Spectroscopic Studies of Arsenic(III) Binding to *Escherichia coli* RI Methyltransferase and to Two Mutants, C223S and W183F†

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Received June 23, 1992

ABSTRACT: The interactions of an arsenic(III) reagent, $(CH_3)_2AsSCH_2CONH_2$, with two *Escherichia coli* RI methyltransferase mutants, W183F and C223S, have been studied by phosphorescence, optically detected magnetic resonance, and fluorescence spectroscopy. The phosphorescence spectrum of the W183F mutant containing only one tryptophan at position 225 reveals a single 0,0-band that is red-shifted by 9.8 nm upon binding of As(III). Fluorescence titration of W183F with $(CH_3)_2AsSCH_2CONH_2$ produces a large tryptophan fluorescence quenching. Analysis of the quenching data points to a single high-affinity As(III) binding site that is associated with the fluorescence quenching. Triplet-state kinetic measurements performed on the perturbed tryptophan show large reductions in the lifetimes of the triplet sublevels, especially that of the T_x sublevel. As(III) binding to the enzyme at a site very close to the Trp225 residue induces an external heavy-atom effect, showing that the perturber atom is in van der Waals contact with the indole chromophore. In the case of the C223S mutant, a single tryptophan 0,0-band also is observed in the phosphorescence spectrum, but no change occurs upon addition of the As(III) reagent. Fluorescence titration of C223S with As(III) shows essentially no quenching of tryptophan fluorescence, in contrast with W183F. These results, along with previous triplet-state and biochemical studies on the wild-type enzyme [Tsao, D. H. H., & Maki, A. H. (1991) *Biochemistry 30,* 4565–4572], show that As(III) binds with high affinity to the Cys223 residue and that the Trp225 side chain is located close enough to that of Cys223 to produce a heavy-atom perturbation when As(III) is bound.

Bacteria are known to contain small amounts of methylated adenine or cytosine bases (Razin et al., 1984). Sequence-specific methylation of DNA is believed to (1) direct mismatch repair systems to repair incorrect strands in newly replicated DNA and (2) enable it to distinguish between foreign and its own DNA. The *Escherichia coli* RI (*Eco*RI) DNA methyltransferase belongs to a type II prokaryotic DNA restriction–modification system that is composed of a restriction endonuclease and a modification methylase, both of which recognize the sequence 5′-GAATTC-3′ in duplex DNA. The *Eco*RI DNA methyltransferase (methylase)[1] catalyzes the transfer of a methyl group from the cofactor *S*-adenosylmethionine (AdoMet) to the second adenine residue, forming N^6-methyladenine. The *Eco*RI restriction endonuclease cleaves the duplex DNA at a site between the two adenine residues in this sequence unless the site is protected by previous methylation. The methylase is a monomer with a molecular mass of 38 kDa. It contains two tryptophan residues at positions 183 and 225, as well as seven cysteines, but it lacks disulfide bridges (Jack et al., 1981; Modrich, 1982).

Phosphorescence and optically detected magnetic resonance (ODMR) techniques using tryptophan as an intrinsic probe have been applied successfully to the study of many protein–ligand interactions including external heavy-atom effects in enzyme–methylmercury(II) complexes (Hershberger & Maki, 1980; Zang et al., 1988). An external heavy-atom effect (HAE) is induced in tryptophan if the heavy perturber atom lies in van der Waals contact with an indole chromophore within the protein. The HAE enhances spin-orbit coupling and thereby increases the transition rates between the singlet and triplet manifolds. Consequently, the HAE causes quenching of fluorescence, an increase in the triplet-state yield, and an increase in the rate constants for triplet-state decay to the ground state.

It has been shown that arsenic(III) compounds bind to cysteine residues, contributing in some cases to the inhibition of enzymes (Rosenthal, 1932). In a previous study of the binding of As(III) to the wild-type *Eco*RI methylase (Tsao & Maki, 1991), the trivalent arsenic compound $(CH_3)_2AsSCH_2CONH_2$ was shown to produce a tryptophan HAE in this enzyme. The same HAE was produced by cacodylate buffer containing a thiol reducing agent. Fluorescence quenching, a large red shift in the phosphorescence 0,0-band, and a reduction in the tryptophan triplet-state lifetime indicated that at least one of the two tryptophans was affected upon As(III) binding to a single high-affinity cysteine residue of methylase. The enzymatic activity of methylase was found to be unaffected by As(III) binding to this site. Since the two tryptophans could not be optically resolved, it was not possible to determine whether one or both of the tryptophans of the wild-type methylase are perturbed. Also, the high-affinity cysteine residue could not be assigned. It has been suggested, however (Tsao, 1991), that Cys223 might be the high-affinity site since the heavy-atom effect is reduced when DNA and sinefungin, an analog of the AdoMet cofactor (Reich & Mashhoon, 1990), are bound to form a ternary complex before As(III) treatment. *N*-Ethylmaleimide (NEM) modification of Cys223 leads to inactivation of the methylase while both

† This research was partially supported by NIH Grant ES-02662 (A.H.M.) and NSF Grant MCB-9018474 (N.O.R.).

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; HAE, heavy-atom effect; methylase, *Eco*RI methyltransferase; MIDP, microwave-induced delayed phosphorescence; NEM, *N*-ethylmaleimide; ODMR, optically detected triplet-state magnetic resonance; SLR, spin–lattice relaxation.

0006-2960/92/0431-10438$03.00/0

the cofactor and DNA protect this residue from modification (Reich & Everett, 1990), indicating that Cys223 may be near the active site.

The purpose of this study is to assign the cysteine residue that binds As(III) with high affinity, as well as the tryptophan residue that undergoes an HAE as a consequence of the modification. Two mutants of the *Eco*RI methylase that were prepared by site-directed oligonucleotide mutagenesis were investigated: one without Cys223, the suspected high-affinity As(III) binding site (C223S), and the other without Trp183 (W183F). The tryptophan residues of these mutants are used as intrinsic probes for heavy-atom effects that may result from As(III) binding. Our measurements on these mutant enzymes allow us unequivocally to identify Cys223 as the single high-affinity As(III) binding site of the methylase and Trp225 as the residue that undergoes an HAE, thus establishing the proximity of the side chains of these residues in the complex. Since the HAE requires van der Waals contact between the As atom and the indole chromophore, it implies specific configurational restraints in this region of the enzyme that are not merely a consequence of the proximity of Cys223 and Trp225 in the primary sequence.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using mutant synthetic oligonucleotides to prime synthesis on single-stranded M13 DNA containing the methylase gene (Kunkel, 1985). The mutations were confirmed by sequencing ss-M13 DNA. The mutated methylase genes were then subcloned into the expression vector pDR540R, creating plasmids pDRC223S and pDRW183F as previously described (Maegley et al., 1992).

Preparation and Purification of Mutant Proteins. *E. coli* harboring either plasmid pDRC223S or plasmid pDRW183F were grown as previously described (Maegley et al., 1992). The mutant proteins were purified by the same procedure used to purify the WT protein (Reich & Mashhoon, 1991; Greene et al., 1978) and shown to be >95% pure by SDS–PAGE analysis.

Kinetic Analysis. Enzyme activity was determined as previously described (Maegley et al., 1992) using an assay that monitors the transfer of the tritiated methyl group from [*methyl*-^{3}H]AdoMet to DNA (Reich & Mashhoon, 1990).

Spectroscopy. Low-temperature phosphorescence, ODMR, and triplet sublevel decay kinetic measurements were performed on the native enzyme (obtained from Dr. Paul Modrich) and on its mutants in either 20 mM phosphate buffer (pH 7.4), 100 mM NaCl, and 0.2 mM EDTA containing 25% (v/v) glycerol (wild type and C223S mutant) or 5 mM phosphate buffer (pH 7.1), 200 mM NaCl, and 0.2 mM EDTA containing 25% (v/v) glycerol (W183F mutant). The thioarsenite reagent, $(CH_3)_2AsSCH_2CONH_2$, was prepared from cacodylic acid and thiolacetamide as described previously (Barber, 1932; Tsao & Maki, 1991).

The methylase and its mutants were allowed to react with excess thioarsenite reagent, and in each case, the sample was loaded into a 1-mm i.d. Suprasil quartz sample tube that was then placed inside a copper helix attached to the end of a coaxial microwave transmission line contained inside a dewar at the desired temperature. The experimental apparatus and methods have been described earlier (Tsao et al., 1989). The sample was excited at 295 nm for measurements of unperturbed tryptophan and at 305 nm for heavy-atom-perturbed tryptophan. Phosphorescence measurements were made at 77 K while the sublevel decay and ODMR measurements were made at 1.2 K where spin–lattice relaxation (SLR) is quenched. The sublevel decay constants were obtained by the microwave-induced delayed phosphorescence (MIDP) technique (Schmidt et al., 1969) for the unperturbed tryptophan and for the two slower decaying sublevels of the perturbed tryptophan. The decay constant of the fastest decaying sublevel of the perturbed tryptophan was obtained from a microwave fast-passage experiment (Winscom & Maki, 1971).

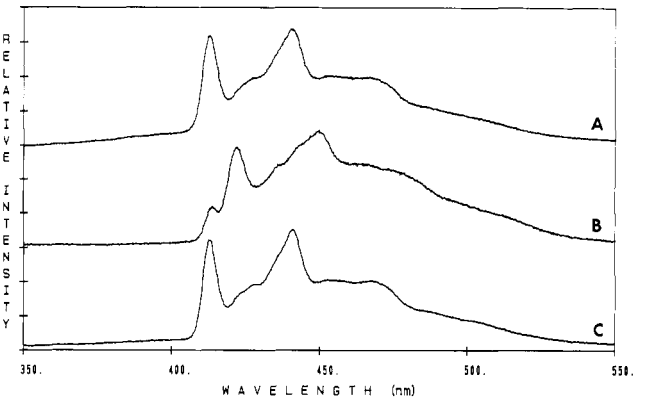

FIGURE 1: Phosphorescence spectra at 77 K of (A) *Eco*RI methylase (1.0×10^{-4} M). (B) As in (A), but with the addition of $(CH_3)_2AsSCH_2CONH_2$ (4×10^{-4} M). (C) *Eco*RI methylase C223S mutant (1.8×10^{-4} M) with $(CH_3)_2AsSCH_2CONH_2$ (2×10^{-3} M). Excitation was at 295 nm for all samples. [(A) and (B) from Tsao and Maki (1991).]

Fluorescence Titrations. A Perkin-Elmer MPF-44B spectrofluorometer was used to carry out the fluorescence titrations as described previously (Khamis et al., 1987). The fluorescence intensity of the sample within a 1-cm quartz cell was monitored upon addition of thioarsenite in small aliquots (2–4 μL) and was corrected for dilution. Excitation was at 295 nm (2-nm bandwidth), and emission was monitored at the peak wavelength of the fluorescence emission (5-nm bandwidth). A plot of the fluorescence intensity vs the ligand concentration was made, and the binding stoichiometry was obtained from the intersection of two lines extrapolated from the initial and final slopes of the plot. An exchange constant defining the affinity of the high-affinity binding site of methylase for the thioarsenite reagent relative to that of a "normal" SH group ($HSCH_2CONH_2$) was estimated using a binding model developed below.

RESULTS

Phosphorescence. The phosphorescence spectrum of the wild-type methylase is shown in Figure 1A. The narrow 0,0-band peaking at 412.3 nm originates from the two unperturbed tryptophan residues at positions 183 and 225. The lack of structure suggests that if both tryptophans contribute to the emission, their local environments are similar. Recent spectroscopic measurements on native methylase and W183F (Maegley et al., 1992) have shown that both Trp183 and Trp225 contribute to the fluorescence and that they reside in similar local environments. These environments are hydrophobic, based on a 339-nm fluorescence maximum. The well-structured phosphorescence and red-shifted origin also suggests a relatively hydrophobic environment with little exposure to the aqueous solvent medium (Hershberger et al., 1980), in agreement with the fluorescence study. Addition of a 4-fold excess of $(CH_3)_2AsSCH_2CONH_2$ produces the spectrum in Figure 1B. The unperturbed 0,0-band at 412 nm is diminished in intensity, while a new phosphorescence 0,0-band peaking at 420.5 nm appears (Tsao & Maki, 1991). The red-shifted

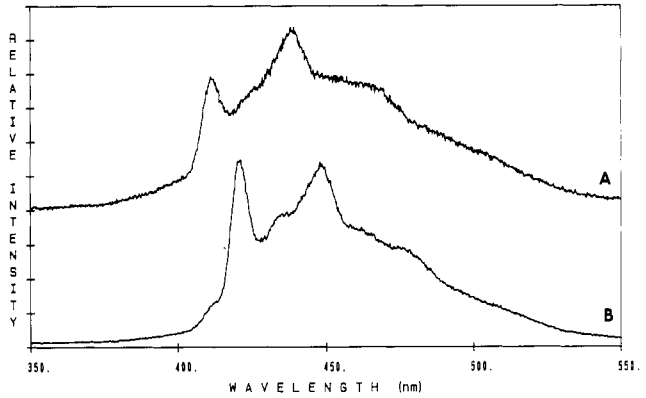

FIGURE 2: Phosphorescence spectra at 77 K of (A) *Eco*RI methylase W183F mutant (4×10^{-5} M) in 5 mM phosphate buffer, pH 7.1, 0.2 M NaCl, 0.2 mM EDTA, and 25% glycerol. (B) As in (A), but with the addition of a 10-fold molar excess of $(CH_3)_2AsSCH_2CONH_2$. Excitation was at 305 nm for both samples.

phosphorescence, which decays with a much reduced lifetime (Tsao & Maki, 1991), is assigned to a tryptophan residue that is perturbed by the As(III) atom via an external HAE.

The mutant methylase, C223S, with the addition of a 10-fold excess of the arsenic reagent produces the phosphorescence spectrum shown in Figure 1C. This spectrum is indistinguishable from that of the C223S mutant in the absence of added arsenic reagent (data not shown). These well-resolved spectra also are indistinguishable from that of the wild-type methylase, suggesting that the C223S mutation has no noticeable effect on the environments of the emitting tryptophans. In contrast with the wild-type methylase, however, addition of an excess of the arsenic reagent does not perturb the phosphorescence spectrum of the C223S mutant, as shown above.

The phosphorescence spectrum of the methylase mutant W183F is shown in Figure 2A. The 0,0-band that now originates only from Trp225 peaks at 410.9 nm and thus is slightly blue-shifted relative to the native enzyme. Addition of excess arsenic reagent causes a large reduction in the unperturbed Trp225 phosphorescence intensity and the appearance of a new red-shifted phosphorescence with a 0,0-band at 420.7 nm as shown in Figure 2B. This red-shifted emission, that must originate from Trp225, has a greatly reduced lifetime relative to unperturbed tryptophan (see below), consistent with an As(III)-produced external HAE. The spectrum of the perturbed W183F mutant is similar to that of the perturbed wild-type methylase (Figure 1B), suggesting similar arsenite–tryptophan interactions in both enzymes.

Triplet Lifetime and Sublevel Kinetics. The phosphorescence lifetimes were measured at 77 K by selectively exciting and monitoring the decay of the perturbed and unperturbed tryptophan phosphorescence 0,0-bands. The perturbed Trp225 phosphorescence lifetime, monitored at the 420.7-nm peak of the W183F mutant (75 ms, 65%), is about 2 orders of magnitude shorter than that of the unperturbed Trp225 monitored at the 410.9-nm peak in the absence of As(III) binding (6.3 s, 70%). These results are consistent with those obtained from the wild-type methylase (Tsao & Maki, 1991). MIDP and rapid-passage transient measurements at 1.2 K have yielded the sublevel decay constants for both the unperturbed and As(III)-perturbed Trp225. These results are summarized in Table I. For the perturbed Trp225, the decay constants of all sublevels are significantly larger than those of the unperturbed tryptophan. It should be noted that the two longer sublevel lifetimes of unperturbed tryptophan

Table I: Triplet-State Decay Constants (s^{-1}) for Some Perturbed and Unperturbed Tryptophans

sample	k_x	k_y	k_z	k_{calc}[a]	k_{obs}(77 K)
W183F	0.21	0.13	0.084	0.14	0.159
W183F–As(III) complex	44.2	0.77	2.33	15.8	13.3
wild-type–As(III) complex[b]					
perturbed Trp	37.5	1.56	1.8	13.6	14.6
unperturbed Trp	0.296	0.099	0.066	0.132	0.136
Trp[c]	0.240	0.12	0.038	0.154	0.172
YYW–CH_3Hg[d]	6.2	6.9	72.1	28.8	30.1

[a] $k_{calc} = (k_x + k_y + k_z)/3$. [b] From Tsao and Maki (1991). [c] From Zuclich et al. (1974). [d] Perturbed Trp158 from doubly mutated phage T4 lysozyme (Zang et al., 1988).

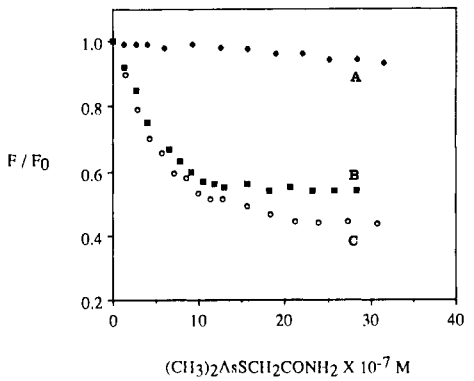

FIGURE 3: Fluorescence titration data of (A) *Eco*RI methylase C223S mutant (1 μM), (B) *Eco*RI methylase (1 μM) [from Tsao and Maki (1991)], and (C) *Eco*RI methylase W183F mutant (0.84 μM) with $(CH_3)_2AsSCH_2CONH_2$. The first two titrations were done in 20 mM phosphate buffer, pH 7.4, with 100 mM NaCl and 0.2 mM EDTA, and the third was done in 5 mM phosphate buffer, pH 7.1, with 200 mM NaCl and 0.2 mM EDTA. Excitation was at 295 nm (2-nm band-pass), and emission was monitored at 335 nm (5-nm band-pass).

are possibly influenced slightly by SLR processes that could remain partially unquenched at 1.2 K. Thus, the reported lifetimes could be somewhat shorter than the actual sublevel lifetimes. The T_x sublevel decay is observed to be the most enhanced of the three sublevels upon As(III) binding. The triplet sublevel decay constants for a normal tryptophan and a methylmercury(II)-perturbed tryptophan of mutated T4 lysozyme (Zang et al., 1988) are listed for comparison. The triplet sublevel kinetic data clearly reveal the presence of an external heavy-atom perturbation of Trp225 in the W183F mutant upon As(III) binding. No short-lived phosphorescence components were induced in the C223S-mutated methylase on treatment with excess As(III) reagent.

Fluorescence Titration. Fluorescence titration curves for the C223S mutant and of the W183F mutant are shown in Figure 3. The data for the wild-type methylase (Tsao & Maki, 1991) are included for comparison. The binding stoichiometry (moles of ligand per moles of enzyme) for the W183F mutant obtained from the fluorescence titration is found to be 0.96 ± 0.04. This is close to the stoichiometry previously found (0.84 ± 0.09) for a single "high-affinity" site in the wild-type methylase (Tsao & Maki, 1991). The level of fluorescence quenching is very small (<6%) for the C223S mutant but is found to be 58% for the W183F mutant under the conditions of our measurements. The limiting quenching for the wild-type methylase (Tsao & Maki, 1991) is somewhat smaller, ca. 46%. This difference could reflect an additional contribution of Trp183 to the wild-type enzyme fluorescence, consistent with recent measurements (Maegley et al., 1992).

Analysis of Fluorescence Titration. The fluorescence titration data for both wild-type methylase and the W183F

Table II: Experimental Values for the Equilibrium Constant for As(III) Binding to Methylase

	K^a		
sample	$n = 0$	$n = 1$	$n = 2$
wild type[b]	7 ± 1.5	13 ± 2	19 ± 4
W183F	7.7 ± 0.5	15 ± 1	22 ± 2

[a] Average of values obtained from intercepts and slopes of data analyzed by eq 3. See text for details. [b] Data taken from Tsao and Maki (1991).

mutated methylase (Figure 3) indicate that the fluorescence is quenched by $(CH_3)_2AsSCH_2CONH_2$ with 1:1 stoichiometry. This stoichiometry shows that there exists a single high-affinity quenching site in the enzyme. We assume that the quenching occurs by exchange of the ligand SCH_2CONH_2 with a single high-affinity cysteine binding site in the methylase and thus the reaction can be written

$$(CH_3)_2AsSR + HSE \overset{K}{\rightleftharpoons} (CH_3)_2AsSE + HSR \quad (1)$$

In eq 1, HSE represents the high-affinity cysteine binding site that is associated with the fluorescence quenching. HSR, however, refers not only to $HSCH_2CONH_2$ but also to accessible cysteine residues of the methylase that do not affect the fluorescence, and that we assume to have "normal" affinity for As(III), i.e., similar to that of thiolacetamide. All of these assumptions are consistent with the absence of quenching upon titrating the C223S mutant methylase with the As(III) reagent (Figure 3), which, furthermore, identifies the high-affinity cysteine as Cys223. The equilibrium constant, K, can be expressed as

$$K = x(x + nz_0)/[(y - x)(z_0 - x)] \quad (2)$$

where z_0 is the total enzyme concentration, y is the concentration of added $(CH_3)_2AsSCH_2CONH_2$, x is the concentration of $(CH_3)_2AsSE$, and n is the number of accessible cysteine residues in the methylase that have normal affinity for As(III). We define F as the corrected fluorescence intensity and let F_0 and F_∞ refer to the initial and limiting intensities, respectively, in the titration. In terms of the F's, eq 2 can be converted to

$$(\Delta F/\Delta F_\infty + n)/(1 - \Delta F/\Delta F_\infty) = K(\Delta F/\Delta F_\infty)^{-1}s - K \quad (3)$$

where $\Delta F = F_0 - F$, $\Delta F_\infty = F_0 - F_\infty$, and $s = y/z_0$. A plot of the left-hand side of eq 3 vs $(\Delta F/\Delta F_\infty)^{-1}s$ is expected to be linear. The slope of the regression can be identified with K, and the intercept with $-K$. The dimensionless equilibrium constant depends on the choice of n.

Reich and Everett (1990) identified three cysteine residues of wild-type methylase (Cys25, Cys116, and Cys223) that undergo modification by NEM. Thus, $n = 2$ would be a reasonable assumption for our study since Cys223 has already been identified as the high-affinity binding site and is not included in n. We have plotted the data of Figure 3 according to eq 3, and have found linear behavior for $n = 0$, 1, and 2 with good agreement between the K's obtained from the slope and intercept. In Table II, we list the values of the equilibrium constant obtained for both wild-type and W183F methylase. Although the uncertainties are larger for the wild-type methylase data than for W183F, the agreement between the K's is satisfactory for these enzymes, indicating that the W183F mutated enzyme binds As(III) at the Cys223 site in the same manner as the wild-type methylase. We note here that the values of K quoted previously for wild-type methylase (Tsao & Maki, 1991) were too large by a factor of 4 due to an inexact mathematical model used in that work to analyze the quenching.

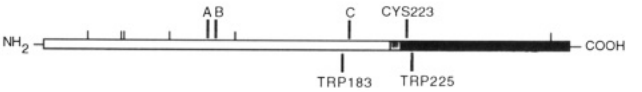

FIGURE 4: Schematic diagram of the methylase polypeptide. The open and solid boxes denote the first and second domains, respectively, as determined by limited proteolysis (Reich et al., 1991), and the dotted box denotes the AdoMet binding (Reich & Everett, 1990) and hinge regions (Reich et al., 1991). The short lines represent the location of the six additional cysteines throughout the protein. "A" signifies the N-terminus of the potentially active 26-kDa methylase fragment (Reich et al., 1991). "B" shows the location of the conserved tetrapeptide NPPF, which has been implicated in DNA recognition and binding (Chandrasegaran & Smith, 1987). "C" represents the GXGXXG motif found in AdoMet binding proteins (Ingrosso et al., 1989).

DISCUSSION

There are no three-dimensional structures available for any AdoMet-dependent enzyme, including the large class of methyltransferases that modify nucleic acids. Indirect results from proteolysis (Reich et al., 1991), affinity labeling (Reich & Everett, 1990), and protein modification (Everett et al., 1990) experiments with *Eco*RI methylase have provided some insight into the organization of folding domains and location of critical amino acids (see Figure 4). Thus, the methylase has two domains: an N-terminal 25-kDa segment and a 13-kDa carboxy region connected by a highly flexible hinge (residues 206–221). This flexible region overlaps the AdoMet site and is adjacent to Cys223. The importance of Cys223 is supported by modification (Everett et al., 1990) and mutagenesis analysis (Maegley et al., submitted for publication). These results and numerous protection studies (Reich et al., 1991; Reich & Everett, 1990; Everett et al., 1990) suggest that the flexible hinge region and the adjacent Cys223 form part of the active site.

Additional indirect evidence describing the placement of amino acids within the folded methylase structure was provided by our demonstration of an HAE with an arsenical-derivatized methylase (Tsao & Maki, 1991). However the presence of seven cysteines and two tryptophans (Figure 4) precluded direct assignment. Our use in the present study of two methylase mutants (C223S and W183F) has allowed direct assignment of amino acids responsible for this HAE.

The binding of As(III) to the W183F mutant methylase produces a perturbation of Trp225 as observed in the red-shift of its phosphorescence 0,0-band, the decrease in its triplet lifetime, and the quenching of fluorescence. Each is an expected consequence of an external HAE that enhances intersystem crossing. For an external HAE to be observed, the tryptophan must be in close proximity (within van der Waals distance) of the perturbing atom (As). Since As(III) is three-coordinate, direct access of the As atom to the indole ring is possible, in contrast with tetrahedral four-coordinate As(V) where such access would be sterically inhibited. The large red-shift observed in the tryptophan phosphorescence can be attributed to the stabilization of its triplet-state electric dipole moment by the increased polarizability of the local environment that now contains the polarizable As atom (Hershberger et al., 1980). The increased spin-orbit coupling between the singlet and the triplet manifolds in the presence of the heavy atom produces an increase in singlet–triplet mixing, thereby enhancing the triplet-state decay rate to the ground state by about 2 orders of magnitude. Quenching of fluorescence results from enhanced intersystem crossing to

the triplet manifold. The magnitude of these effects suggests that the As(III) atom lies very close, indeed within van der Waals contact of the indole chromophore of Trp225.

Trivalent arsenic is known to bind to SH groups in proteins. When Cys223 is mutated to serine in the C223S mutant, thus eliminating this SH group, no external heavy-atom effect is observed. This result confirms that Cys223 is required in order for Trp225 to undergo a heavy-atom perturbation upon As(III) binding, and thus defines the high-affinity binding site of methylase. The similarity of the triplet sublevel decay kinetics of perturbed tryptophan in the wild-type methylase and in the W183F mutant (Table I) suggests that Trp225 is the affected residue in the wild-type enzyme as well. This is also suggested by the similarity in the K's (Table II).

The proximity of the high-affinity As(III) binding site to the AdoMet and DNA binding domains is in accord with the previous study on the wild-type methylase (Tsao & Maki, 1991) where the heavy-atom effect was found to be reduced when a ternary complex between the enzyme, DNA, and a cofactor analog, sinefungin, was formed before the addition of the arsenic compound. Fluorescence titration data on the ternary complex with As(III) showed that the binding of DNA and sinefungin provides protection against As(III) binding to the site that quenches Trp225. It has been demonstrated that binding of NEM to Cys223 interferes with DNA methylation (Reich & Everett, 1990). Enzymatic activity is retained, however, upon binding of As(III) to Cys223 (Tsao & Maki, 1991), suggesting that NEM binding to this residue inhibits enzymatic activity by a steric effect, perhaps by reducing cofactor binding, rather than by the requirement of Cys223 in the catalytic mechanism. This is further supported by the kinetic analysis of C223S (Maegley et al., submitted for publication) which showed that the mutation of Cys223 to Ser (Ala, Gly) caused insignificant (~2-fold) changes in the apparent k_{cat}, K_m^{DNA}, and K_m^{AdoMet} values. It is likely that there are cysteine binding sites for As(III) other than Cys223 that are not in close proximity to a tryptophan residue and, therefore, are not probed in experiments designed to reveal an HAE. In previous work (Tsao & Maki, 1991), we found that the wild-type methylase gradually loses enzymatic activity when treated with a stoichiometric excess of the arsenic reagent. Thus, loss of activity may be associated with As(III) binding to a relatively inaccessible cysteine residue.

The triplet sublevel kinetic measurements on the unperturbed tryptophan in the W183F mutant produce rate constants in the expected range for normal tryptophan. For the perturbed tryptophan, all three sublevel decay constants, especially k_x, are larger than that of a normal tryptophan. As suggested previously (Tsao & Maki, 1991) using the model of Weinzierl and Friedrich (1981) on the effects of external heavy-atom perturbation on the sublevel dynamics of an aromatic chromophore, we suggest that the As atom is located in the xz plane of the tryptophan magnetic axes system near the edge of the indole ring. This location of the perturber atom predicts the preferential enhancement of k_x, as is observed. On the other hand, the selective enhancement of k_z by methylmercury binding to phage T4 lysozyme (Table I) implies that the Hg atom is bound close to the z-axis of indole. In the work reported here on the W183F mutant, we observed the same triplet sublevel kinetics for the arsenic-perturbed tryptophan as in the wild-type methylase, suggesting that it is Trp225 that is perturbed in both cases.

The fact that the As(III) atom induces a significant HAE on Trp225 when bound to Cys223 implies that there is van der Waals contact between the perturber atom and the indole ring. This contact implies a rather specific folding of the peptide sequence Cys223-Leu224-Trp225. Molecular modeling studies are in progress to shed light on the local peptide conformation.

In conclusion, our spectroscopic experiments with the two methylase mutants have clearly shown the site of arsenical attachment to be Cys223. Attachment of this probe has minimal conformational consequences in the vicinity of Trp225 based on the retention of enzymatic activity, and interaction with this residue is the major source of the observed HAE. Our results further characterize this region of the methylase, previously shown to be adjacent to amino acids that form part of the AdoMet binding site and a highly flexible hinge region. Cys223 and Trp225 lie at the carboxyl end of this region, and our results show that the side chains of the two residues are in close proximity to each other.

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