 ($A_{260}$ = 38.56 for 1 mg/ml [17]).

DSC experiments to investigate thermal stability of repressor protein, DNA, and their complexes were performed using a Microcal MC-2D differential scanning calorimeter at a nominal scan rate of 60°C · $h^{-1}$, using an appropriate equilibration buffer in the reference cell [18]. Both sample and reference solutions were degassed under vacuum with gentle stirring for about 1 min prior to loading. ITC studies of the energetics of protein : DNA : co-repressor (SAM) binding were carried out at 15, 25 and 40°C using a Microcal OMEGA titration microcalorimeter [18,19]. In a typical titration experiment, degassed DNA solution (10–15 μM, single strand) was loaded into the reaction cell (1.4 ml) and titrated with up to 25 × 10 μl injections of MetJ (0.1–0.2 mM) from a 250 μl stirrer syringe, allowing a 3 min equilibration time between injections. Some titrations were also performed by addition of concentrated DNA aliquots to MetJ solutions under similar conditions. Co- repressor binding was studied by injection of concentrated SAM into protein or protein/DNA mixtures in the ITC cell. Integrated heats of reaction data were corrected for the effects of mixing and dilution obtained from appropriate control experiments performed under identical conditions. DSC and ITC data were analysed in terms of standard models using the Microcal ORIGIN software package. Stoichiometric complex formation between MetJ and consensus DNA was confirmed by calibrated gel-filtration measurements (Superose 12 FPLC) under the same conditions as used for calorimetric titrations.

## 3. Results and discussion

Isothermal titration calorimetry experiments for the binding of specific DNA 16-mer and SAM to MetJ are illustrated in Figs. 1 and 2. In all cases the calorimetric data are consistent with exothermic stoichiometric complex formation, but with significant differences in the enthalpies of formation of binary and ternary complexes (Table 1). Formation of either MetJ:DNA (Fig. 1) or MetJ:SAM (Fig. 2) binary complex liberates very little heat energy, whereas completion of the MetJ:SAM:DNA ternary complex by whatever route is considerably more exothermic. Analysis of the MetJ–DNA binding thermograms (Fig. 1) gives a stoichiometry of 2 MetJ dimers per DNA 16-mer duplex (average $n = 2.04 \pm 0.14$, from 11 separate experiments), i.e. one repressor molecule bound per met box, consistent with previous structural and genetic studies. Similarly, for MetJ–SAM binding in the presence of DNA (Fig. 2), calorimetric titration shows a stoichiometry of 2 mol of SAM per MetJ dimer, with an apparent binding constant ($K$) of about $9 \times 10^4$ $M^{-1}$. The latter is consistent with the 'unexpectedly low' $K$ of about 5,000 $M^{-1}$ ($K_{Diss} \simeq 200$ μM) found by equilibrium dialysis in the absence of DNA, coupled with the roughly one order of magnitude enhancement in affinity in the presence of DNA [8]. We are unable so far to measure the binding affinity of SAM to MetJ alone using calorimetric methods, because of the low affinity and small enthalpy in the absence of DNA. Binding constants for the DNA–MetJ interaction are also difficult to estimate with certainty because of the relatively high concentrations required for the calorimetric binding experiments, but values ($K_{app}$) obtained from these titrations generally lie in the region $1–5 \times 10^7$ $M^{-1}$, corresponding to a standard free energy of MetJ:DNA complexation ($\Delta G°$) of −40 to −44 kJ · $mol^{-1}$ at 25°C. Slight deviations of the titration data from ideal binding curves (Fig. 1) may indicate some cooperative interaction between MetJ dimers at adjacent DNA (met) sites, as suggested by previous work [6,7]. Saint-Girons et al. [8] showed, using gel retardation and filter binding techniques on longer DNA fragments, that $K_{app}$ for the repressor–operator complex is about $10^9$ $M^{-1}$ in the presence of 10 mM SAM, and about one order of magnitude less in the absence of SAM. This difference in absolute magnitudes of $K_{app}$ may reflect different experimental conditions or the inherent reduced stability of shorter DNA fragments used here. Nevertheless, our results are consistent with the relatively small (ca. 10-fold) effect of SAM on the affinity of MetJ for DNA.

Binding to specific DNA also affects the thermal stability of the MetJ protein. DSC experiments show that, in the absence of specific DNA, MetJ undergoes a reversible thermal unfolding transition comparable to that shown previously in this laboratory [14], with $T_m$ = 54.0°C and $\Delta H_m$ = 530 kJ · $mol^{-1}$ under these conditions. Addition of SAM (up to 3 mM) has only a small effect on this thermal stability (though such experiments are complicated by the poor thermal stability of SAM itself in aqueous buffers at the elevated temperatures encountered in the DSC). However, addition of increasing amounts of specific DNA 16-mer, with or without SAM, gives a progressive increase in apparent $T_m$ of the transition, reaching a maximum of 59.4°C ($\Delta H_m \simeq 750$ kJ · $mol^{-1}$) with stoichiometric amounts of DNA (Fig. 3). The DNA duplex itself shows a typically broad DSC melting profile at even higher temperature ($T_m \simeq 71–$

Table 1
Mean apparent heats of formation of MetJ : DNA : SAM complexes in 25 mM potassium phosphate 0.1 M KCl buffer, pH 7.0, obtained by direct calorimetric titration of DNA with MetJ

| Complex | T (°C) | $\Delta H$ (kJ · $mol^{-1}$)* |
|---|---|---|
| MetJ:SAM:DNA | 10 | −88 ± 10 |
| | 25 | −99 ± 6 |
| | 40 | −95 ± 10 |
| MetJ:DNA | 25 | −10 ± 5 |
| MetJ:SAM | 25 | ≈−8 |

*Expressed per mol of MetJ dimer (4.184 J = 1 calorie).

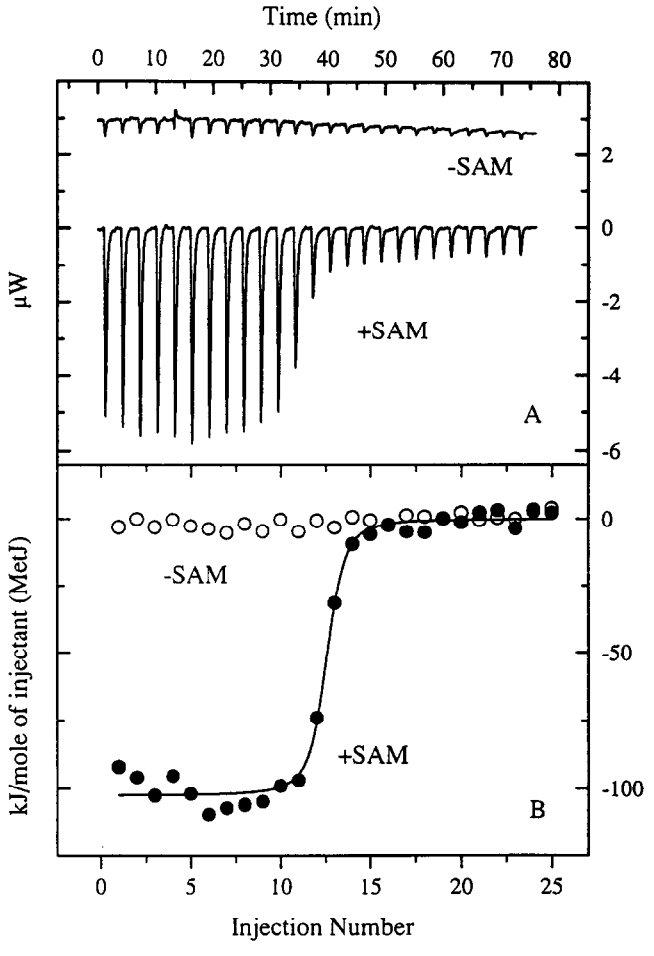


Fig. 1. Microcalorimetric titration data for the binding of MetJ to a specific 16 bp DNA fragment comprising two consensus met boxes. Data were obtained at 25°C in 25 mM phosphate buffer, 0.1 M KCl, pH 7, from 25 × 10 μl injections of MetJ (0.132 mM) into DNA (12.7 μM), either in the absence (○) or presence (●) of SAM (0.288 mM). (A) Calorimeter output showing raw titration data. (B) Normalized heats of reaction obtained by integration of injection pulses from A, corrected for heat of dilution. The solid line is the result of non-linear least squares fit for non-cooperative binding of 2 MetJ dimers per DNA duplex with $K_{app} = 5.4 \times 10^7\ M^{-1}$ and $\Delta H = -102\ kJ \cdot mol^{-1}$.

74°C, depending on concentration) under these conditions (Fig. 3). Fluorescence measurements (McAlpine, Cooper and Stockley, unpublished) show no changes in tryptophan environment in the protein–DNA complex, consistent with lack of conformational changes seen by X-ray crystallography [5]. Control experiments for non-specific (non-operator sequence) DNA effects on MetJ (± SAM) showed no effect on stability of the protein in DSC, and only weak ($\Delta H \simeq -8\ kJ \cdot mol^{-1}$) non-stoichiometric binding in ITC measurements.

Unlike several other protein–DNA systems [12,20,21], the enthalpies of formation of the MetJ:SAM:DNA complex show no significant variation with temperature over a 10–40°C range (Table 1; $\Delta C_p = -0.2 \pm 0.3\ kJ \cdot K^{-1} \cdot mol^{-1}$). This appears to be consistent with the lack of significant conformational change revealed by X-ray crystallography [5] and the interpretation of negative $\Delta C_p$ in terms of local folding effects [12]. However, it leaves unresolved the question as to why there are such large differences in enthalpy between MetJ binary and ternary complexes, or why co-repressor (SAM) has such a large effect on the enthalpy of the MetJ–DNA interaction, despite the relatively small effect on binding affinity. Specifically, our measurements show that formation of the ternary MetJ:SAM:DNA complex is almost an order of magnitude more exothermic than either of the binary complexes of MetJ:DNA or MetJ:SAM alone. The associated change in Gibbs free energy is comparatively much less (a 10-fold increase in affinity corresponding to $\Delta\Delta G = -5.7\ kJ \cdot mol^{-1}$ at 25°C), implying that the major decrease in overall enthalpy of the system ($\Delta\Delta H \approx -90\ kJ \cdot mol^{-1}$) occurs with a concomitant de-

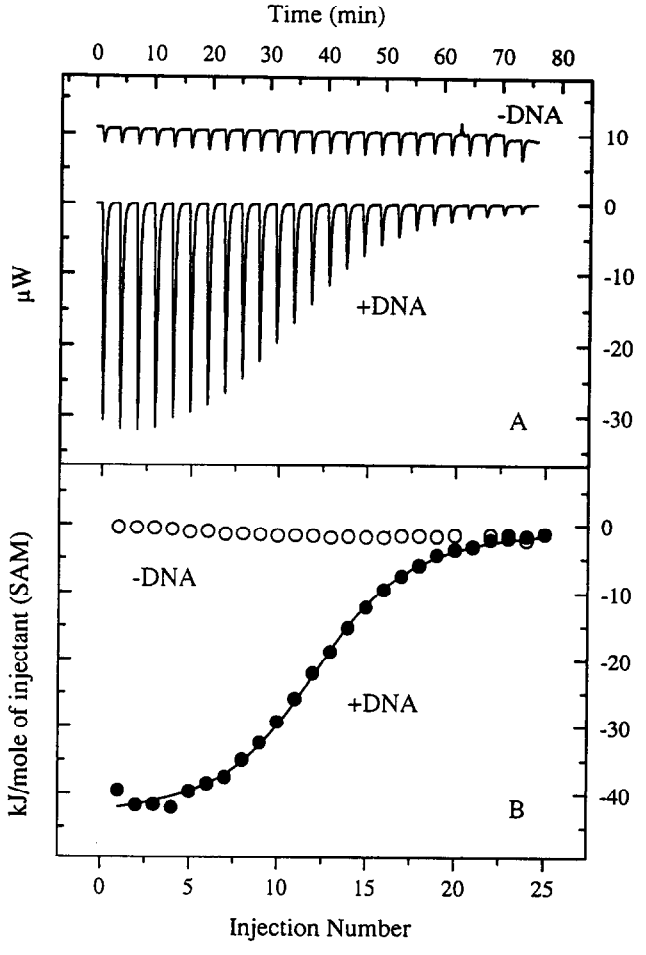


Fig. 2. Isothermal titration data for the binding of SAM to MetJ in the presence or absence of DNA. 25 × 10 μl injections of SAM (2.2 mM) into a mixture of MetJ (85 μM) and DNA (150 μM), using the same buffer conditions as in Fig. 1. (A) Raw titration data. (B) Normalized heat of reaction data obtained by integration of raw data from A, corrected for heat of dilution. The solid line is the result of a non-linear least squares fit for non-cooperative binding of SAM to MetJ:DNA with $K_{app} = 9.3 \times 10^4\ M^{-1}$ and $\Delta H = -44\ kJ \cdot mol^{-1}$, assuming two SAM binding sites per repressor.

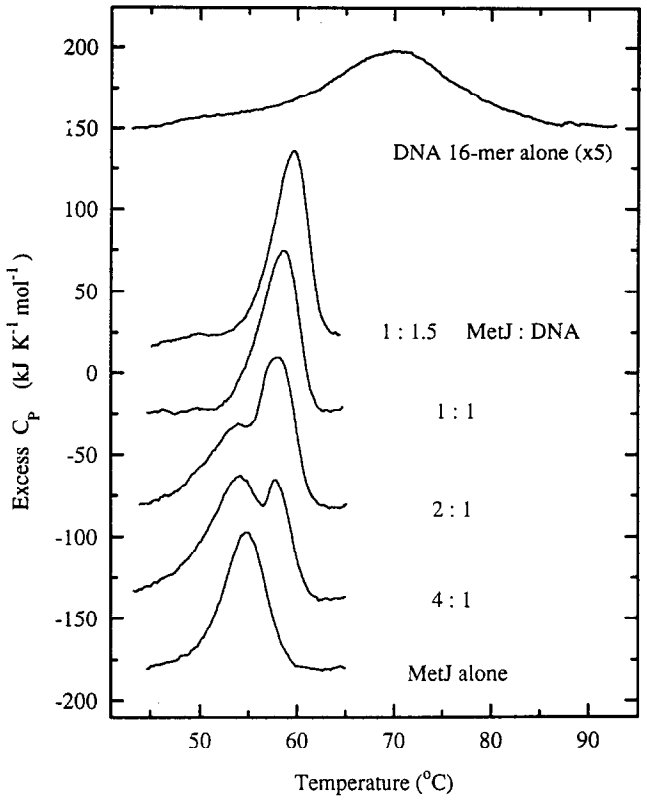


Fig. 3. DSC thermograms for MetJ, repressor DNA 16 bp fragment, and protein–DNA mixtures in 25 mM phosphate, 0.1 M KCl, pH 7, containing 1 mM DTT. Data have been corrected for control buffer baseline and concentration normalized. Stoichiometric ratios of MetJ dimer to DNA duplex in the mixtures are indicated where appropriate. The data for DNA alone have been expanded (×5) for clarity.

crease in entropy ($\Delta\Delta S \approx -280$ J·K$^{-1}$·mol$^{-1}$). This large decrease in entropy is unlikely to arise solely from electrostatic effects. Nor is it likely to be due to displacement of counter-ions or solvent from the protein–DNA interface, since no such effect is seen during complex formation in the absence of co-repressor. It is tempting, therefore, to suggest that formation of the ternary DNA:MetJ:SAM complex results in a reduction in dynamic flexibility of the entire system, i.e. binding of SAM 'stiffens' the protein–DNA complex, and that the observed enthalpy and entropy changes arise mainly from global changes in molecular dynamic fluctuations rather than any specific set of interactions.

This dynamic interpretation could be pictured as follows. Imagine that, in the absence of SAM, MetJ is relatively flexible and undergoes scissor-like or hinge-bending motions about some notional 'pivot'. These motions might, indeed, facilitate the 'walking' of MetJ (possibly in a crab-wise fashion?) along the DNA during diffusion along the DNA chain postulated to explain repressor kinetics in some systems [22–25]. It might also make it easier for repressor molecules to be shunted along or elbowed out of the way when required to prevent traffic control problems during the passage of RNA polymerase, for example. The binding of SAM, on the opposite side of the hinge/pivot from the DNA, might jam this motion. Although the consequent effect of such dynamic changes on the free energy might be relatively small, since there is little or no change in average conformation and because the reduction in overall enthalpy would be offset by the concomitant decrease in entropy [26,27], we might anticipate that the effects on the kinetics of displacement of protein from the DNA should be much greater. A more rigid complex would allow less dynamic flexibility, with consequently higher kinetic activation barriers to the various conformational fluctuations required for displacement or diffusion of the repressor from its operator site.

Our proposal that co-repressor binding leads to reduced repressor flexibility is consistent with recent comparisons of the high resolution X-ray crystal structures of the apo- and co-repressor bound forms of MetJ in different crystal lattices and the ternary complex [28], which show that binding of co-repressor leads to a slight tightening and shrinking of the repressor molecule, which increases still further when the operator fragment is bound. These results may also be compared to recent calorimetric studies of other repressor systems. The only other equivalent system in which relevant thermodynamic measurements have been reported to date for protein–DNA interactions is the Cro protein–DNA complex, studied by pulsed-flow microcalorimetry under saturating conditions to give the heats of complex formation [20]. The thermodynamics of co-repressor and operator DNA binding by the *E. coli* TrpR repressor have also been studied [21], although the protein–DNA complex data could only be obtained by indirect Van't Hoff methods. In both cases, and in contrast to the MetJ situation described here, the enthalpies of complex formation show an anomalously high temperature dependence, which has been attributed to major DNA-induced changes in protein conformation or dynamic flexibility. The basic trends of the TrpR observations have been confirmed independently in Glasgow by direct titration microcalorimetry (A. Cooper and A.N. Lane, work in progress), though the nature of the temperature dependence is somewhat more complicated than could be determined by the indirect techniques of Jin et al. [21]. Binding of L-tryptophan to the *trp* repressor (TrpR) results in a significant structural change around the region responsible for DNA binding [29–31] which may be responsible for the large enthalpy changes observed. No such structural change is seen in MetJ [10]. In the Cro protein studies the unusual enthalpic properties have been attributed to the renaturation of partially unfolded protein [20]. This is unlikely to be the case with TrpR since this protein does not begin to unfold till around 75°C [32], well above the temperature range investigated. Similarly, with MetJ under the conditions used here for binding studies, thermal unfolding of the protein does not begin

until about 45°C [14], and melting of the DNA fragment occurs at even higher temperature (Fig. 3). Consequently, major conformational changes do not seem to play a significant role in the thermodynamics of protein–DNA interactions in the methionine repressor system.

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