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# Additions and Corrections

**Vol. 274 (1999) 31272–31278**

**Thermodynamic analyses reveal role of water release in epitope recognition by a monoclonal antibody against human guanylyl cyclase C receptor.**

*Chittoor P. Swaminathan, Animesh Nandi, Sandhya S. Visweswariah, and Avadhesha Surolia*

**Page 31274:** Equation 1 should be corrected to read as follows.

$$d\ln K_b/d[\text{solute}]_{\text{osmolal}} = -\Delta n_w/55.56 \qquad \text{(Eq. 1)}$$

Consequently, the number of water molecules released during the interaction of the antibody with its complementary peptides will be 5.304-fold higher than reported. However, the original interpretation of the role of water molecules involved therein remains the same.

**Vol. 277 (2002) 4042–4049**

**Water-assisted dual mode cofactor recognition by *Hha*I DNA methyltransferase.**

*Chittoor P. Swaminathan, Umesh T. Sankpal, Desirazu N. Rao, and Avadhesha Surolia*

**Page 4044:** Equation 5 should be corrected to read as follows.

$$d\ln K_b/d[\text{solute}]_{\text{osmolal}} = -\Delta n_w/55.56 \qquad \text{(Eq. 5)}$$

Consequently, the number of water molecules taken up by *Hha*I DNA methyltransferase for binding to the cofactors will be 5.304-fold higher than reported. However, the orginal interpretation of the role of water molecules involved therein remains the same.

**Vol. 277 (2002) 2951–2957**

**Differential effect of ik3-1/Cables on p53- and p73-induced cell death.**

*Keitaro Tsuji, Kiyohisa Mizumoto, Tadanori Yamochi, Ikuo Nishimoto, and Masaaki Matsuoka*

**Page 2951:** In the Summary, the phrase "ik3-1-ΔC, an ik3-1 deletion mutant lacking the C-terminal 134 amino acids" should read "ik3-1-ΔC, an ik3-1 deletion mutant lacking the C-terminal 139 amino acids."

**Page 2952:** In the lefthand column under "Plasmids and Adenoviral Vectors," second paragraph, the phrase "ik3-1-ΔC is an ik3-1 partial cDNA in which the C-terminal 134-amino acid region of ik3-1 is deleted" should read "ik3-1-ΔC is an ik3-1 partial cDNA in which the C-terminal 139-amino acid region of ik3-1 is deleted."

**Page 2954:** In the lefthand column under "ik3-1 Activates p53-induced Cell Death," third paragraph, the phrase "ik3-1-ΔC, an ik3-1 deletion mutant lacking the C-terminal 134 amino acids," should read "ik3-1-ΔC, an ik3-1 deletion mutant lacking the C-terminal 139 amino acids."

These errors do not affect the conclusions of this study.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

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# Water-assisted Dual Mode Cofactor Recognition by *Hha*I DNA Methyltransferase*

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**Energetically competent binary recognition of the cofactor *S*-adenosyl-L-methionine (AdoMet) and the product *S*-adenosyl-L-homocysteine (AdoHcy) by the DNA (cytosine C-5) methyltransferase (M.*Hha*I) is demonstrated herein. Titration calorimetry reveals a dual mode, involving a primary dominant exothermic reaction followed by a weaker endothermic one, for the recognition of AdoMet and AdoHcy by M.*Hha*I. Conservation of the bimodal recognition in W41I and W41Y mutants of M.*Hha*I excludes the cation−$\pi$ interaction between the methylsulfonium group of AdoMet and the $\pi$ face of the Trp$^{41}$ indole ring from a role in its origin. Small magnitude of temperature-independent heat capacity changes upon AdoMet or AdoHcy binding by M.*Hha*I preclude appreciable conformational alterations in the reacting species. Coupled osmotic-calorimetric analyses of AdoMet and AdoHcy binding by M.*Hha*I indicate that a net uptake of nearly eight and 10 water molecules, respectively, assists their primary recognition. A change in water activity at constant temperature and pH is sufficient to engender and conserve enthalpy−entropy compensation, consistent with a true osmotic effect. The results implicate solvent reorganization in providing the major contribution to the origin of this isoequilibrium phenomenon in AdoMet and AdoHcy recognition by M.*Hha*I. The observations provide unequivocal evidence for the binding of AdoMet as well as AdoHcy to M.*Hha*I in solution state. Isotope partitioning analysis and preincubation studies favor a random mechanism for M.*Hha*I-catalyzed reaction. Taken together, the results clearly resolve the issue of cofactor recognition by free M.*Hha*I, specifically in the absence of DNA, leading to the formation of an energetically and catalytically competent binary complex.**

Methyltransferase-catalyzed modification of substrate DNA, RNA, or protein serves as a key signal in the regulation and maintenance of diverse biological processes in a range of organisms from prokaryotes to eukaryotes (1, 2). Subsequent to its isolation from *Hemophilus haemolyticus* nearly a quarter century ago, *Hha*I DNA methyltransferase (M.*Hha*I)[1] has been subjected to incisive investigations yielding detailed insights into the reaction chemistry (3–7). M.*Hha*I is an *S*-adenosyl-L-methionine (AdoMet) dependent DNA methyltransferase that catalyzes the covalent attachment of a methyl group at the C-5 position of the aromatic ring of the first cytosine in the specific sequence 5′-GCGC-3′. Its ability to perform the methyl transfer within the B-DNA helix was rationalized by a localized "DNA backbone rotation" by nearly 180° (base flipping) without significantly bending or kinking of the rest of the duplex (8–11). Base flipping serves to deliver the base into a concave catalytic site in the enzyme (8). The Gln$^{287}$ side chain fills up the gap generated concomitantly in the DNA bound with M.*Hha*I, leading to a stable and specific interruption of the aromatic $\pi$-stacked base pairs, a feature recently utilized to evaluate long range electron migration through DNA (12). M.*Hha*I methylates all of the cytosines in poly(dG-dC) DNA (3), with hemimethylated DNA being the preferred substrate (13). Both the reaction product *S*-adenosyl-L-homocysteine (AdoHcy) (14) and, by implication, the cofactor AdoMet (7), appear to share the ability to drive M.*Hha*I to trap the target cytosine in the catalytic site to form a productive complex. These results portray AdoMet and AdoHcy as important modulators of the multiple binding modes of the DNA-bound enzyme.

Despite a wealth of information on the structural, dynamic, and kinetic aspects of the M.*Hha*I mechanism, little attention has been paid to the energetics of AdoMet and AdoHcy recognition reactions. No molecule other than ATP serves as a cofactor in more diverse reactions than does AdoMet (15). It was, therefore, of interest to understand the thermodynamic basis of recognition of the ubiquitous cofactor AdoMet by methyltransferases, a feature shared among structurally related enzymes that catalyze reactions with diverse substrate specificities (16–18). For example, a snapshot of a step that follows methyl transfer has been elucidated by crystallographic analyses of the DNA intermediate covalently bound to M.*Hha*I (8) and M.*Hae*III (19). Despite its absence in the original crystallization mix of the ternary complex, it was AdoHcy that was found, fortuitously, to reside within the cofactor binding pocket (8) shared by AdoMet (16). This illustrates that the transfer of the methyl group from AdoMet to the target cytosine results in the formation not only of the modified (*i.e.* methylated) DNA but also the transformation of AdoMet into AdoHcy. On the other

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[1] The abbreviations used are: M.*Hha*I, *Hha*I DNA methyltransferase; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; DSC, differential scanning calorimetry; EEC, enthalpy entropy compensation; ITC, isothermal titration calorimetry; $\Delta G^0_b$, Gibbs free energy change; $\Delta H^0_b$, enthalpy change; $\Delta S^0_b$, entropy change.

hand, the same experiment with M.*Hae*III failed to reveal any electron density for either AdoMet or AdoHcy (19) yet resulted in the formation of the DNA intermediate covalently bound to the M.*Hae*III, indicating that AdoHcy had formed but diffused out of the crystal. Together, these observations point to the existence of subtle differences in free energies of AdoMet and AdoHcy binding that determine their complexation status with different methyltransferases.

AdoHcy competitively inhibits subsequent AdoMet binding. There have been suggestions that steps after methyl transfer are rate-limiting (7) and that the M.*Hha*I molecule in the absence of DNA does not have the capacity to bind either AdoMet or AdoHcy (3). However, results to the contrary have been reported recently (7, 16). To gain further insight into the energetic basis of the recognition of AdoMet and AdoHcy by M.*Hha*I, we have pursued a thorough characterization of the thermodynamics of the interaction including the changes in the solvation. Results presented herein demonstrate that M.*Hha*I binds to AdoHcy with greater affinity than to AdoMet. Both recognition processes reveal an unusual, hitherto unobserved, dual mode of recognition wherein a primary dominant exothermic reaction is followed by a weaker endothermic one. These results provide a new perspective on the mechanism of one of the best characterized methyltransferases and have implications for the driving force responsible for low turnover of M.*Hha*I. The results provide information not only on the energetics of the intermolecular binding reaction between AdoMet and M.*Hha*I as well as AdoHcy and M.*Hha*I but also allow one to glean further insight into the role of water molecules involved in the recognition process.

## EXPERIMENTAL PROCEDURES

*Materials*—All reagents were of analytical or ultrapure grade. *S*-Adenosyl-L-methionine and *S*-adenosyl-L-homocysteine were procured from Sigma. Both of these cofactors were further purified on a cation exchange (SP-Sepharose) chromatography. [$^3$H-methyl]AdoMet was purchased from PerkinElmer Life Sciences (74 Ci/mmol). Glycerol and ethylene glycol were of ultrapure grade from Sigma. Deionized Milli-Q water was used for all of the studies. M.*Hha*I was prepared according to a method described previously (20). The following oligonucleotides were synthesized (New England Biolabs) and used as substrates in the methylation reactions: 5′-GACTGGTACAGTATCAG**GCGC**TGACCCA-CAACATCCG-3′ and 5′-TCGGATGTTGTGGGTCA**GCGC**CTGATACT-GTACCAGT-3′. The *Hha*I recognition sequence is in boldface type.

These complementary oligonucleotides were annealed to form duplexes by heating to 80 °C and gradually cooled to room temperature.

*Construction of W41I and W41Y Mutants, Enzyme Expression, and Purification*—Site-directed mutagenesis was performed to replace tryptophan at position 41 by isoleucine and tyrosine using the oligonucleotides 5′-TTCTAATGAAATCGATAAATATG-3′, and 5′-TTCTAAT-GAATACGATAAATATG-3′, respectively. A single-stranded DNA template containing uracil residues was made from *Escherichia coli* strain ER2880 that harbored the plasmid pGEM3Zf(−)M.*Hha*I. Primers were hybridized to this single-stranded DNA, and oligonucleotide-directed mutagenesis was performed essentially according to the method of Kunkel (21). The W41I mutant was screened by the creation of a restriction site *Cla*I, whereas W41Y was screened by a loss of the same site. The resultant plasmids containing the mutations were sequenced on both strands to confirm that no undesired codon changes had occurred. Mutant M.*Hha*I genes were subcloned into the overexpression vector pUHE25, and the mutant methyltransferases were purified according to the protocol followed for the wild type enzyme (20). Both of the mutant methyltransferases, W41I and W41Y, were as active as the wild type M.*Hha*I with no significant changes in their kinetic parameters (data not shown).

*Preparation and Analysis of Solutions for Calorimetry*—M.*Hha*I solution was prepared in 10 mM phosphate buffer at pH 7.4 ± 0.01, dialyzed overnight in a large volume of the same buffer, and centrifuged to remove any insoluble M.*Hha*I. The AdoMet and AdoHcy solutions were prepared by weight in the dialysate to minimize differences between the protein buffer solution and ligand buffer solution in the ITC measurements. For osmotic stress studies, the M.*Hha*I solution was dialyzed extensively against glycerol and ethylene glycol solutions in the above buffer. AdoMet and AdoHcy solutions were then prepared with the final dialysate. Care was taken that the neutral solute osmolalities used were not significantly different from their ordinary molal concentrations (22–25).

*ITC Measurements and Analyses*—The titration calorimetric measurements were performed with a Microcal Omega titration calorimeter (26). Briefly, the titration calorimeter consists of a 1.347-ml cell containing the protein solution and a matched reference cell in an adiabatic enclosure. Aliquots of AdoMet and AdoHcy solutions at 15−40 times the M.*Hha*I concentration were added via a 250-$\mu$l rotating stirrer microsyringe operated with a plunger driven by a stepper motor under computer control. Samples were carefully scrutinized for precipitate after the titration. No precipitate was observed after the titration either in the presence or in the absence of the osmolytes. The unitless quantity $c$, defined as the product of the initial macromolecule concentration ([M.*Hha*I]$_0$) and the equilibrium association constant ($K_b$) (*i.e.* $c = K_b$[M.*Hha*I]$_0$), was in the range of $2 < c < 200$, as required for ITC studies (26). This corresponds to a binding regime that is best suited for the most precise measurements of the stoichiometry ($n$), $K_b$, and $\Delta H^0_b$ simultaneously in a single ITC experiment. The total concentration of the M.*Hha*I, [M.*Hha*I]$_t$, was from 0.02 to 0.04 mM, whereas the total concentrations of either AdoMet or AdoHcy ligands, [L]$_t$, were from 0.8 to 1.2 mM. The titration of ligand solution in this concentration range with the buffer solution alone gave negligible values for the heat of dilution both in the presence and absence of osmolytes at 293.2 K. Nonetheless, for every experiment, the heat of dilution of the ligand was measured and subtracted from the runs conducted with the M.*Hha*I. The time duration between the injections was at least 3 min to allow the peak to return to base line. The number of additions of AdoMet as well as AdoHcy titrant was fixed such that the area below the peak was reduced by at least an order of magnitude or until M.*Hha*I binding sites were saturated >95%. All measurements were made at a constant stirrer speed of 395 rpm.

*Tandem ITC Experiments*—In the first tandem ITC experiment, a low concentration ligand regime (1–2 mM), injected in small volume aliquots, was utilized. The goal was to measure thermodynamic parameters for the high affinity mode in a single experiment, at the end of which only an insignificant fraction of the secondary binding mode was occupied. Having saturated the primary mode fully, the system was poised for characterizing the weaker binding mode, with a substantially high concentration of AdoMet or AdoHcy (10 mM) injected in higher volume aliquots. Thus, in the second experiment the detected heat changes emerge almost entirely from the interaction of the ligand with M.*Hha*I in the secondary binding mode. The contributions from one mode to the other are negligible in these tandem ITC experiments, despite the fact that they were performed under two separate ligand concentration regimes.

To achieve $c$ values in the correct range for an ideal titration of the secondary weak mode, an unrealistically high concentration of *M.Hha*I in the millimolar range is required, which would necessitate prohibitively high concentrations of the 1–2 M of AdoMet and AdoHcy to be injected. However, at these concentration ranges, the $c$ values for the primary high affinity mode would overshoot the upper limit of the ideal range. The first titration experiment was performed at an optimum $c$ value for fully characterizing the primary binding mode, which was followed by a second one with suboptimal $c$ value for characterizing the secondary binding mode. Consequently, the thermodynamic parameters obtained for the secondary weak binding mode should be viewed as approximate.

The titration data were analyzed using a single site fitting model. The identical site model was fitted to the data utilizing a monomeric M.*Hha*I concentration. The heat content of a solution, $Q_t$, has been shown to be related to the total concentrations of the methyltransferase (M.*Hha*I) and either AdoMet or AdoHcy ligand (L) through the equation (26),

$$Q_t = n[\text{M.}Hha\text{I}]_t\Delta H^0_b V(1 + [\text{L}]_t/n[\text{M.}Hha\text{I}]_t + 1/nK_b[\text{M.}Hha\text{I}]_t - [(1 + [\text{L}]_t/n[\text{M.}Hha\text{I}]_t + 1/nK_b[\text{M.}Hha\text{I}]_t)^2 - 4[\text{L}]_t/n[\text{M.}Hha\text{I}]_t]^{1/2})/2 \quad \text{(Eq. 1)}$$

where $n$ is the stoichiometry, $K_b$ is an intrinsic binding constant, $\Delta H^0_b$ is an intrinsic heat of binding, [M.*Hha*I]$_t$ is the total M.*Hha*I concentration, and $V$ is the cell volume. The expression for the heat released per $i$th injection, $\Delta Q_i$, is then the following,

$$\Delta Q_i = Q_i + \text{d}V_i/2V(Q_i + Q_{i-1}) - Q_{i-1} \quad \text{(Eq. 2)}$$

where $\text{d}V_i$ is the volume of titrant added to the solution. A least squares fit of $\Delta Q_i$ to Equation 2, obtained by each run of titration calorimetry,

gives values for stoichiometry ($n$), $\Delta H^0_b$ and $K_b$. Since M.*Hha*I at pH 7.4 exists as a monomeric protein, an identical site model utilizing a concentration of the protein monomer was the simplest binding model found to provide the best fit to the ITC data. Values for $\Delta S^0_b$ were obtained from the basic equation of thermodynamics,

$$\Delta G^0_b = \Delta H^0_b - T\Delta S^0_b \quad \text{(Eq. 3)}$$

where

$$\Delta G^0_b = -nRT\ln K_b \quad \text{(Eq. 4)}$$

and $n$ represents the number of moles, $T$ is the absolute temperature, and $R = 8.315$ J mol$^{-1}$ K$^{-1}$.

*Coupled Osmotic-ITC Measurements and Analyses*—Coupled osmotic-ITC experiments were performed essentially as described in Ref. 24. The magnitude of the effect of osmotic stress depends both on the solute osmolal concentration, which is equivalent to the bulk water chemical potential, and on the difference in the number of solute-excluding water molecules associated with the ligand-M.*Hha*I complex and the number associated with the free M.*Hha*I and ligand molecules. This osmotic effect, when quantitated analogously to the conventional analysis of the effect of salt activity on protein binding, yields information on the effect of change in activity of water molecules upon ligand binding free energy (23, 27). The slope of log $K_b$, *versus* ln $a_w$, where $a_w$ is the water activity, is 2.303 $\Delta n_w$.

This value $\Delta n_w$ gives the change in number of solute-excluding water molecules coupled to the binding process. Since ln $a_w = -[\text{solute}]_{\text{osmolal}}/55.56$, where $-[\text{solute}]_{\text{osmolal}}$ is the solute osmolal concentration and 55.56 is the number of moles of water in 1 kg, the slope of the plot of log $K_b$ as a function of solute osmolality is given by the following,

$$d\log K_b/d[\text{solute}]_{\text{osmolal}} = -2.303\Delta n_w/55.56 \quad \text{(Eq. 5)}$$

In a true osmotic effect, an increase in osmotic strength of the medium manifests as a decrease in water activity in the vicinity of the respective binding sites and vice versa by the relationship shown in Equation 5. Linearity of the plots of logarithm of binding constants *versus* neutral solutes' osmolality for the binding of AdoMet and AdoHcy to *M.Hha*I indicates a linkage to a well defined and constant difference in the number of water molecules involved in ligand binding (24, 27). Control experiments in which aliquots of glycerol or ethylene glycol were injected into M.*Hha*I, AdoMet, or AdoHcy solutions did not result in any measurable heats of binding. This rules out the possibility of specific interactions between the stressing solute and either M.*Hha*I, AdoMet, or AdoHcy, confirming the neutrality of the chosen osmolytes.

*DSC Measurements and Analysis*—DSC measurements were performed with a VP-DSC scanning calorimeter from Microcal™ Inc., consisting of two fixed 0.5-ml cells, a reference cell and a sample cell. The measurements were usually made at a scan rate of 60 K h$^{-1}$. To determine any dependence of the parameters on the scan rate, scans were also performed between 2 and 90 K h$^{-1}$. From each sample *versus* buffer experiment, the corresponding buffer *versus* buffer base-line run was subtracted. The data were analyzed by Origin™ program supplied by the manufacturer. The raw data were normalized for concentration and scan rate. The pre- and post-transition base lines were connected by a progress base line, and the resultant processed data were used for nonlinear least-squares curve fitting analyses based on Levenberg-Marquardt methods. A two-state transition model with direct measurement of heat capacity $\Delta C_p$ was found to be the simplest one capable of fitting all the DSC data. The thermal transition was irreversible at all scan rates examined. This was attributable to a step after the reversible two-state transition, according to the model,

$$\text{N} \leftrightarrow \text{U} \rightarrow \text{D}$$

wherein the native protein (N) undergoes a reversible transition to an unfolded (U) state, which, subsequently, undergoes an irreversible transition leading to the oligomerization of the denatured polypeptides (D). The DSC scans were found to be essentially independent of protein concentration as well as scan rate, thus validating the two-state assumption (24).

*Methylation Assay*—Methylation assays for M.*Hha*I was carried out in methylase buffer (50 mM Tris, pH 8.0, 10 mM EDTA, and 5 mM 2-mercaptoethanol) using [$^3$H-methyl]AdoMet (20) After completion of the reaction, the reaction mixture was spotted onto a DE81 filter (anion exchange). The filter was washed three times with cold 0.2 M ammonium bicarbonate and once with ethanol. The filter was dried, and the radioactivity was measured in 3 ml of liquid scintillation fluid.

*Isotope Partitioning Analysis*—To analyze isotope partitioning by

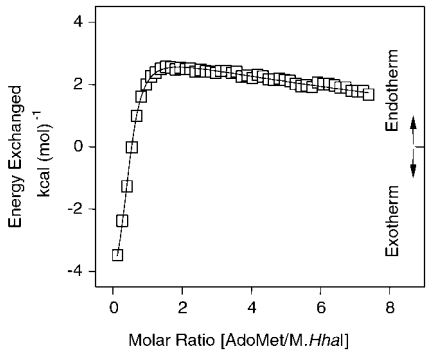


FIG. 1. **Calorimetric titration of AdoMet solution with *Hha*I DNA methyltransferase solution.** Shown is the nonlinear least squares fit (*line*) of the incremental heat per mole of added AdoMet (□) (49 automatic injections of 2 mM AdoMet solution in aliquots of 6.7 μl into 0.062 mM M.*Hha*I solution in 10 mM phosphate buffer, pH 7.4, at 283.2 K). The data points represent average values of three independent measurements. The S.D. values were well within the size of the *symbols*.

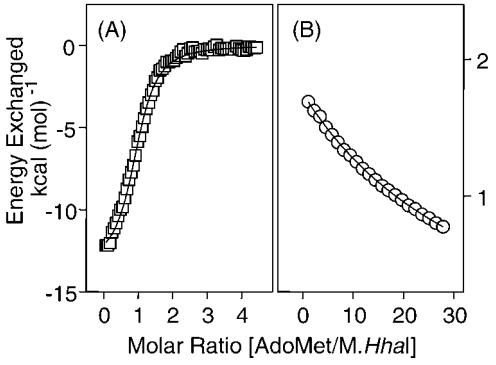


FIG. 2. **Tandem calorimetric titration.** Shown is tandem ITC titration of AdoMet solution with M.*Hha*I at 283.2 K in 10 mM phosphate buffer, pH 7.4. *a*, nonlinear least squares fit (*line*) of the incremental heat change per mole of added AdoMet (□) (66 automatic injections of 1 mM AdoMet solution in aliquots of 5.0 μl into 0.098 mM M.*Hha*I solution). *b*, nonlinear least squares fit (*line*) of the incremental heat change per mole of added AdoMet (○) (22 automatic injections of 10 mM AdoMet solution in aliquots of 15.0 μl into 0.098 mM M.*Hha*I solution).

M.*Hha*I, 400 nM *Hha*I was preincubated with 400 nM [*methyl*-$^3$H]AdoMet at 37 °C. 10 μl of this preincubated mix was brought to a final volume of 200 μl with methylase buffer containing 400 nM [*methyl*-$^3$H]AdoMet and 100 nM DNA. Aliquots of 30 μl each were removed at 10-, 20-, 30-, 40-, 50-, and 60-s time intervals, and the reaction was stopped by snap chilling the samples in liquid nitrogen. Samples were then analyzed for radiolabeled product formation using a DE81 filter binding assay (as mentioned above). In a parallel reaction, the above preincubated mix was brought to 200 μl with methylase buffer containing 400 nM unlabeled AdoMet and DNA. This corresponds to a 20-fold dilution of the label.

In another set of reactions, 100 nM *Hha*I was preincubated with 400 nM of labeled AdoMet at 37 °C. 40 μl of this preincubated mix was brought to 200 μl with methylase buffer containing either 400 nM labeled AdoMet or unlabeled AdoMet and DNA (a 5-fold dilution of the label). Aliquots of 30 μl were removed at 10-, 20-, 30-, 40-, 50-, and 60-s time intervals, and the reaction was stopped by snap chilling the samples in liquid nitrogen. Samples were then analyzed for radiolabeled product formation using a DE81 filter binding assay (as mentioned above). The final concentrations of *Hha*I, DNA, and AdoMet in both sets were 20, 95, and 400 nM, respectively.

*Preincubation Studies*—Preincubation experiments were carried out by incubating 20 nM M.*Hha*I with either 500 nM [*methyl*-$^3$H]AdoMet or 220 nM of DNA at 37 °C for 5 min. The reaction was initiated by adding DNA or [*methyl*-$^3$H]AdoMet, respectively. At 10-, 20-, 40-, 60-, and 120-s time intervals or 15-, 25-, 35-, 40-, 60-, and 125-s time intervals, 30-μl aliquots were removed and analyzed for product formation, using the DE81 filter binding assay.

TABLE I

*Thermodynamic quantities for the primary mode recognition of AdoMet and AdoHcy by wild type (WT), W41I, and W41Y mutants of M.HhaI*

Values in parenthesis indicate S.D.

| Ligand | M.*Hha*I | $T^a$ | $K_b$ | $-\Delta G^0_b$ | $-\Delta H^0_b$ | $-T\Delta S^0_b$ | $-\Delta S^0_b$ |
|---|---|---|---|---|---|---|---|
| | | °C | $M^{-1} \times 10^{-3}$ | | *kcal mol*$^{-1}$ | | *cal mol*$^{-1}$ *K*$^{-1}$ |
| AdoMet | WT | 10.0 | 157.9 (± 5.1) | 6.71 (± 0.018) | 13.87 (± 0.21) | 7.16 (± 0.23) | 25.28 (± 0.80) |
| | WT | 20.0 | 110.6 (± 3.2) | 6.74 (± 0.017) | 14.76 (± 0.24) | 8.01 (± 0.26) | 27.36 (± 0.87) |
| | WT | 25.0 | 95.4 (± 2.1) | 6.76 (± 0.013) | 15.41 (± 0.39) | 8.64 (± 0.40) | 28.98 (± 1.35) |
| | WT | 30.0 | 76.7 (± 1.9) | 6.75 (± 0.015) | 16.32 (± 0.42) | 9.57 (± 0.43) | 31.56 (± 1.43) |
| | WT | 37.0 | 61.5 (± 2.0) | 6.77 (± 0.019) | 17.16 (± 0.33) | 10.39 (± 0.35) | 33.5 (± 1.13) |
| | W41I | 10.0 | 78.3 (± 2.1) | 6.32 (± 0.015) | 16.33 (± 0.25) | 9.99 (± 0.28) | 35.31 (± 0.99) |
| | W41Y | 10.0 | 82.4 (± 1.6) | 6.34 (± 0.012) | 15.77 (± 0.39) | 9.42 (± 0.40) | 33.28 (± 1.42) |
| AdoHcy | WT | 10.0 | 807.1 (± 12.2) | 7.62 (± 0.008) | 15.62 (± 0.41) | 7.99 (± 0.42) | 28.23 (± 1.48) |
| | WT | 20.0 | 563.4 (± 11.4) | 7.68 (± 0.012) | 16.81 (± 0.46) | 9.12 (± 0.47) | 31.12 (± 1.61) |
| | WT | 25.0 | 431.9 (± 12.1) | 7.66 (± 0.016) | 17.21 (± 0.46) | 9.55 (± 0.57) | 32.03 (± 1.86) |
| | WT | 30.0 | 348.7 (± 10.2) | 7.66 (± 0.017) | 17.79 (± 0.54) | 10.13 (± 0.54) | 34.41 (± 1.77) |
| | WT | 37.0 | 251.8 (± 6.3) | 7.63 (± 0.015) | 18.53 (± 0.52) | 10.89 (± 0.49) | 35.13 (± 1.59) |
| | W41I | 10.0 | 124.1 (± 3.3) | 6.58 (± 0.015) | 18.13 (± 0.48) | 11.55 (± 0.62) | 40.81 (± 2.20) |
| | W41Y | 10.0 | 109.4 (± 3.6) | 6.50 (± 0.018) | 17.83 (± 0.54) | 11.32 (± 0.56) | 40.00 (± 1.97) |

[a] Variation in temperature was within ±0.1 °C.

TABLE II

*Thermodynamic quantities for the secondary mode recognition of AdoMet and AdoHcy by wild type (WT), W41I, and W41Y mutants of M.HhaI*

Values in parenthesis indicate S.D.

| Ligand | M.*Hha*I | $T^a$ | $K_b$ | $-\Delta G^0_b$ | $-\Delta H^0_b$ | $-T\Delta S^0_b$ | $-\Delta S^0_b$ |
|---|---|---|---|---|---|---|---|
| | | °C | $M^{-1} \times 10^{-3}$ | | *kcal mol*$^{-1}$ | | *cal mol*$^{-1}$ *K*$^{-1}$ |
| AdoMet | WT | 10.0 | 0.6 (± 0.05) | 3.58 (± 0.047) | −1.38 (± 0.11) | −4.96 (± 0.16) | −17.53 (± 0.55) |
| | WT | 20.0 | 0.7 (± 0.06) | 3.80 (± 0.050) | −1.49 (± 0.12) | −5.29 (± 0.17) | −18.04 (± 0.58) |
| | WT | 25.0 | 0.8 (± 0.05) | 3.94 (± 0.037) | −1.58 (± 0.10) | −5.52 (± 0.14) | −18.53 (± 0.46) |
| | WT | 30.0 | 1.0 (± 0.07) | 4.14 (± 0.042) | −1.73 (± 0.09) | −5.87 (± 0.13) | −19.38 (± 0.44) |
| | WT | 37.0 | 1.2 (± 0.07) | 4.35 (± 0.036) | −1.82 (± 0.14) | −6.22 (± 0.13) | −20.06 (± 0.41) |
| | W41I | 10.0 | 0.8 (± 0.06) | 3.74 (± 0.042) | −1.34 (± 0.10) | −5.08 (± 0.14) | −17.96 (± 0.50) |
| | W41Y | 10.0 | 0.7 (± 0.05) | 3.67 (± 0.040) | −1.37 (± 0.06) | −5.04 (± 0.10) | −17.80 (± 0.353) |
| AdoHcy | WT | 10.0 | 0.5 (± 0.04) | 3.48 (± 0.045) | −1.49 (± 0.12) | −4.97 (± 0.16) | −17.56 (± 0.58) |
| | WT | 20.0 | 0.7 (± 0.07) | 3.80 (± 0.058) | −1.61 (± 0.14) | −5.41 (± 0.19) | −18.45 (± 0.67) |
| | WT | 25.0 | 0.9 (± 0.06) | 4.01 (± 0.039) | −1.76 (± 0.13) | −5.77 (± 0.17) | −19.36 (± 0.57) |
| | WT | 30.0 | 1.0 (± 0.09) | 4.14 (± 0.054) | −1.85 (± 0.15) | −5.99 (± 0.20) | −19.77 (± 0.67) |
| | WT | 37.0 | 1.4 (± 0.11) | 4.45 (± 0.048) | −1.96 (± 0.14) | −6.40 (± 0.18) | −20.91 (± 0.61) |
| | W41I | 10.0 | 0.7 (± 0.06) | 3.67 (± 0.048) | −1.51 (± 0.08) | −5.18 (± 0.13) | −18.29 (± 0.45) |
| | W41Y | 10.0 | 0.7 (± 0.04) | 3.67 (± 0.032) | −1.45 (± 0.07) | −5.12 (± 0.10) | −18.09 (± 0.36) |

[a] Variation in temperature was within ±0.1 °C.

## RESULTS AND DISCUSSION

*Tandem ITC Analyses Reveal That M.HhaI Utilizes a Dual Mode of Recognition of AdoMet as Well as AdoHcy*—The calorimetric titrations detected directly the heat change upon binding of the ligands AdoMet as well as AdoHcy to M.*Hha*I (Fig. 1). The results reveal an initial phase of rapid decrease in the exothermic heat of binding with successive injections, followed by a heat sign reversal that culminates in a phase involving a

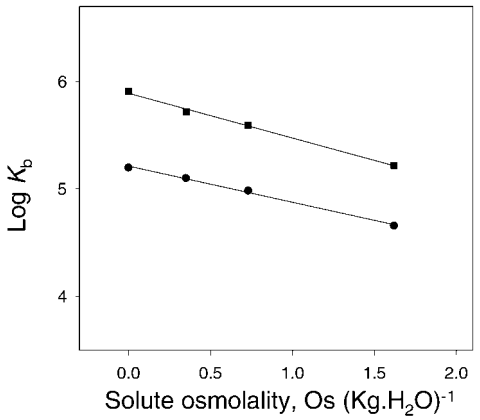


FIG. 3. **Osmotic stress effects on the binding of cofactors to *Hha*I DNA methyltransferase.** Osmotic sensitivity of logarithm of binding constant as a function of neutral solute osmolality for the binding of AdoMet (●) and AdoHcy (■) to M.*Hha*I under glycerol stress at 283.2 K. The *straight lines* were obtained by linear regression analysis of the data. The lines have a slope of −0.336 with a correlation coefficient of 0.99 for AdoMet and −0.417 with a correlation coefficient of 0.99 for AdoHcy. The data represent the average of four independent measurements. *Os*, osmoles.

monotonic decrease in the endothermic heat of binding. The results suggest the existence of a primary high affinity binding mode predominantly exothermic in nature, followed by a secondary low affinity one of endothermic origin. Qualitatively similar ITC profiles were obtained for AdoHcy binding to M.*Hha*I. The existence of a large difference in the net heat change as a function of the added ligand emanating during the primary *vis-à-vis* the secondary regimes of this dual binding mode, together with their widely disparate affinities, precluded the extraction of thermodynamic data reliably in a single titration experiment. This difficulty was overcome by performing sequential ITC experiments aimed at determining the parameters for both modes independently (Fig. 2). Despite their greater precision, the results of the latter ITC experiments appear qualitatively similar to those obtained from the ITC experiments in which both the binding modes are observed simultaneously in a single experiment. The temperature dependence of the changes in binding enthalpy ($\Delta H^0_b$), entropy ($\Delta S^0_b$), and free energy ($\Delta G^0_b$) is shown in Tables I and II. The heat capacity change at constant pressure ($\Delta C_p$), measured for both AdoMet−M.*Hha*I and AdoHcy−M.*Hha*I interactions are small and independent of temperature. Hence, large conformational changes in these interactions are ruled out. The primary binding mode is driven by dominant favorable enthalpic contributions, whereas the secondary weak binding mode is driven by entropic factors (Tables I and II). Although the two binding modes could be attributed to impurities present in the AdoMet preparations, as we have used highly purified AdoMet (>99%), this possibility is unlikely. More importantly, since AdoHcy also showed similar behavior, the presence of impurities is ruled out.

*Cation-π Interaction Is Not Involved in the Origin of Dual Mode AdoMet and AdoHcy Recognition by M.HhaI*—The π face of the $Trp^{41}$ indole ring is involved in a cation-π interaction with the methylsulfonium cation of AdoMet in the "unprimed" orientation in the binary AdoMet−M.*Hha*I complex (5, 16). This cation-π interaction is absent in the "primed" orientation of AdoMet in the binary AdoMet−M.*Hha*I complex crystallized in the presence of nonspecific DNA (5). That the dual mode recognition of AdoMet by M.*Hha*I could be attributed to the crystallographically observed diametrically opposite "primed" and "unprimed" orientations of AdoMet captured within the cofactor binding site of the enzyme is ruled out from its conservation in AdoHcy recognition. AdoHcy is incapable of cation-π interaction, since it lacks the sulfonium group and for which only a single orientation has been noted in the crystal structure. However, it has been pointed out that not all cation-aromatic contacts represent energetically favorable interactions, and therefore a cation-π interaction can either be attractive or repulsive (28). The possible role of a cation-π interaction in the dual mode recognition was tested experimentally by performing titration calorimetric analysis on the two mutants of M.*Hha*I, *viz*. W41I and W41Y. The W41Y mutation is, in principle, a conservative change for the retention of cation-π interaction, whereas W41I mutation is a disruptive one. Conservation of dual mode recognition in both AdoMet and AdoHcy recognition by both of these mutants rules out a role for cation-π interactions, be it energetically favorable or otherwise, in the origin of the dual mode of cofactor recognition in M.*Hha*I (Table I).

*Dual Mode of Cofactor Recognition as a Thermodynamic Signature*—Formation of specific cofactor-bound M.*Hha*I complexes is characterized by a relatively larger favorable enthalpy change for the primary binding mode (Table I). The small values of $\Delta C_p$ for these interactions rule out very large conformational changes in the reacting species. This suggests a role for hydrogen bonding and van der Waals interactions in the net binding free energy in the primary recognition mode (29). During the secondary weak binding mode, there appears a reversal in the signs of changes in binding enthalpies and entropies for both AdoMet and AdoHcy recognition by M.*Hha*I, and these reactions now become entropically driven ones (Table II). This suggests the establishment of an association by burial of nonpolar residues from solvent and also implicates the involvement of hydrophobic forces in driving the binding process. These results are consistent with a combined usage of hydrogen bonding and hydrophobic interactions as energetic signatures noted previously in ligand recognition by proteins (25).

Methyltransferases have perhaps evolved from dehydrogenases, with their structural fold being highly conserved, followed by function and, last, sequence (17, 30). M.*Hha*I shares an AdoMet binding site architecture (Rossman fold) as found in the cofactor binding site of NADPH dependent enzymes (16). The NAD(P)H:flavin oxidoreductase, ferredoxin−$NADP^+$ reductase, *E. coli* dihydrofolate reductase, and R67 dihydrofolate reductase all have the capability to recognize ligands in multiple binding modes (31–34) The dual energetic mode observed for the recognition of AdoMet and AdoHcy by M.*Hha*I, therefore, does not appear surprising. The dual mode recognition, therefore, implies plasticity in the recognition of bipartite molecules such as AdoMet and AdoHcy at concentrations high enough to saturate the cofactor binding site of M.*Hha*I. It is tempting to speculate that this dual mode energetic feature utilized by M.*Hha*I perhaps serves as a means for a distinctive recognition of AdoMet within the intracellular milieu and could hold the thermodynamic key to its conservation as a methyl group donor in the evolution of methyltransferases.

*Functional Role of Water in Primary AdoMet and AdoHcy Recognition by M.HhaI*—The net free energy change for the interaction of two hydrated species coming into contact is the difference between the free energy of M.*Hha*I and the cofactor in the bound complex *vis-a-vis* those of M.*Hha*I and water as well as the cofactor and water. Crystal structural results on water-mediated interactions in AdoMet as well as AdoHcy recognition by M.*Hha*I, although important in identifying invariant water molecules, provide little information on the net energetic role of these water molecules. The crystallographic water molecules in the active site are known not to retain their position throughout a 1 ns molecular dynamics simulation (35).

TABLE III

*Thermodynamic quantities for the primary mode recognition of AdoMet and AdoHcy by M.HhaI under osmotic stress at 10 °C*

Errors for the binding constant, $K_b$, $-\Delta H^0_b$ and for $-\Delta G^0_b$ were within 3%, while that for $-\Delta S^0_b$ was within 5%. Os, osmoles.

| Ligand | [Glycerol] | $K_b$ | $-\Delta G^0_b$ | $-\Delta H^0_b$ | $-T\Delta S^0_b$ | $-\Delta S^0_b$ |
|---|---|---|---|---|---|---|
| | *Os (kg $H_2O$)$^{-1}$* | *$M^{-1} \times 10^{-3}$* | *kcal mol$^{-1}$* | | | *cal mol$^{-1}$ K$^{-1}$* |
| AdoMet | 0 | 157.9 | 6.74 | 13.87 | 7.13 | 25 |
| | 0.35 | 126.1 | 6.61 | 13.14 | 6.53 | 28 |
| | 0.73 | 96.5 | 6.46 | 12.85 | 6.39 | 28 |
| | 1.62 | 45.5 | 6.04 | 9.71 | 3.67 | 13 |
| AdoHcy | 0 | 807.1 | 7.65 | 15.62 | 7.97 | 28 |
| | 0.35 | 528.4 | 7.41 | 14.69 | 7.28 | 26 |
| | 0.73 | 391.5 | 7.25 | 13.08 | 5.83 | 21 |
| | 1.62 | 165.1 | 6.76 | 10.24 | 3.48 | 12 |

Reorganization of water can influence the interaction between a protein and its ligand in a variety of ways (36–39). The precise nature of the reorganization of solvent, which could involve the uptake, release, or restructuring of water molecules within and around the binding pocket of M.*Hha*I with cofactors, was therefore determined.

Calorimetric titrations of AdoMet and AdoHcy solution into M.*Hha*I solution in the presence of glycerol or ethylene glycol were performed (data not shown). For each solute, binding free energies showed a linear dependence on solute osmolal concentration. The negative sign of the slope defines the net uptake of water molecules by the AdoMet–M.*Hha*I and the AdoHcy–M.*Hha*I complexes as a function of osmotic stress (Fig. 3 and Table III). Differential scanning calorimetric analyses demonstrate that in the presence of osmolytes, the thermal unfolding transition temperature as well as the enthalpy of the transition increase for AdoMet- or AdoHcy-bound M.*Hha*I, as compared with the respective transitions in the absence of osmolytes (Fig. 4). This suggests that the decrease in the free energies for cofactor binding by M.*Hha*I as a function of increase in osmotic strength of the medium is not due to osmolyte-induced destabilization of either the M.*Hha*I molecule or its cofactor-bound complexes.

The dual mode of binding is conserved, in the presence and absence of osmolyte, for the recognition of both AdoMet and AdoHcy by M.*Hha*I. However, there is a differential uptake of water molecules, 8 and 10, respectively, for AdoMet and AdoHcy (Table IV). These water molecules are critical in both the AdoMet recognition and/or methyl transfer reaction. This is consistent with the proposal that a set of conserved residues $Gln^{82}$ and $Asn^{304}$ (1, 40), stabilize a water network in the active site (41). This stabilization of a water network provides a means for activation of a water molecule to abstract the C-5 proton of cytosine, with the water channel also serving as a means for this proton to pass into the bulk solvent (35). Relatively fewer numbers of water molecules were observed in the crystal structures, perhaps due to their short residence time(s) and/or limiting resolution.

*Energetics of AdoMet–M.Hha*I *and AdoHcy–M.Hha*I *Recognition Reactions Display an Enthalpy–Entropy Compensation Effect Mediated by Solvent Reorganization*—The temperature dependence of the change in binding free energy ($\Delta G^0_b$) is remarkably negligible (Tables I and II). This occurs because of enthalpy-entropy compensation (EEC), a relationship observed in several molecular recognition reactions. Its origin has been associated with solvent reorganization accompanying protein–ligand interactions (42, 43) and/or due to weak intermolecular interactions (44). The thermodynamics of binding of AdoMet as well as AdoHcy to M.*Hha*I as a function of temperature exhibits compensatory changes in $\Delta H^0_b$ and $T\Delta S^0_b$ (Fig. 5). Also, a linear relationship between $\Delta H^0_b$ and $T\Delta S^0_b$, for both the primary and the secondary modes of recognition of AdoMet and AdoHcy by M.*Hha*I, is a good indication for a single interaction mechanism for the specific recognition, namely solvent reorganization (Fig. 5).

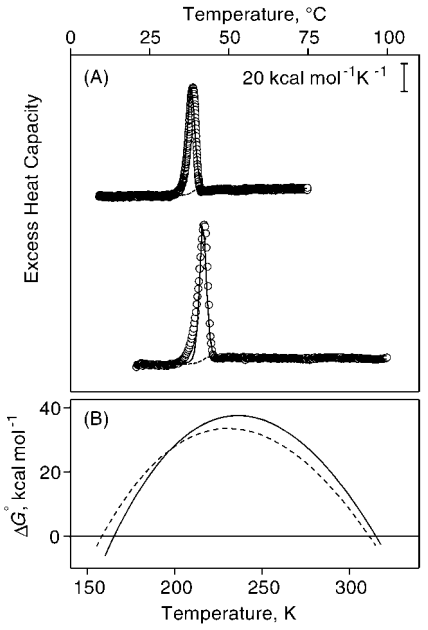


FIG. 4. **Differential scanning calorimetric analysis.** *A*, typical differential scanning calorimetric scan at 20 K h$^{-1}$ showing the apparent excess heat capacity for the thermal denaturation of M.*Hha*I, (0.05 mM) in 10 mM phosphate buffer (pH 7.4) in the absence (*top panel*) and presence of 10% glycerol (*bottom panel*). The buffer-buffer base line-subtracted and concentration-normalized DSC curves are displayed as *open circles*, and the fits of nonlinear least-squares regression to the two-state model with heat capacity are shown as *solid lines*. The progress base-line connections for the pre- and post-transition base line are displayed as *dotted lines*. *B*, estimation of free energy of unfolding of M.*Hha*I in the absence (*dashed line*) and presence of glycerol (*solid line*), as a function of temperature using the Gibbs-Helmholtz equation, $\Delta G_u(T) = \Delta H_m(1 - T/T_m) + \Delta Cp[T - T_m - T \ln(T/T_m))$. The $\Delta G_u$(max) for M.*Hha*I in the absence and presence of glycerol was 33.52 and 37.51 kcal mol$^{-1}$, respectively, indicating that osmolytes do not induce any destabilization of the protein.

TABLE IV

*Changes in the number of solute excluding water molecules ($\Delta n_w$) coupled to the binding of AdoMet and AdoHcy to M.HhaI as a function of osmotic stress*

The uncertainties in parenthesis represent S.D. of the mean values determined from four independent measurements.

| Ligand | Glycerol | Ethylene glycol |
|---|---|---|
| AdoMet | 8.12 (± 0.36) | 8.43 (± 0.27) |
| AdoHcy | 10.05 (± 0.44) | 10.39 (± 0.35) |

The origin of EEC itself appears to be intimately associated with solvent reorganization. Results from osmotic-calorimetric measurements of the primary binding mode display an induction of EEC that falls on the same line as those obtained from

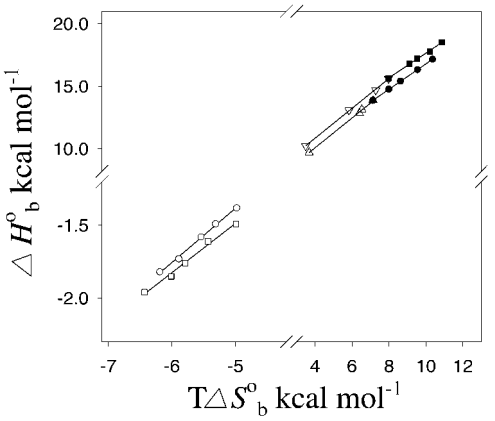


FIG. 5. **Enthalpy-Entropy compensation plot.** Enthalpy-entropy compensation plot of $-\Delta H^o_b$ as a function of $-T\Delta S^o_b$ for the binding AdoMet (●, ○) and AdoHcy (■, □) to M.*Hha*I. The *closed* and *open symbols* represent the primary and secondary binding modes, respectively. The EEC plots obtained from temperature dependence of thermodynamic parameters for the primary mode recognition of AdoMet and AdoHcy follow the same linear dependence for those obtained from the osmotic dependence of thermodynamic parameters for the primary mode recognition of AdoMet (△) and AdoHcy (▽). The EEC plot for the secondary mode recognition of AdoMet and AdoHcy displays small deviations in slopes from those obtained from the primary mode cofactor recognition due to greater uncertainty in the measurement of the parameters in weaker secondary mode. The *straight lines* were obtained by linear regression analysis of the data and show a linear relationship with slope of 1.11 and correlation coefficient of 0.99 for AdoMet and a slope of 1.12 and a correlation coefficient of 0.99 for AdoHcy (for the primary binding mode).

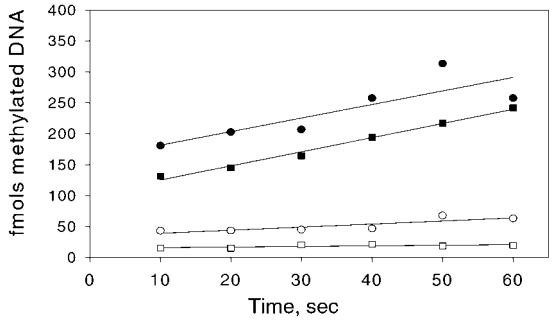


FIG. 6. **Isotope partitioning analysis of *Hha*I DNA methyltransferase.** *Hha*I DNA methyltransferase was preincubated with [*methyl*-$^3$H]AdoMet, and then the reaction was started by adding DNA and either unlabeled AdoMet (*open symbols*) or [*methyl*-$^3$H]AdoMet (*closed symbols*). The reaction was done under different dilution conditions 5-fold (●, ○) and 20-fold (■, □) as described under "Experimental Procedures."

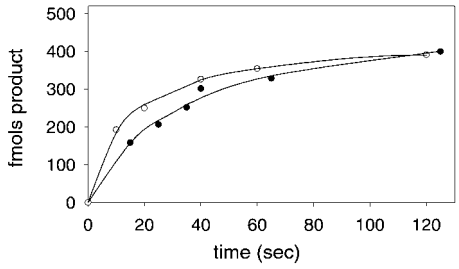


FIG. 7. **Time course of methylation reaction under different preincubation conditions.** *Hha*I methyltransferase was preincubated with either [*methyl*-$^3$H]AdoMet (●) or DNA (○) as described under "Experimental Procedures." The reaction was then initiated with the addition of DNA or [*methyl*-$^3$H]AdoMet, respectively. Aliquots were removed at the indicated time points, and the incorporation of methyl groups was measured by the filter-binding assay.

temperature-dependent calorimetric measurements of primary binding mode involving no osmolytes (Tables I–III). This serves as a diagnostic of true osmotic effect consistent with a change in water activity leading to a conservation of EEC in AdoMet−M.*Hha*I and AdoHcy−M.*Hha*I recognition reactions (24). It is noteworthy that a change in water activity alone, at the same temperature and pH, was sufficient to induce and conserve EEC in AdoMet−M.*Hha*I and AdoHcy−M.*Hha*I dual mode recognition reactions. These results directly implicate solvent reorganization as providing the major contribution to the origin of the ubiquitous isoequilibrium phenomenon of EEC in AdoMet−M.*Hha*I and AdoHcy−M.*Hha*I interactions.

*Catalytic Competence of the M.HhaI-AdoMet Complex*—The kinetic mechanism as proposed by Wu and Santi (3) suggested that the methylase reaction proceeds in an ordered manner, with DNA binding being a prequisite for AdoMet binding. Such a mechanism, however, failed to explain the binary M.*Hha*I-AdoMet crystal structure (16) or the presence of AdoMet-bound enzyme during the purification (20). The reinvestigation of the kinetic mechanism using isotope partitioning studies (6) demonstrated the incompetence of the M.*Hha*I-AdoMet complex, whereas the M.*Hha*I-DNA complex was shown to be catalytically competent. Such a scheme fitted well with the Wu and Santi mechanism. However, a more recent and rigorous study (7) using stopped flow and isotope partitioning analysis points toward a random mechanism for M.*Hha*I. Therefore, based on the concentration of the substrate used (6, 7), isotope partitioning analysis (45) can give different results. We therefore repeated the isotope partitioning experiment (6) under different dilution conditions (Fig. 6). As expected, we observe a decrease in the burst of product formation in the chase reaction. However, this decrease is in accordance with the dilution factor used in the chase reaction, suggesting that the decrease in burst rate is a manifestation of the labeled AdoMet being diluted. Our results, therefore, indicate that the interpretation of the catalytic competence of the M.*Hha*I-AdoMet complex based on isotope partitioning experiments should be viewed with caution. Despite the limitation of the isotope partitioning technique, it has been used successfully in a number of systems, *Eco*RI DNA methyltransferase (46), *Msp*I DNA methyltransferase (47), the murine DNA (C-5 cytosine) methyltransferase (48), and *Rsr*I DNA methyltransferase (49), to decipher the order of substrate binding. The requirement of DNA binding for the formation of competent complex was found in the case of murine DNA (C-5 cytosine) methyltransferase (48), whereas for the *Eco*RI DNA methyltransferase (46) and *Rsr*I DNA methyltransferase (49) the enzyme-AdoMet complex was found to be catalytically active.

The competence of the M.*Hha*I-AdoMet complex was further probed by measuring the rate of product formation under different preincubation conditions. M.*Hha*I was preincubated with [*methyl*-$^3$H]AdoMet or with DNA for 5 min, and the reaction was initiated by adding DNA and [*methyl*-$^3$H]AdoMet, respectively. Preincubation of M.*Hha*I with either AdoMet or DNA did not have any significant effect on the rate of product formation (Fig. 7). Earlier studies (6) had also indicated that increasing concentration of AdoMet did not affect the burst in product formation, which was expected if the M.*Hha*I-AdoMet complex were to be catalytically incompetent. Therefore, our results for both isotope partitioning and preincubation studies favor a random mechanism for M.*Hha*I whereby either DNA or AdoMet would be capable of binding M.*Hha*I and forming a catalytically active complex.

In summary, we have presented herein unequivocal evidence for the binding of AdoMet as well as AdoHcy to M.*Hha*I in solution state. This clearly resolves the issue of their recognition by free M.*Hha*I specifically in the absence of DNA, leading

to the formation of an energetically and catalytically competent binary complex. An unusual dual mode of recognition was demonstrated that could serve as a thermodynamic key to the molecular recognition of the ubiquitous methyl group donor AdoMet and the reaction product AdoHcy by M.*Hha*I and serve to distinguish them from related molecules in the intracellular milieu. The results from these analyses provide new insights into our understanding of the overall catalytic cycle of the M.*Hha*I DNA methyltransferase.

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