Investigation of Ionizable Residues Critical for Sequence-Specific Enzymatic DNA Modification: Protein Modification and Steady-State and Pre-Steady-State Kinetic pH Analyses of *Eco*RI DNA Methyltransferase†

Neda Mashhoon‡ and Norbert O. Reich*

Department of Chemistry and Interdepartmental Biochemistry Program, University of California, Santa Barbara, California 93106

Received December 17, 1993; Revised Manuscript Received April 5, 1994®

ABSTRACT: Steady- and pre-steady-state pH kinetic analyses are widely used methods to investigate important ionizable groups in enzyme-catalyzed reactions. The first such analysis to identify ionizable residues critical for sequence-specific modification of DNA is presented. *Eco*RI DNA methyltransferase uses *S*-adenosyl-L-methionine (AdoMet) to catalyze the N^6 methylation of the second adenine in the double-stranded DNA sequence GAATTC. The kinetic mechanism was previously shown to be steady-state-ordered bi bi in which AdoMet binds first followed by DNA addition [Reich, N. O., & Mashhoon, N. (1991) *Biochemistry 30,* 2933–2939]. Steady-state parameters are strongly dependent on pH and implicate at least four residues with pK_a values between 8.2 and 8.9 in the free enzyme and AdoMet–Bound enzyme and one residue with an apparent pK_a of 6.0. The data obtained are consistent with the enzyme binding the form of AdoMet in which the α amino group is protonated. Two protein residues with an apparent pK_a between 8.9 and 9.2 were implicated within the central complex (enzyme–DNA–AdoMet). The general insensitivity of all steady-state parameters to pH changes between pH 6.0 and 8.0 suggests that no critical protein residues undergo ionization-state changes in this range. The lack of significant pH-dependent changes in protein fluorescence and DNA thermal stability suggests minimal structural changes in either macromolecule. In support of the steady-state results single-turnover experiments reveal minimal pH dependence of the methylation rate constant between pH 5.53 and 8.6. Thus, no amino acids critical for catalysis undergo ionization-state changes in this range. The previously identified site of NEM-mediated inactivation of the methyltransferase, cysteine 223 [Everett et al. (1990) *J. Biol. Chem. 265,* 17713–17719], has a pK_a of 7.9; on the basis of the steady- and pre-steady-state pH dependences this cysteine does not directly contribute to catalysis and substrate binding. The results are discussed in the context of the requirement for significant enzymic activation of the poorly nucleophilic N^6 position.

DNA (N^6-adenosine) methyltransferase (EC 2.1.1.72) catalyzes the AdoMet[1]-dependent methylation of the second adenine in the sequence 5′GAATTC3′ to give N^6-methyladenine and AdoHcy. Bacterial DNA methyltransferases (MTases) are a component of restriction-modification systems and serve as valuable tools for the manipulation of DNA structure and analysis of protein–nucleic acid interactions [for reviews see Razin et al. (1984), Modrich and Roberts (1982), and Ferrin and Camerini-Otero (1991)]. DNA methylation serves the bacterial host by making the methylated site resistant to cleavage by the corresponding restriction endonucleases (Rubin & Modrich, 1977). A variety of other important biological processes including genetic recombination, eukaryotic gene regulation, and DNA repair are also dependent on DNA methylation.

DNA methylation is catalyzed by a diverse group of enzymes that are uniformly dependent on AdoMet as a methyl donor. Although much is known about the biology of DNA methylation, little is known about the chemical mechanism of the methylation reaction, in particular the identity of critical amino acids. *Eco*RI DNA MTase, like most DNA MTases, is a monomer (MW 38 050) and binds asymmetrically to its symmetrical site (Rubin & Modrich, 1977; Reich & Danzitz, 1991, 1992). The MTase binds AdoMet and noncanonical DNA randomly, but AdoMet binding is required for canonical site recognition (Reich & Mashhoon, 1991). The MTase is highly efficient, with a specificity constant (k_{cat}/K_m) for plasmid DNA of greater than 10^8 s^{-1} M^{-1}, and a large component of the extreme efficiency derives from a facilitated diffusion mechanism.[2] The enzyme catalyzes methylation of one strand of duplex DNA and dissociates from the DNA prior to any subsequent catalytic events[2] (Rubin & Modrich, 1977; Reich & Mashhoon, 1991). Furthermore, only one strand can be methylated from a single binding orientation (Reich & Mashhoon, 1993). The *Eco*RI MTase reaction occurs by direct attack of the N^6 nitrogen onto the methylsulfonium and not via the attack by the more nucleophilic N^1 center in the purine ring (Santi et al., 1988) (see Figure 1). Model studies suggest aliphatic amines require significant activation in the nucleophilic attack onto methylsulfonium compounds (Coward & Sweet, 1971). Thus, the *Eco*RI MTase is required to "activate" the exocyclic amine of the adenine; the mechanism by which this activation occurs is yet

† This work was supported by NSF Grant DMB-90-18474 to N.O.R.

* Author to whom correspondence should be addressed.

‡ Present address: Department of Molecular Biology, University of Southern California, Los Angeles, CA.

® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; MTase, DNA methyltransferase; mDNA, methylated DNA; NEM, *N*-ethylmaleimide; HPLC, high-performance liquid chromatography; DTT, Dithioreitol; BSA, bovine serum albumin; Tris (T) tris(hydroxymethyl)aminometane; Bis Tris (BT), [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; Bis Tris propane (BTP), 1,3-bis[tris(hydroxymethyl)methylimino]propane; *I*, ionic strength.

[2] M. A. Surby and N. O. Reich, submitted for publication.

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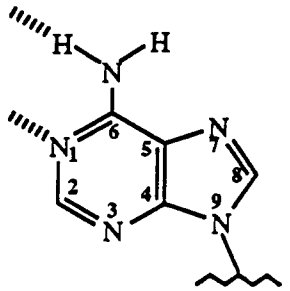

FIGURE 1: Adenine structure showing the numbering system; the dashed lines indicate hydrogen bonds to the base-pair thymine.

to be defined. We previously demonstrated that, in the absence of DNA and AdoMet, the cysteines at positions 25, 116, and 223 were modified by NEM, resulting in complete activity loss (Everett et al., 1990). In the presence of DNA and an AdoMet analog, sinefungin, Cys^{223} was essentially unmodified, suggesting that NEM modification of Cys^{223} is responsible for the MTase activity loss. Cys^{223} is preceded by an asparagine, and this dipeptide is conserved in most adenine MTases, all N^4 cytosine MTases, and no cytosine C^5 MTase (Everett et al., 1990). However, C^5 cytosine MTases do have a conserved cysteine that activates the pyrimidine through the formation of a MTase–DNA covalent intermediate (Santi et al., 1988). We recently demonstrated that Cys^{223} in the *Eco*RI MTase is not catalytically essential since MTase mutants with glycine, serine, or alanine at this position retained wild-type activity.[3]

We undertook steady- and pre-steady-state pH kinetic analysis and chemical modification studies of the MTase to identify amino acids that contribute to catalysis or binding. Although rarely used with proteins that interact with DNA (Strauss et al., 1980; Barkley et al., 1981; Lohman et al., 1980), these methods have frequently led to more detailed assignment of critical residues for numerous enzymes. Here we report the pK_a value for Cys^{223}, the pH dependencies of all steady-state parameters, and the pH dependence of the rate constant for the methyl-transfer step (k_{meth}).

MATERIALS AND METHODS

Chemicals. [*methyl*-^{3}H]AdoMet, 73 and 85 Ci/mmol, was purchased from New England Nuclear and Amersham, respectively. Unlabeled AdoMet (chloride salt), crystalline BSA, Trizma base, and 2-mercaptoethanol were purchased from Sigma. Bis-Tris and Bis-Tris propane were from Research Organics. Dithiothreitol was from Bethesda Research Laboratories. Glycerol (enzyme grade) was from Fisher Scientific. *N*-Ethylmaleimide (NEM) was from Aldrich. DE81 anion-exchange filter papers were from Whatman. Liquiscint scintillation fluid was from National Diagnostics.

*Eco*RI DNA MTase was purified from *Escherichia coli* strain MM294 harboring plasmid pDRCW (Reich et al., 1992). The concentration of *Eco*RI MTase was determined spectrophotometrically [$E^{1\%}$ at 278 nm = 10.8 (Rubin & Modrich, 1977)]. Synthetic oligonucleotides 5′GGCGGAATTCGCGG3′ and 5′CCGCGAATTCCGCC3′ were prepared on a BioSearch 3810 DNA synthesizer using β-cyanoethyl phosphoramidites. Oligonucleotides were purified on a C_{18} reversed-phase HPLC column (Reich & Danzitz, 1991). The concentrations of single strands were determined spectrophotometrically. Confirmation and concentration determination of the double-stranded form was through autoradiography with nondenaturing polyacrylamide gel electrophoresis (Reich & Danzitz, 1991). Thermal stability of the double-stranded DNA (14-mer, above-mentioned sequence) was determined spectrophotometrically (Reich & Danzitz, 1991). Unlabeled AdoMet purchased from Sigman was approximately 87% pure; this was further purified by chromatography on a cation-exchange column (Reich & Mashhoon, 1991).

MTase Assay. All MTase assays monitored the incorporation of tritiated methyl groups into DNA at 37 °C by using an ion-exchange filter binding assay as previously described (Reich & Mashhoon, 1991). [*methyl*-^{3}H]AdoMet was 3000–65 000 cpm/pmol. Substrates (DNA and AdoMet) and enzyme concentrations were as noted in each experiment. After incubation at 37 °C, reactions (50 μL each) were stopped by transferring 40-μL aliquots to 2 × 2 cm square Whatman DE81 filter papers (unless specified otherwise). Filters were washed and dried as described previously (Reich & Mashhoon, 1991), and tritium content was determined in 4 mL of Liquiscint using a Beckman Model LS 1701 scintillation counter.

Steady-State pH Studies. pH measurements were performed with a Corning Model 145 pH meter and a calomel combination electrode at 37 °C. Ionic strength (I) measurements were with a Bio-Rad conductivity monitor using KCl as a standard. Three standard buffers were used for calibration at 37 °C, and the precision was 0.01 pH unit. A tribuffer system of Bis Tris, Tris, and Bis Tris propane (BT/T/BTP) was used. BT/T/BTP (0.075 M) at constant ionic strength of 60 mM was used for the pH range 5.5–8.5. The buffer concentration was increased to 0.2 M (I = 60 mM) for pH 9.0 and 9.5. The tribuffer system was adjusted to the desired pH by using hydrochloric acid; KCl was added to bring the total ionic strength to 60 mM. Reactions contained 0.075 or 0.2 M BT/T/BTP (I = 60 mM), 10 mM EDTA, 10 mM DTT, and 200 μg/mL BSA. The MTase was diluted in protein dilution buffer (PD): 20 mM potassium phosphate, pH 7.4, 0.2 mM EDTA, 200 μg/mL BSA, 2 mM DTT, and 10% (v/v) glycerol. The minimal pH changes observed after addition of AdoMet to the reaction mixture at high pH values were corrected.

Determination of k_{cat} and k_{cat}/K_m for AdoMet and 14-mer at different pH values was carried out by varying the level of the desired substrate at several different fixed concentrations of the other substrate at each pH buffer. The reactions were initiated by the addition of 14-mer. An aliquot of the sample was assayed for activity using the MTase assay.

The pH stability of the MTase was examined by incubating the enzyme in the presence of AdoMet for 15 min on ice at the pH of interest and assaying an aliquot for activity at pH 8.0.

NEM Modification Experiments. The MTase was dialyzed overnight at 4 °C against nonreducing buffer (NR): 10 mM potassium phosphate, pH 7.0, 10 mM NaCl, 1 mM EDTA, and 10% glycerol. The MTase (3 μM) was incubated at 37 °C with 15 μM NEM in 0.1 M BT/BTP tribuffer system (I = 50 mM) at pH 7.0–9.0. An aliquot taken out at different time points was diluted 1:25 in NR buffer containing 7 mM β-mercaptoethanol and 200 mM NaCl to stop the NEM reaction. An additional 1:15 dilution was made in PD buffer, an aliquot of which was assayed for residual activity using the MTase assay as described above.

Pre-Steady-State pH Studies. These experiments were performed at 37 °C on a PQ-53 rapid quench machine from Hi-Tech Scientific (Reich & Mashhoon, 1993). MTase was

[3] K. A. Maegley, and N. O. Reich, submitted for publication.

dialyzed in a modified PD buffer containing 10 mM potassium phosphate, pH 7.4, 200 mM NaCl, 2.5 mM EDTA, 5 mM DTT, and 5% (v/v) glycerol. The MTase (4 μM) was preincubated with [^{3}H]AdoMet (40 μM) in 0.12 M BT/T/BTP tribuffer system (I = 60 mM), pH 5.5–8.6. The reactions were initiated by the simultaneous mixing of the MTase–AdoMet complex (80 μL) and 0.8 μM 14-mer (80 μL). The reactions were quenched with 1.0 M HCl (210 μL) after time intervals ranging from 3 ms to several seconds. An aliquot of the collected sample was transferred to 2 × 2 in. square Whatman DE81 filter papers. Filters were washed as mentioned before, and tritium content was determined.

Data Analysis. All steady-state pH data are the average of at least duplicate determinations. All steady-state and chemical modification pH data were analyzed by using a BASIC version of the Cleland (1979) programs which weight the data by assuming equal variance for the velocities of the fitted parameter. Reciprocal plots at each pH were analyzed with eq 1, a modified version of Cleland's SEQUEN program, where the K_{ia} term is replaced by the K_d for AdoMet (30 μM) (Reich & Mashhoon, 1991). In eq 1, A and B are AdoMet

$$v = V_m AB/ (30K_b + K_b A + K_a B + AB) \quad (1)$$

and DNA concentrations, respectively, K_a is K_mAdoMet, and K_b is K_m^{DNA}. Standard error for each parameter was calculated by the program. The pH plots were analyzed with two different methods. Initial analysis was done graphically using slopes of tangents to the log X vs pH plots, where X is the steady-state pH parameter of interest (e.g., $1/K_m$). The slope of the descending segment implicates the number of ionizing residues, while the intersection of the tangents indicates the pK_a (Segel, 1975). We used the number of implicated residues from this method as a starting point for the second method, which relies on fitting the data to equations with various [H$^+$] dependencies (Cleland, 1979). This computer fitting was done with MINSQ software from MicroMath Science which calculates standard deviation and correlation coefficients (R^2). The k_{cat} data were fit to eq 2,

$$\log y = \log[C/(1 + KH + K^2/H^2)] \quad (2)$$

$$\log y = \log[C/(1 + H/K + K/H + K^2/H^2 + K^3/H^3)] \quad (3)$$

where K represents the ionization constant (K_a), y is k_{cat}, and C is the pH-independent value of y (Cleland, 1979). $(1/K_m)^{AdoMet}$ data were fit to eq 3. $(1/K_m)^{DNA}$ and $(k_{cat}/K_m)^{AdoMet}$ data were fit to eq 3 with a [H]^4 term. Finally, $(k_{cat}/K_m)^{DNA}$ data were fit to eq 2 with terms up to [H]^5.

Results of NME inactivation experiments were plotted as the log (% activity) remaining as a function of time. The fast inactivation phase (initial 2 min) was fit to a straight line, and the slope of this line is k_{inact}. The k_{inact} data for each pH were fit to eq 4 using MINSQ. In eq 4, Y_L and Y_H are the values

$$\log k_{inact} = \log[(Y_L + Y_H (K/H))/(1 + (K/H))] \quad (4)$$

of k_{inact} at low and high pH, respectively; K is the ionization constant, and H is the hydrogen ion concentration.

In the pre-steady-state pH experiments, picomoles of product were plotted as a function of time. The data were fit to a single exponential, eq 5, using MINSQ (Reich & Mashhoon, 1993). In eq 5, k_{meth} is the methylation rate constant, p is picomoles of methylated DNA (mDNA) formed, D_0 is the maximum amount of mDNA that was formed, and t is the time.

$$p = D_0(1 - e^{-k_{meth}t}) \quad (5)$$

RESULTS

The pH dependency of kinetic parameters was determined to obtain information on the acid–base chemistry of the reaction catalyzed by the *Eco*RI MTase. We first determined the pH stability of the enzyme and DNA (14-mer, sequence shown under Materials and Methods) and the pH dependency of the kinetic mechanism. The MTase was preincubated at all pH values for 15 min, and aliquots were removed and assayed for activity at pH 8.0. No irreversible loss of activity was detected in the pH range 5.5–9.5 (data not shown). The kinetic mechanism was previously shown to be steady-state-ordered bi bi in which AdoMet binds first followed by DNA addition (Reich & Mashhoon, 1991). The double-reciprocal patterns obtained for both substrates throughout the pH range were similar to those obtained under optimal conditions, suggesting that the kinetic mechanism is independent of pH in the range 5.5–9.5 (data not shown).

We addressed the MTase conformational stability by using fluorescence spectroscopy (Maegley et al., 1992). Fluorescence emission spectra of the MTase (0.3 μM) were monitored at 37 °C at an excitation wavelength of 295 nm in the presence or absence of AdoMet (100 μM) in 0.12 M BT/T/BTP buffer system, pH 8.0 and 9.5, with a Perkin-Elmer LS50 luminescence spectrophotometer utilizing a xenon lamp (Maegley et al., 1992). The shape of the spectra, λ_{max} (335.8 ± 1.7 nm), and the maximal fluorescence intensity for all four conditions were within error from each other (data not shown).

The stability of double-stranded 14-mer was studied at pH 9.5 using DNA melt analyses to ensure that the duplex DNA is intact and the effective duplex concentration is accurate (Reich & Danzitz, 1992). The melting temperature for 14-mer (4 μM) in 0.12 M BT/T/BTP buffer system at pH 9.5 was determined to be 64.6 °C (data not shown), within a few degrees from that of 14-mer at pH 8.0 (68 °C). Although known to be unstable at alkaline pH values, AdoMet is not expected to undergo decomposition in the short time of our assays (Borchardt, 1979).

pH Dependency of Steady-State Kinetic Parameters. Plots of the data for k_{cat}, $1/K_m$, and k_{cat}/K_m for AdoMet vs pH are shown in parts A, B, and C of Figure 2, respectively. The decrease observed in log(k_{cat}) (Figure 2A) has a slope of –2, and the tangents intersect at pH 8.9. The best fit to the data was obtained with eq 2, which has a [H$^+$]^2 term, with an apparent pK_a of 9.2. Thus, two residues with pK_a values between 8.9 and 9.2 are implicated from the k_{cat} plot. Graphical analysis of the log$(1/K_m)^{AdoMet}$ data as a function of pH (Figure 2B) gives a slope of –1.5 and a pK_a of 8.2; fitting these data to eq 3 gave an apparent pK_a of 8.5. We interpret the results from the graphical analysis and fitting with eq 3 at the higher pH values to suggest that at least two protein residues with pK_a values between 8.2 and 8.5 may be critical. On the basis of a comparison of correlation coefficients, worse fits were obtained when lower and higher [H$^+$] dependencies were used. This was generally observed with the other steady-state parameters described below, although in some cases minor improvements in the correlation coefficients resulted from the higher order [H$^+$] fits. Equation 3 takes into account the slight drop in $1/K_m^{AdoMet}$ at lower pH, which indicates a slope of 1 and an apparent pK_a of 6.0.

Graphical analysis of the log$(k_{cat}/K_m)^{AdoMet}$ plot (Figure 2C) shows a slope of –3 and an apparent pK_a of 8.3. The best

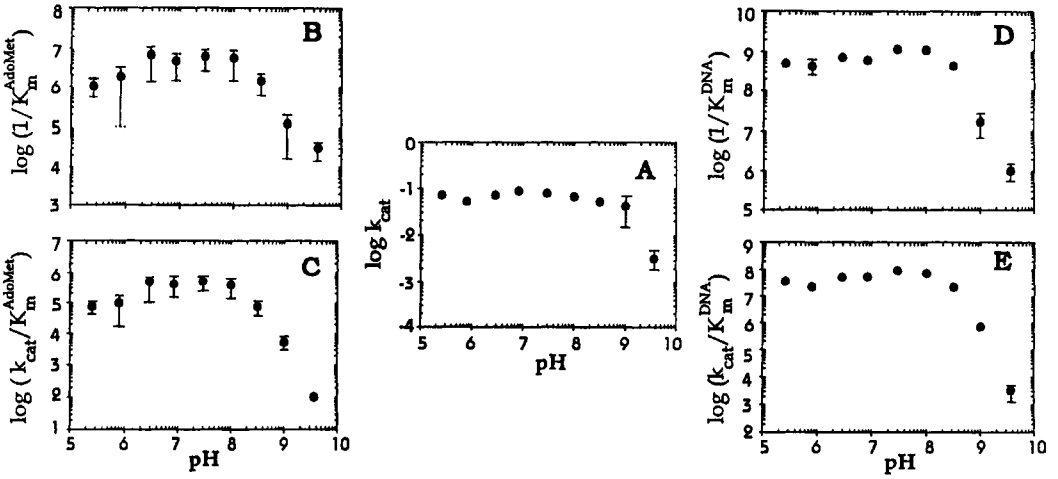

FIGURE 2: pH dependency of the steady-state kinetic parameters for the *Eco*RI MTase with DNA (14-mer) and AdoMet. Initial velocity experiments were performed at each pH buffer. True K_m and k_{cat} values were obtained for each pH data set by fitting the double-reciprocal data to eq 1. For experiments in which the pH ranged from 5.5 to 8.5, reactions contained 0.2 nM MTase, 10–100 nM 14-mer, and 0.2–3.0 μM [^{3}H]AdoMet in 0.075 M BT/T/BTP (I = 60 mM) buffer system. For pH 9.0 experiments, the reactions contained 0.6 nM MTase, 50–300 nM 14-mer, and 1–6 μM [^{3}H]AdoMet in 0.2 M BT/T/BTP (I = 60 mM) buffer system. For pH 9.5 experiments, reactions contained 20 nM MTase, 0.25–5.0 μM 14-mer, and 1–12 μM [^{3}H]AdoMet in 0.2 M BT/T/BTP (I = 60 mM) buffer system. In all experiments the reaction components were preincubated at 37 °C and reactions were started by the addition of 14-mer. Error bars indicate one standard deviation and were calculated by the computer program as described under Data Analysis. (A) log k_{cat} vs pH; (B) log$(1/K_m^{AdoMet})$ vs pH; (C) log$(k_{cat}K_m^{AdoMet})$ vs pH; (D) log$(1/K_m^{DNA})$ vs pH; (E) log(k_{cat}/K_m^{DNA}) vs pH.

fit for these data with eq 3 occurred using a $[H^+]^4$ term; the calculated apparent pK_a is 8.5. Thus, at least three residues contribute to this pH dependency with pK_a values between 8.3 and 8.5. The changes at lower pH show a slope of 1 and an apparent pK_a of 6.0, identical to that observed in Figure 2B with the log$(1/K_m)^{AdoMet}$ plot. The log$(1/K_m)^{DNA}$ plot in Figure 2D shows a significant decrease at higher pH, with a graphically determined slope of –2.5 and an apparent pK_a of 8.4. Upon fitting to eq 3 with a $[H^+]^4$ term, an apparent pK_a of 8.8 was obtained. Again, comparison of goodness of fit (R^2) using lower and higher order $[H^+]$ terms showed that no significant gain in fit was achieved beyond $[H^+]^4$. Thus, at least three residues are implicated in the pH dependency of $1/K_m^{DNA}$. Figure 2E shows the log$(k_{cat}/K_m)^{DNA}$ plot, and graphical analysis indicates a slope of –3.6 with an apparent pK_a of 8.4. The best fit with eq 2 occurred when terms up to $[H^+]^5$ were used; the calculated apparent pK_a is 8.9. Therefore, at least four ionizing residues are implicated by our data for k_{cat}/K_m^{DNA}.

Inactivation of EcoRI MTase by NEM. Incubation of the MTase with various concentrations of NEM at pH 8.0 results in a time-dependent inactivation of the MTase, with the rate of inactivation dependent on NEM concentration (data not shown). A plot of log(k_{inact}) vs log[NEM] gave a straight line with a slope of 0.94, suggesting that the inactivation is first order in NEM. A second-order constant for inactivation, 3.1×10^2 s^{-1} M^{-1}, was determined from a plot of k_{inact} vs [NEM]. The MTase (3 μM), when incubated with 15 μM NEM at pH 8.0, loses ~90% activity in the first 2 min, while ~10% activity is gradually lost over a period of 4 min. A pH-dependency study of this fast inactivation phase (first 2 min) gave a pK_a value of 7.9 ± 0.1 when the data were fit to eq 4 (Figure 3).

pH Dependency of the Single-Turnover Rate Constant. Single-turnover experiments were performed to measure how the methylation rate constant (k_{meth}) (Reich & Mashhoon, 1993) is affected by pH. Preincubation of the *Eco*RI MTase (4 μM) with 40 μM [^{3}H]AdoMet results in product (methylated 14-mer) formation under addition of DNA (14-mer). The magnitude of the initial rise was AdoMet concentration

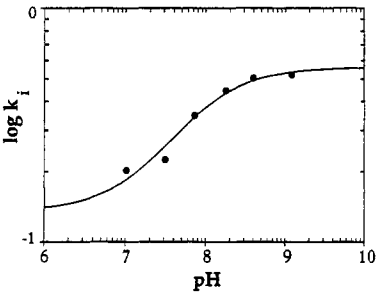

FIGURE 3: pH dependency of NEM-mediated inactivation rate of the *Eco*RI MTase. MTase (3 μM) was incubated at 37 °C with 15 μM NEM in 0.1 M BT/T/BTP (I = 50 mM) buffer system at different pH buffers. Samples withdrawn at designated time intervals were assayed for enzyme activity using the MTase assay (see Materials and Methods). The observed pseudo-first-order inactivation rate constants, K_{inact}, were obtained for different pH buffers. A pK_a of 7.9 ± 0.1 was obtained by fitting the k_{inact} vs pH data to eq 4.

dependent; a K_d for MTase–AdoMet complex of 30 μM determined previously (Reich & Mashhoon, 1991) was used to design these experiments. Fitting the data (picomoles of mDNA formed as a function of time) to eq 5 gives k_{meth} at each pH buffer condition. A comparison of k_{meth} as a function of pH is shown in Table 1. There is a 4-fold linear enhancement of k_{meth} between pH 5.5 and 8.5. Experimental limitations (lower affinity of the MTase for both AdoMet and DNA) prevented us from performing these experiments above pH 8.5.

DISCUSSION

Chemical modification and the pH dependency of kinetic parameters are two widely used techniques for the identification of amino acids essential for catalysis and binding in enzyme-catalyzed reactions. A pH-dependent change in the k_{cat}/K_m, k_{cat}, and $1/K_m$ profiles can provide information regarding groups on the enzyme or substrates that are involved in either binding or catalysis. For a bisubstrate reaction the k_{cat}/K_m profile of the first substrate (S_1) reveals information concerning changes in the ionization state of essential functional groups on the free enzyme (E) and/or free S_1. Similar studies with the second substrate (S_2) probe residues

Table 1: Methylation Rate Constant Variation with pH for the *Eco*RI MTase[a]

pH	k_{meth} ± error (1 SD)	pH	k_{meth} ± error (1 SD)
5.53	7.24 ± 0.89	7.49	15.00 ± 4.00
6.10	9.90 ± 4.49	8.06	26.00 ± 4.00
6.63	16.04 ± 7.49	8.6	28.14 ± 8.33
7.01	14.60 ± 5.20		

[a] MTase (4 μM) was preincubated at 37 °C with [^{3}H]AdoMet (40 μM) in 0.12 M BT/T/BTP tribuffer system (I = 60 mM), pH 5.5–8.6. The reactions were initiated by the simultaneous mixing of the MTase–AdoMet complex (80 μL) and 0.8 μM 14 mer (80 μL). The reactions were stopped with 1.0 M HCl (210 μL) after 3 ms to several seconds. An aliquot of the collected sample was assayed for activity (see Materials and Methods). Picomoles of methylated 14-mer were plotted vs time; these data were fit to eq 5 using MINSQ software. Error is represented as one standard deviation and was calculated using least-squares analysis by the software program.

on the binary enzyme–first substrate (E–S_1) complex and/or free S_2. The pH dependency of k_{cat} follows the ionization constant(s) of the ternary complex (E–S_1–S_2), while the pH dependency of $1/K_m$ for S_1 follows the ionization constant(s) of the free E and S_1 (Fersht, 1985; Cleland, 1990). The pH dependency of k_{cat}/K_m and $1/K_m$ for nonsticky substrates (substrates that dissociate more rapidly than they react to form products) are most valuable to consider since they represent changes from the free enzyme and free substrate only, and the presence of additional intermediates or nonproductive binding modes in the chemical mechanism does not affect these parameters (Cleland, 1990).[4]

Surprisingly, previous pH analyses involving protein–DNA systems have been limited to RNA polymerase (Strauss et al., 1980), *lac* repressor (Barkley et al., 1981), and pentalysine (Lohman et al., 1980). Thus, there is no previous characterization of the acid–base chemistry of a sequence-specific DNA-modifying enzyme. A significant concern in any pH analysis of enzymic function is the stability of the substrates and enzyme. We tested the impact on DNA stability of changing the pH from 8.0 to 9.5 and showed that the melting temperature (T_m) decreases only 3.4 ± 1 °C in this range. In comparison, similar variations in T_m result from single base-pair sequence changes, while single mismatches lower the T_m by 20 (Aboul-ela et al., 1985) and 30 °C (Brown et al., 1989). Therefore, although thermal stability analysis does not provide a direct probe of localized structural changes, it seems unlikely that this small stability difference could cause a decrease in steady-state parameters of up to 5 orders of magnitude. No *irreversible* loss of MTase activity was observed at any of the pH values used in our study. Finally, we addressed the more subtle question of *reversible* pH-dependent MTase conformational changes by comparisons of fluorescence spectra. The MTase contains two tryptophans, and both contribute to the observed fluorescence (Maegley et al., 1992). We previously demonstrated that ligand-induced conformational changes within the MTase are readily detectable by changes in the fluorescence spectra (Maegley et al., 1992). Thus, our inability to detect any pH-dependent changes in the fluorescence of the free enzyme and AdoMet-bound enzyme suggests either that no significant structural changes occurred or at least that the protein environment in the vicinity of both tryptophans remains unchanged.

The pH dependencies of steady-state parameters shown in Figure 2A–E indicate a plateau in the pH range 5.5–8.0. As pH is raised from 8.5 to 9.5, this plateau is followed by a sharp decline in all steady-state kinetic parameters (k_{cat}, $1/K_m$, and k_{cat}/K_m) for both substrates. The general lack of significant pH dependence of the steady-state kinetic parameters between pH 5.5 and 8.0 suggests either that no residues with pK_a values in this range are involved in catalysis and binding or that the functional consequences of ionization changes of critical residues are masked (Knowles, 1976) (see discussion later). The sharp decrease in the steady-state kinetic parameters between pH 8.5 and 9.5 indicates that amino acid residues with pK_a values in this range are responsible for both catalysis and binding. The pK_a values calculated from the data in Figures 2A–E range from 8.2 to 9.2 and represent apparent or average values because of the large number of implicated residues. Although this precludes direct assignment of pK_a values to individual functionalities within various forms of the enzyme, the implicated residues must have pK_a values near the identified apparent value.

$\text{Log}(1/K_m)^{\text{AdoMet}}$ (Figure 2B) and $\log(k_{cat}/K_m)^{\text{AdoMet}}$ (Figure 2C) both reflect the ionization state of the free enzyme. Figure 2B is for the free enzyme forming the binary MTase–AdoMet complex and implicates at least two residues with pK_a values between 8.2 and 8.5 and one residue with a pK_a near 6.0. The most likely protein residues with the higher pK_a values are cysteine, lysine, tyrosine, and arginine. In addition, the α amino moiety of AdoMet may in part be responsible for this effect, suggesting that the protonated form of AdoMet is bound by the enzyme. Interestingly, our recent demonstration that adenosine binds as well as AdoMet to the MTase (Maegely et al., 1992) suggested that neither the carboxylate nor the α amino groups are essential for binding, although removal of both moieties may have compensatory consequences. Figure 2C shows the pH dependency of the free enzyme and substrates forming products and implicates at least three residues with pK_a values between 8.3 and 8.5 as well as the single residue with a pK_a of 6.0. We recently showed that modification of the MTase by the histidine-selective reagent diethyl pyrocarbonate leads to complete loss of activity, and the pK_a for this inactivation is also 6.0.[5] At least one histidine residue is implicated by the protein modification results,[5] and the combined data suggest the unprotonated form of histidine may be critical for AdoMet binding.

$\text{Log}(1/K_m)^{\text{DNA}}$ (Figure 2D) and $\log(k_{cat}/K_m)^{\text{DNA}}$ (Figure 2E) implicate residues within the MTase–AdoMet complex which are critical for the formation of the central complex (MTase–DNA–AdoMet) and products, respectively. Figure 2D shows that at least three residues with pK_a values between 8.4 and 8.8 are essential, again implicating amino acid residues similar to those mentioned for Figure 2B,C. The lack of a significant shift in pK_a values suggests that AdoMet binding does not perturb critical amino acids undergoing ionization-state changes. This is consistent with our fluorescence results which showed that minimal structural changes occur within the MTase upon AdoMet binding (Maegley et al., 1992). Figure 2E implicates at least four residues with pK_a values between 8.4 and 8.9. A pH dependency of such severity is highly unusual for an enzyme-catalyzed reaction.[6] However,

[4] We previously showed that both AdoMet and DNA (14-mer) are nonsticky substrates in the MTase-catalyzed reaction (Reich & Mashhoon, 1991).

[5] N. O. Reich, S. Hobson, and A. Falick, submitted for publication. A novel LC–MS electrospray method was developed to identify DEPC-modified histidines. Application of this strategy to the MTase has identified the two critical histidines.

[6] A single report by Viola and Cleland (1978) for yeast hexokinase implicates five protein groups with pK_a of 5.05 whose protonation caused loss of enzyme activity.

the extreme pH dependency seen with the *Eco*RI MTase is perhaps not unexpected for a DNA-modifying enzyme given the high content of basic amino acids and the requirement for binding a highly negatively charged substrate. We recently demonstrated that the MTase forms three ionic interactions (Record et al., 1991) with its canonical site,[7] some of which may involve amino acid interactions with the DNA backbone. Thus, a significant number of the residues implicated in Figure 2C,E may be involved in these ionic interactions.

Log(k_{cat}) (Figure 2A) shows the pH dependency for the conversion of the central complex to form products. The apparent pK_a of the implicated residues in this complex is between 8.9 and 9.2, which is above the pK_a values obtained from Figure 2B–E. This upward shift in pK_a in the ternary complex most likely derives from the binding of the negatively charged DNA; nevertheless, cysteines, lysines, arginines, and tyrosines remain the probable candidates. Steps subsequent to catalysis such as product dissociation were previously shown to be rate limiting (Reich & Mashhoon, 1991, 1993). Since the affinity of the MTase for AdoMet and DNA decreases with increasing pH (Figure 2B,D), it is unlikely that dissociative steps contribute to the decrease in k_{cat} detected with increasing pH. Thus, the significant decrease in k_{cat} between pH 9 and 9.5 most likely derives from ionization changes in amino acids essential for catalysis. The MTase presumably uses a cysteine, protonated lysine or arginine, and/or tyrosine to activate the adenine for nucleophilic attack onto the methylsulfonium of AdoMet.[8]

We extended the previously characterized NEM-mediated MTase inactivation to determine if cysteines were important for catalysis and substrate binding. The rate of NEM inactivation exhibited a pH dependency (Figure 3), indicating that modification of a titratable residue with a pK_a value of 7.9 ± 0.1 is responsible for activity loss. These data further implicate cysteine(s) in this inactivation. Our previous modification studies confirmed that Cys^{223} was the primary site of NEM-mediated MTase inactivation, while our subsequent mutagenesis of Cys^{223} suggests this residue is not critical for catalysis.[3] The pK_a obtained from the steady-state kinetic parameters is clearly higher than that obtained from the NEM inactivation studies, suggesting that either none of the seven cysteines within the MTase are involved in catalysis and binding or a cysteine other than Cys^{223}, with a pK_a between 8.2 and 9.2 which is inaccessible to NEM-mediated inactivation, may be critical for catalysis/binding.

We utilized rapid kinetic techniques to investigate the involvement of specific residues that ionize in the pH range 5.5–8.5 (the plateau region of the steady-state pH curve) whose ionization may be important in catalysis but may have been masked (Knowles, 1976). Our recent single-turnover experiments (Reich & Mashhoon, 1993) showed that k_{meth} has a value of 41 s^{-1} and is over 300-fold greater than k_{cat}, 0.12 s^{-1}. The 4-fold increase in k_{meth} as a function of pH, shown in Table 1, argues against the involvement in catalysis of residues undergoing ionization-state changes in this pH range. The sharp decrease in DNA and AdoMet binding affinity at higher pH values precluded the measurement of k_{meth} above pH 8.5. Thus, although the decrease in k_{cat} above pH 8.5 is most likely due to corresponding decreases in k_{meth}, we were unable to demonstrate this directly.

CONCLUSION

Our steady-state pH analyses suggest that a single protein residue with a pK_a of 6.0 is important for AdoMet binding; no other ionizable residues are indicated between pH 5.5 and 8.2. In contrast, at least four amino acid residues with pK_a values between 8.2 and 9.2 are involved in substrate binding and catalysis. The most likely protein residues are lysine, cysteine, tyrosine, and arginine, in addition to the α amino group of AdoMet. Previous analyses of protein–DNA interactions with RNA polymerase (Strauss et al., 1980), *lac* repressor (Barkley et al., 1981), and pentalysine (Lohman et al., 1980) all showed fewer pH-sensitive groups. This may derive from the significant effects on k_{cat} observed with the MTase, while these past studies were limited to binding interactions. The lack of catalytically critical residues undergoing ionization-state changes between pH 5.5 and 8.5 was verified by single-turnover measurements of k_{meth}. Finally, Cys^{223}, which was previously shown to be the site of the NEM-mediated inactivation, has a pK_a of 7.9, strongly suggesting that this residue is not catalytically essential; the involvement of other cysteines with pK_a values above 8.5 is not excluded. The implication of several basic residues in catalysis suggests that the MTase may use acid–base chemistry to activate the poorly nucleophilic N^6 center.[8]

ACKNOWLEDGMENT

We thank Mark Surby and Lino Gonzalez, Jr., for purification of MTase and Dr. Mike Danzitz and John Murphy for DNA synthesis. We also thank Karen Maegley for assistance in fluorescence experiments. We especially thank Dr. H. Boyer for the use of his Rapid Quench instrument, Joe Day for his patience and skillful tutorial on how to use the instrument, and Dr. Vernon Anderson for helpful discussions.

REFERENCES

Aboul-ela, F., Koh, D., & Tinoco, I., Jr. (1985) *Nucleic Acids Res. 13*, 4811–4825.

Barkley, M. D., Lewis, P. A., & Sullivan, G. E. (1981) *Biochemistry 20*, 3842–3851.

Borchardt, R. T. (1979) *J. Am. Chem. Soc. 101*, 458–463.

Brown, T., Leonard, G. A., Booth, E. D., & Chambers, J. (1989) *J. Mol. Biol. 207*, 455–457.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–136.

Cleland, W. W. (1982) *Methods Enzymol. 87*, 390–405.

Cleland, W. W. (1990) *Enzymes 19*, 99–158.

Coward, J. K., & Sweet, W. D. (1971) *J. Org. Chem. 36*, 2337–2346.

Everett, E. A., Falick, A. M., & Reich, N. O. (1990) *J. Biol. Chem. 265*, 17713–17719.

Ferrin, L. J., & Camerini-Otero, R. D. (1991) *Science 254*, 1494–1497.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, New York.

Knowles, J. R. (1976) *CRC Crit. Rev. Biochem.* 165–172.

[7] M. A. Surby and N. O. Reich, unpublished results.

[8] One possible mechanistic involvement of a protonated side chain is to catalyze the tautomeric distribution of the normally prevalent keto–amine pair observed within a thymine–adenine base pair. This type of acid–base catalysis would presumably involve another residue to remove a proton at another site. For example, protonation at the N^3 position of adenine with concomitant proton removal of the target N^6 proton could result in the stabilization of the activated imino tautomer; subsequent proton abstraction from N^3 would generate a transiently negatively charged nitrogen at N^6 which could attack the methyl sulfonium. Evidence supporting such a mechanism includes the following: (a) replacement of the N^3 nitrogen within the target adenine results in a greater than 100-fold drop in $k_{methylation}$ (Reich *et al.*, unpublished results); (b) the MTase is known to interact with the minor groove (Reich & Danzitz, 1991); and (c) the N^3 position of adenine is both accessible and reactive, as demonstrated by its reactivity with various minor groove binding antimicrobial compounds.

Lohman, T. M., de Haseth, P. L., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3522–3530.
Maegley, K. A., Gonzalez, L., Jr., Smith, D. W., & Reich, N. O. (1992) *J. Biol. Chem. 267*, 18527–18532.
Modrich, P., & Roberts, R. J. (1982) in *Nucleases* (Linn, S. M., & Roberts, R. L., Eds.) pp 109–154, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Razin, A., Cedar, H., & Riggs, A. D., Eds. (1984) *DNA Methylation: Biochemistry and Biological Significance*, Springer-Verlag, New York.
Record, M. T., Jr., Ha, J.-H., & Fisher, M. A. (1991) *Methods Enzymol. 208*, 291–343.
Reich, N. O., & Danzitz, M. J. (1991) *Nucleic Acids Res. 19*, 6587–6594.
Reich, N. O., & Danzitz, M. J. (1992) *Biochemistry 31*, 1937–1945.
Reich, N. O., & Mashhoon, N. (1991) *Biochemistry 30*, 2933–2939.
Reich, N. O., & Mashhoon, N. (1993) *J. Biol. Chem. 268*, 9191–9193.
Reich, N. O., Olsen, C., Osti, F., & Murphy, J. (1992) *J. Biol. Chem. 267*, 15802–15807.
Rubin, R. A. & Modrich, P. (1977) *J. Biol. Chem. 252*, 7265–7272.
Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
Santi, D. V., Pogolotti, A. L., Jr., Ono, A., & Subramaniam, R. (1988) *J. Biol. Chem. 263*, 7461–7464.
Segel, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3504–3515.
Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111–4117.
Wolfenden, R. V. (1969) *J. Mol. Biol. 40*, 307–310.