Optically Detected Magnetic Resonance Study of the Interaction of an Arsenic(III) Derivative of Cacodylic Acid with *Eco*RI Methyl Transferase†

Désirée H. H. Tsao and August H. Maki*

Department of Chemistry, University of California, Davis, California 95616

Received August 29, 1990; Revised Manuscript Received January 28, 1991

ABSTRACT: The interaction of the enzyme *Escherichia coli* RI methyl transferase (methylase) with an arsenic(III) derivative of cacodylic acid has been investigated by optical detection of triplet-state magnetic resonance (ODMR) spectroscopy in zero applied magnetic field. The reactive derivative $(CH_3)_2AsSR$ is formed by the reduction of cacodylate by a thiol. The As(III) derivative binds to the enzyme by mercaptide exchange with a cysteine (Cys) residue located close to a tryptophan (Trp) site. The arsenical binding selectively induces an external heavy-atom effect, perturbing the nearby Trp residue in the enzyme. Zero-field splittings (ZFS) and total decay rate constants of the individual triplet-state sublevels of the Trp residue in the presence and absence of perturbation by As(III) have been determined. The perturbed Trp shows a large reduction in the overall decay lifetime compared with unperturbed Trp residue, exhibiting a high selectivity for the T_x sublevel. This selectivity suggests that the As atom lies in the xz plane of the principal magnetic axis system of Trp, but not directly along the z (out-of-plane) axis. The accessibility of this enzyme binding site to the arsenical is decreased upon forming a ternary complex of methylase with sinefungin and a DNA oligomer, d[GCGAA(BrU)(BrU)CGC], containing two 5-bromouracil (BrU) bases in place of thymine within the hexadeoxynucleotide recognition sequence. This result indicates that the arsenical binding site in methylase which produces the Trp heavy-atom effect is protected from this ligand by ternary complex formation or the enzyme undergoes a conformation change, removing the Cys from the Trp site. This protection is also observed in fluorescence quenching experiments. The As(III) reagent, upon binding to methylase, quenches the Trp fluorescence by 46%. When the ternary complex is formed, the quenching of Trp fluorescence is only 17%. A binding constant for the arsenical to the high-affinity enzyme site was obtained, which is at least 27 times that of binding to a free sulfhydryl residue. The addition of a 1:1 molar ratio of the arsenical to methylase did not affect the activity of the enzyme, but incubation with excess arsenical quenches the activity, suggesting that the high-affinity Cys residue is not involved in the DNA methylation process. In the ternary complex methylase–sinefungin–DNA, no heavy-atom perturbation of the two Trp residues in the enzyme by BrU was observed, demonstrating that Trp residues are not involved in close-range interactions with the two heavy-atom-derivatized nucleic acid bases. A similar result was observed previously with the analogous *E. coli* RI endonuclease–decanucleotide complex [John, N.-I., Casas-Finet, J. R., Maki, A. H., & Modrich, P. (1988) *Biochim. Biophys. Acta 949*, 189–194].

The primary biological function of DNA methylation is to affect directly sequence-specific interactions of proteins with DNA, adding an additional level of information to the DNA helix. Methylation serves no essential function in DNA replication, but it has two other well-substantiated functions, modification and mismatch repair (Razin et al., 1984). DNA modification in host-specific restriction–modification systems consists of two functional components: (1) a restriction endonuclease which recognizes sequence specific sites in DNA and produces double-strand cleavage; (2) a modification enzyme recognizing the same DNA sequence as the restriction enzyme and protecting it from cleavage by methylation. Consequently, foreign DNA from a species with a different methylation pattern is destroyed by the restriction system, reducing the efficiency of gene transfer between unrelated species. The second type of function is mismatch repair, where the newly replicated (or repaired) strand is transiently unmethylated, so it can be distinguished from the old strand in the mismatch repair system, resulting in a strand selectivity for mismatch repair.

Restriction–modification systems have been classified into types I, II, and III based on differences in enzyme structure and mechanisms. The *Escherichia coli* RI (*Eco*RI) methylase is part of the prokaryotic type II DNA restriction and modification system. Both component proteins recognize the same hexanucleotide sequence in duplex DNA (Jack et al., 1981; Modrich, 1982):

5′...G-A-A-T-T-C...3′

3′...C-T-T-A-A-G...5′

The restriction endonuclease is a dimer, requires Mg^{2+} for activity, and cleaves the phosphodiester bond between the guanine and adenine residues. The modification methylase[1] is a monomer and catalyzes the transfer of a methyl group from the cofactor *S*-adenosylmethionine (SAM) to the 6-amino group of the central adenine residue at position 3, from the 5′ end, making the DNA sequence resistant to restriction.

† This work has been supported in part by a grant from the National Institutes of Health (ES-02622).

* Author to whom correspondence should be addressed.

[1] Abbreviations: BAL, British anti-Lewisite; *D* and *E*, triplet-state zero-field splitting parameters; DTT, dithiothreitol; HAE, heavy-atom effect(s); ME, β-mercaptoethanol; methylase, *Escherichia coli* RI methyl transferase; MIDP, microwave-induced delayed phosphorescence; MSR, microwave saturation recovery; NEM, *N*-ethylmaleimide; ODMR, optically detected magnetic resonance; SAM, *S*-adenosylmethionine; ZFS, zero-field splitting(s).

0006-2960/91/0430-4565$02.50/0

The methylase has a molecular weight of 38 050 and contains seven cysteine (Cys) and two tryptophan (Trp) residues (Rubin & Modrich, 1977; Newman et al., 1981).

We have found that when *Eco*RI methylase in cacodylate buffer is treated with a thiol reducing agent such as dithiothreitol (DTT), very unusual Trp triplet-state emission characteristics result. In this paper, we report on the application of low-temperature phosphorescence and optically detected magnetic resonance (ODMR) spectroscopy of the excited triplet state to study the interaction of *Eco*RI methylase with a three-coordinate arsenic(III) compound derived from cacodylate. ODMR spectroscopy (Maki, 1984) has been applied to study a variety of biopolymer interactions, such as protein–nucleic acid binding (Khamis et al., 1987a; Tsao et al., 1989), protein–lipid binding (Mao et al., 1985, 1986), and external heavy-atom effects (HAE) occurring in enzyme–methylmercury(II) complexes (Hershberger & Maki, 1980; Zang et al., 1988). In our present application of the ODMR experiment, the phosphorescence of tryptophan in its excited triplet state is monitored at cryogenic temperatures, while magnetic resonance transitions are induced between its magnetic sublevels. Intensity changes result from population transfer between sublevels which is induced by resonant microwaves. ODMR probes the microenvironment of aromatic residues, with Trp being among the most extensively studied in proteins (Zang et al., 1988; Hershberger & Maki, 1980; Khamis et al., 1987a,b).

The mechanism by which arsenic exerts its toxic effects on living organisms has been the subject of intense interest for quite a number of years. Much of the basis of our current understanding of the mechanism of arsenic toxicity in living systems comes from work done in the late 1800s, from the development of arsenical drugs during the early 1900s, and from work done in the 1940s in response to the need to find effective antidotes for arsenical warfare agents. A major focus of research addressed the question of how arsenicals combine with enzymes and other proteins. A well-known example is Lewisite [(2-chloroethenyl)arsenous dichloride] which exerts its toxic effects by combining with tissue thiol groups. Its toxicity can be antagonized by a dithiol, 2,3-dimercaptopropanol (British anti-Lewisite, BAL) (Stocken & Thompson, 1946). The affinity of BAL for the As(III) adduct is large enough to cause it to dissociate from tissue proteins. Pentavalent arsenic is less active and less toxic than the trivalent form. It is known (Rosenthal, 1932) that trivalent arsenoxides bind to proteins that contain sulfhydryl groups and that no such combination occurs in their absence, thus providing evidence for the specificity of the reaction. Studies on the capacity of trivalent arsenic compounds to interfere with metabolic processes due to their affinity for thiol residues have been conducted (Gordon & Quastel, 1948). Arsenic(III) was found to interfere with the activity of a number of enzymes.

In this paper, we describe the formation of an arsenic(III)–*Eco*RI methylase complex when the enzyme is in the presence of cacodylate buffer and DTT. Cacodylic acid, an arsenical with the molecular formula $(CH_3)_2As(O)OH$, when treated with a reducing agent such as DTT or β-mercaptoethanol (ME), forms an As(III)–thiolate intermediate which in turn reacts with methylase. An As(III)–thiolate compound, $(CH_3)_2AsSCH_2CONH_2$, which is structurally similar to the proposed intermediate, was synthesized by a previously described method (Barber, 1932) and was allowed to react with the enzyme. We propose that the As(III) compounds react with methylase by a thiol exchange process. Fluorescence titrations were performed to obtain binding stoichiometries, and an association constant for the synthetic intermediate in the methylase complex was estimated for the proposed exchange reaction. In the arsenical–enzyme complex, an arsenic atom lies close to (within van der Waals contact with) one of the two Trp residues in the enzyme, thereby inducing an external heavy-atom effect (HAE). The HAE leads to fluorescence quenching, an increase in phosphorescence quantum yield, and a reduction of the triplet-state lifetime of the perturbed Trp residue. Similar effects have been observed previously when Hg(II) binds within van der Waals contact of Trp (Hershberger & Maki, 1980; Zang et al., 1988). We report here the phosphorescence and ODMR measurements on the *Eco*RI methylase–arsenical complex. Slow-passage and transient ODMR experiments were used to characterize the heavy-atom-perturbed chromophore. Lifetimes and triplet-state sublevel kinetics were measured for both the unperturbed and the heavy-atom-perturbed Trp residues, and a mechanism of interaction is discussed. We also show that when a ternary complex is formed between methylase, sinefungin (an analogue of the natural cofactor *S*-adenosylmethionine), and an analogue of its natural DNA substrate d[GCGAA(BrU)(BrU)CGC], which contains a pair of 5-bromouracil substitutions for thymine within the hexameric recognition part, the accessibility of the arsenical to the enzyme is reduced, since fluorescence quenching and the contribution of heavy-atom-perturbed phosphorescence are substantially decreased. It has been demonstrated recently (Reich & Mashhoon, 1990) that sinefungin, in which the methylsulfonium of SAM is replaced with an amine–methine substituent, is a good cofactor analogue. Sinefungin is a potent inhibitor of methylase, with inhibition occurring through formation of a stable, sequence-specific ternary complex of methylase–sinefungin–DNA. Sinefungin displaces SAM when added to the methylase–SAM complex, and introduction of the DNA substrate, forming the ternary complex, enhances the affinity of methylase for sinefungin. Also in Reich's group, it was demonstrated (Everett et al., 1990) that Cys at position 223 is essential for DNA methylation. Modification of this residue by *N*-ethylmaleimide (NEM) causes the enzyme to become inactive. We undertook an enzyme activity measurement of the methylase–As(III) complex to determine if arsenic binding inactivates the enzyme. Our studies suggest that the Cys residue that becomes modified by the arsenical causing a HAE on Trp is not Cys-223, because the enzyme remains fully active upon addition of 1 molar equiv of $(CH_3)_2AsSCH_2CONH_2$. However, incubation with an excess of the As(III) compound inactivates the enzyme, indicating that the arsenical binds to other Cys residue(s), including Cys-223.

The bromine-derivatized uracil base was chosen in the DNA sequence rather than the thymine residue at positions 6 and 7 of the decanucleotide in order to determine whether these residues undergo close-range interactions with Trp residues of the protein. Such interactions which produce HAE have been observed by ODMR (Khamis et al., 1987a,b; Tsao et al., 1989) in the complexes of *Escherichia coli* single-stranded DNA binding protein and heavy-atom-derivatized single-stranded polynucleotides. In the case of *Eco*RI methylase, the enzyme did not show changes in its triplet-state properties upon forming a complex with sinefungin and the heavy-atom-derivatized decamer, suggesting that neither of the two Trp residues is involved in close-range interactions with the brominated DNA bases in the ternary complex.

Materials and Methods

Materials. *Eco*RI methylase was a generous gift from Dr. Paul Modrich. The enzyme was stored in 20 mM potassium

phosphate buffer, pH 7.4, 200 mM NaCl, 5 mM DTT, and 0.2 mM EDTA containing 10% glycerol by volume. Low-temperature phosphorescence studies were made of the enzyme either in 20 mM potassium phosphate buffer or in 20 mM cacodylate buffer, pH 7.4, 100 mM NaCl, 0.2 mM EDTA, and 5 mM DTT containing 25% glycerol (v/v), unless otherwise indicated. Sinefungin was obtained from Calbiochem and was dissolved in water, to make a 53 mM stock solution. The self-complementary DNA oligomer d[GCGAA(BrU)-(BrU)CGC] was synthesized by Synthecell (Rockville, MD), purified by HPLC, and dissolved in 20 mM potassium phosphate buffer, pH 7.4, containing 10 mM NaCl. The thioarsenite, $(CH_3)_2AsSCH_2CONH_2$, was prepared as described by Barber (1932), by adding cacodylic acid (0.5 g) to a hot solution of acetamidothiol (1 g) in water. Acetamidothiol was prepared by bubbling NH_3 into thioglycolic acid (Bhandari et al., 1971). The final product was characterized by its melting point (found 108 °C, reported 107 °C; Barber, 1932) and by proton NMR [General Electric QE-300 (300 MHz) spectrometer]. The solvent used was deuterated chloroform with the chemical shifts expressed relative to tetramethylsilane: The resonances found were at 1.37 (CH_3), 3.32 (CH_2), 6.0 (NH), and 6.5 (NH) ppm. The thioarsenite was dissolved in water, and stock solutions were prepared for phosphorescence measurements (5.5×10^{-3} M) and fluorescence titrations (1×10^{-4} M). Buffer exchange was achieved by ultrafiltration using a Centricon (Amicon Corp.) microconcentrator with a 30-kDa cut-off membrane. [C^3H_3]SAM (73.8 Ci/mmol) was purchased from New England Nuclear and DE81 (2.3-cm diameter) from Whatman. The radioactivity was measured by a Beckman Model LS6800 scintillation counter.

Phosphorescence and ODMR. The low-temperature phosphorescence and ODMR measurements were conducted with the enzyme in the 20 mM potassium phosphate buffer described above. Four different samples were prepared containing methylase. In the first, cacodylic acid was added to the enzyme; the phosphorescence showed contributions from both unperturbed and perturbed Trp residues. Further characterization of these residues was carried out by ODMR and triplet-state sublevel kinetics measurements. In the second sample, methylase was allowed to react with the synthesized thioarsenite, $(CH_3)_2AsSCH_2CONH_2$, in the absence of DTT. In the third sample, the ternary complex of methylase with sinefungin and DNA was formed, and this complex was characterized by ODMR. Finally, the methylase–sinefungin–DNA complex was formed, with subsequent addition of cacodylate, to observe the reactivity of the As(III) intermediate with the enzyme upon complex formation. The samples were measured within 1-mm i.d. Suprasil quartz sample tubes placed within a Cu helix attached to a coaxial transmission line. This was immersed into either liquid nitrogen or liquid helium. The apparatus and methods for performing phosphorescence lifetime and ODMR measurements have been described earlier (Tsao et al., 1989). The samples were excited with a 100-W high-pressure Hg lamp, using an excitation band centered at 295 nm for the unperturbed Trp and at 305 nm for the perturbed Trp. The lifetime and ODMR measurements were performed while monitoring the 0,0 band of the phosphorescence emission of each type of residue. The average phosphorescence lifetimes of the perturbed and unperturbed Trp residues were obtained from phosphorescence decay measurements at 77 K, where rapid spin–lattice relaxation equalizes the sublevel populations and the average sublevel properties are expressed. In order to quench spin–lattice relaxation for ODMR and sublevel decay

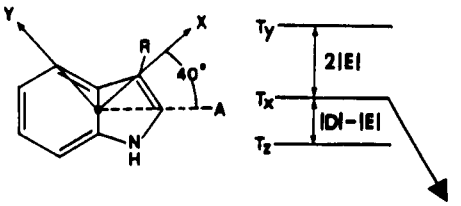

FIGURE 1: Molecular axes convention and triplet sublevel splittings of Trp in zero field. The solid arrow indicates radiative sublevel decay.

kinetics experiments, the sample was immersed in pumped liquid helium, at 1.1–1.2 K. ODMR slow-passage experiments were performed by monitoring the phosphorescence intensity change upon sweeping the microwaves slowly through the transitions between sublevels in order to obtain the ZFS parameters D and E. The values obtained were corrected by extrapolation to zero sweep rate. For the unperturbed Trp, the sweep rate varied from 30 to 60 MHz/s, while for the perturbed Trp, sweep rates ranged from 0.6 to 1.8 GHz/s. Seven scans were averaged, giving a signal-to-noise (S/N) ratio of 8 for $D - E$ and 4 for $2E$, for the unperturbed Trp. For the perturbed Trp, the number of scans varied from 160 to 490 where the S/N ratio varied from ca. 4 to 6 for both transitions. Figure 1 displays the principal axes and the triplet-state sublevel splittings of Trp in zero field. The triplet sublevel dynamics were studied by means of the fast-passage transient method (Winscom & Maki, 1971) and the microwave-induced delayed phosphorescence (MIDP) technique (Schmidt et al., 1969). The total apparent decay rate constants of the T_y and T_z sublevels were measured by MIDP for both perturbed and unperturbed Trp residues. The lifetime of the more rapidly decaying T_x sublevel was obtained by fitting the tail of the MIDP response of the two observed transitions $|D| - |E|$ and $2|E|$; for the heavy-atom-perturbed Trp, the lifetime of the T_x sublevel was obtained by fast-passage response of the $2|E|$ transition and corrected for optical pumping. Microwaves were swept at a rate of 470 GHz/s. The microwave saturation recovery (MSR) experiment was carried out (Shain & Sharnoff, 1973) to obtain the relative radiative decay rate constants of the perturbed Trp sublevels. The microwaves were set at a central frequency of 1.6 or 2.6 GHz (for $|D| - |E|$ and $2|E|$ transitions, respectively), and frequency was modulated at 1 kHz over a bandwidth of 230 MHz. The observed biexponential recovery from microwave saturation of the heavy-atom-perturbed Trp was simulated by fitting values of k_x, k_y, and k_z. The ratio of the preexponential factors gives Q_y/Q_x for the $2|E|$ and Q_z/Q_x for the $|D| - |E|$ transitions, respectively, where $Q_u = k^r_u/k_u$, the sublevel radiative quantum yield. The relative k^r_u could be determined from values of k_u. The $|D| + |E|$ transition is very weak and was not analyzed.

Fluorescence Titration. Fluorescence titrations were performed by using a Perkin-Elmer MPF-44B spectrofluorometer as previously described (Khamis et al., 1987b), in which the intrinsic Trp fluorescence of the free enzyme was monitored upon addition of $(CH_3)_2AsSCH_2CONH_2$. The titration was performed in the phosphate buffer described previously, in the absence of DTT. Aliquots of the thioarsenical were added to the enzyme (1 μM) in a 1-cm cell. The stepwise titration of the fluorescence quenching was monitored at constant emission wavelength and was corrected for dilution. The excitation was carried out at 295 nm with a 2-nm slit width. At 295 nm, the absorption is due primarily to Trp (Teale & Weber, 1956), so Trp can be selectively excited without excitation of tyrosine and subsequent energy transfer from tyrosine to tryptophan. The emission was monitored at 335 nm with 5-nm bandwidth. A plot of the normalized fluorescence intensity versus the

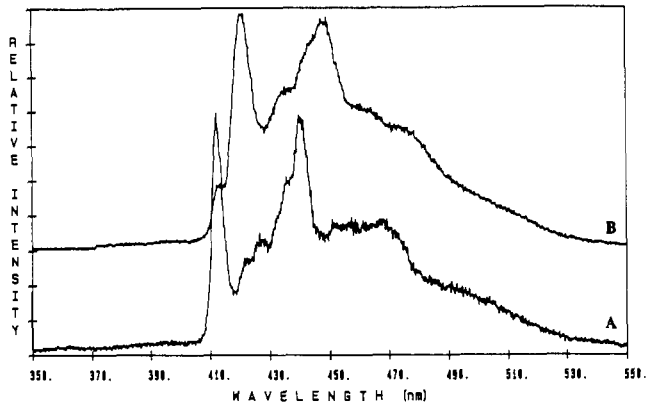

FIGURE 2: Phosphorescence spectra of *Eco*RI methylase at 77 K in pH 7.4 buffer containing 100 mM NaCl, 5 mM DTT, 0.2 mM EDTA, and 25% glycerol (v/v). (A) 20 mM potassium phosphate; (B) 20 mM sodium cacodylate. Excitation is at 295 nm with 16-nm band-pass, and the emission is collected with 3-nm band-pass slits. The enzyme concentration is ca. 8×10^{-5} M.

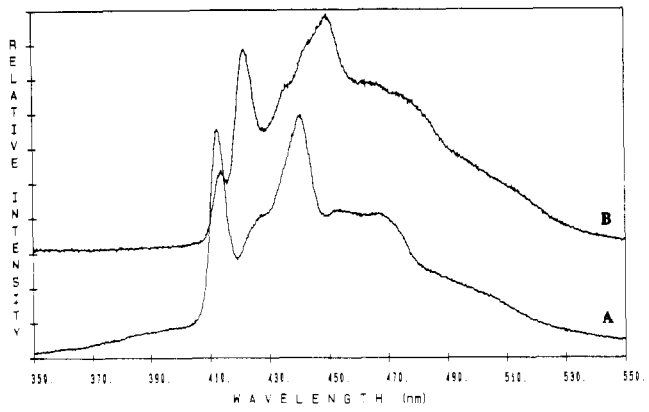

FIGURE 3: (A) Phosphorescence spectra of *Eco*RI methylase (1×10^{-4} M) at 77 K in 20 mM potassium phosphate buffer, pH 7.4, 100 mM NaCl, 0.2 mM EDTA, and 25% glycerol. (B) As in (A), but with the addition of $(CH_3)_2AsSCH_2CONH_2$ (4×10^{-4} M). Excitation is at 295 nm for both samples.

concentration of the ligand added was constructed. The stoichiometry of binding was determined from the intersection of the extrapolated initial and final slopes of the plot. The binding constant was obtained from a modification of the double-reciprocal plot analysis introduced by Kelly et al. (1976). Details are presented under Results. The thioarsenite titration of methylase (1 μM) complexed with sinefungin (9 μM) and DNA (37 μM) was also performed. The ternary complex was formed in the absence of DTT and then further diluted in the titration cell to the final concentrations indicated.

Enzyme Activity Assay. Methylase activity was monitored by the incorporation of tritiated methyl groups of SAM into DNA (Rubin & Modrich, 1977). The enzyme was in 20 mM potassium phosphate, pH 7.4, 0.2 M NaCl, and 10% glycerol. The assay of pure methylase and methylase–As(III) was carried in 50 μL of 0.1 M Tris-HCl, pH 8.0, 5 mM EDTA, and 0.4 mg/mL BSA with 0.1 μM methylase, 1.5 μM SAM, and 8 μM DNA (per strand of oligonucleotide). For the methylase–As(III) complex, the arsenical $(CH_3)_2AsSCH_2CONH_2$ was allowed to react with the enzyme first in a 1:1 molar ratio (for 15 min at 37 °C) as well as in a 1:20 molar (for 45 min at 37 °C followed by ca. 3 h at room temperature) and then added to the reaction buffer, which had been preincubated at 37 °C also. The reaction was monitored for 1 h at room temperature with 2-μL aliquots taken at 10-min time intervals that were spotted onto DE81 paper. The papers were washed 5 times for 7 min with 0.2 M ammonium bicarbonate, once with 95% ethanol, and once with ether. They were air-dried, and the tritium content was measured with a Beckman LS6800 scintillation counter in 5 mL of Aquasol. The concentration of methyl groups transferred to DNA was calculated from the cpm bound to the paper minus that at zero time divided by the total radioactivity per micromolar methyl groups in the mixture (Brennan et al., 1986).

RESULTS

Phosphorescence Spectra. The extremely well-resolved phosphorescence spectrum of *Eco*RI methylase in phosphate buffer upon excitation at 295 nm is shown in Figure 2A. The sharp origin band ($\lambda_{0,0}$ = 412.3 nm) is assigned to the unperturbed Trp residue(s). Figure 2B shows the phosphorescence of the enzyme in cacodylate buffer containing DTT. The spectrum shows the presence of a new intense and relatively broad band at 420.5 nm. We assign this emission to the 0,0 band of Trp perturbed as As(III) binding to the methylase enzyme. The triplet lifetime of this Trp is reduced relative to that in the unperturbed sample (see below); the much weaker band at 412.7 nm is assigned to residual unperturbed Trp. The emission maximum of the first strong vibronic band is red-shifted from 440.5 nm (Figure 2A) to 448.5 nm (Figure 2B). Upon excitation of these samples at 305 nm (data not shown), the phosphorescence of the enzyme in phosphate buffer does not change, still exhibiting a 0,0 band at 412.3 nm. For the enzyme in cacodylate buffer, however, the shoulder at 412.7 nm is now very weak, and the phosphorescence originates predominantly from the heavy-atom-perturbed Trp.

The occurrence of the HAE in Trp can be easily monitored from the appearance of the red-shifted phosphorescence spectrum with a 0,0 band at about 420 nm. The presence of cacodylate and a thiol such as DTT is essential for the observation of the red-shifted Trp spectrum. By use of ME instead of DTT plus cacodylate, the same HAE was observed. However, when cacodylate was added to the enzyme in phosphate buffer in the absence of DTT or ME, no HAE occurred, and the phosphorescence spectra remained the same as in Figure 2A. Upon exchanging the cacodylate buffer of the enzyme reaction product (Figure 2B) to a phosphate buffer containing DTT, the relative intensity of the red-shifted 0,0 band in the phosphorescence spectrum was reduced, indicating that the reaction resulting in the HAE is readily reversible. On the other hand, if the exchanging buffer did not contain a sulfhydryl reagent such as DTT, the relative intensities of the two 0,0 bands did not change, indicating that the mechanism of binding and removal involves mercaptide ligand exchange.

The same perturbation of the Trp phosphorescence is observed when the synthesized thioarsenite, $(CH_3)_2AsSCH_2CONH_2$, is allowed to react with the enzyme in the absence of cacodylate. Figure 3A shows the phosphorescence spectra of methylase in the phosphate buffer described earlier in the absence of DTT. Upon addition of the thioarsenite to the enzyme, the phosphorescence shifts predominantly to that of heavy-atom-perturbed Trp (Figure 3B), with the appearance of the broad 0,0 band at 420.7 nm and a reduced triplet-state lifetime. Again, the HAE was reversed by exchanging the buffer with a DTT-containing phosphate buffer.

On the other hand, the perturbation by the As(III) is not readily accomplished when the enzyme is bound to sinefungin and its DNA substrate. In Figure 4A, we show the phosphorescence of the enzyme in DTT-containing phosphate buffer, to which a small amount of cacodylate has been added to produce a HAE. Both perturbed and unperturbed chromophores contribute to the emission, which is characterized

Table I: Phosphorescence 0,0 Maxima and Zero-Field Splitting Parameters in the Methylase and Methylase–Arsenical Complex

sample[a]	λ_{exc} (nm)	$\lambda_{0,0}$ (nm)	$D - E$[b] (GHz)	$2E$[b] (GHz)	D (GHz)	E (GHz)
methylase	295	412.3	1.65 (50)	2.67 (80)	2.99	1.34
methylase + cacodylic acid	295	412.2	1.63 (50)	2.67 (95)	2.97	1.34
		420.5				
	305	412.7				
		420.5	1.63 (100)	2.64 (140)	2.95	1.32

[a] Enzyme is in phosphate buffer containing DTT. See text. [b] The numbers in parentheses are full-width at half-maxima of the transitions, in megahertz.

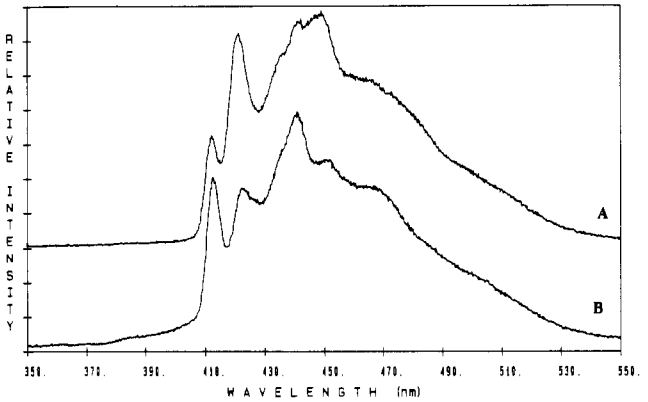

FIGURE 4: Phosphorescence spectra of *Eco*RI methylase (ca. 8×10^{-5} M) in the phosphate buffer indicated in the caption of Figure 2 at 77 K (A) with the addition of cacodylate (1×10^{-3} M) and (B) complexed initially with sinefungin (5×10^{-4} M) and DNA decamer (2×10^{-3} M phosphate) with subsequent addition of cacodylate (1×10^{-3} M). Excitation was at 295 nm for both samples.

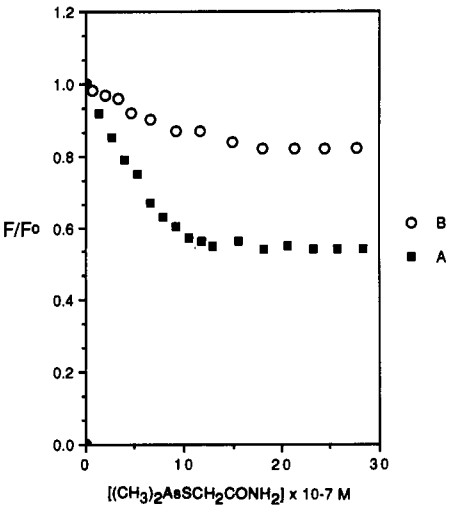

FIGURE 5: Fluorescence titration curve of (A) methylase (1 μM) with thioarsenite $(CH_3)_2AsSCH_2CONH_2$ and (B) methylase (1 μM) complexed with sinefungin (9 μM) and the DNA decamer (37 μM phosphate) titrated with the thioarsenite. Both titrations were done in 20 mM potassium phosphate buffer, pH 7.4, with 100 mM NaCl and 0.2 mM EDTA.

by the 0,0 bands at 412.2 and 420.5 nm. By allowing the methylase to bind first to sinefungin and DNA, and subsequently adding cacodylate, there is a much smaller contribution from the heavy-atom-perturbed Trp, as shown in Figure 4B. The phosphorescence spectrum is predominantly from unperturbed Trp. This result implies that when DNA and sinefungin are bound to methylase, there is less accessibility of the arsenical to the HAE-producing site of the enzyme.

Fluorescence Titration. The steady-state fluorescence titration of methylase with $(CH_3)_2AsSCH_2CONH_2$ is presented in Figure 5 (curve A). It exhibits a limiting Trp fluorescence quenching of 46%. The observation that the fluorescence quenching saturates in the manner that it does is consistent with a static quenching process resulting from complex formation.

The fluorescence titration of methylase complexed with sinefungin and DNA, on the other hand, showed a limiting quenching of only 17%, as displayed in Figure 5 (curve B). From the intersection of the extrapolated initial and final slopes of curve A in Figure 5, the stoichiometry is found to be 0.84 ± 0.09 mol of ligand/mol of enzyme. This result indicates that a single "high-affinity" cysteine is responsible for the fluorescence quenching when As(III) is bound. Thus, the exchange reaction can be represented by

$$\sum_i (CH_3)_2AsSR_i + E\text{-}S^*H \overset{K}{\rightleftharpoons} E\text{-}S^*As(CH_3)_2 + \sum_i HSR_i \quad (1)$$

The index i ranges over all accessible "low-affinity" sulfhydryl binding species, both in solution and in the enzyme. The single high-affinity enzyme binding species associated with the fluorescence quenching is represented by E–S*H. The exchange constant for reaction 1 was obtained by using a modification of the double-reciprocal plot analysis introduced by Kelly et al. (1976) to account for the difference between eq 1 and a simple ligand binding equation. The double-reciprocal plot consists of $1/\Delta F$ vs $\sum_i[HSR_i]/\sum_i[(CH_3)_2AsSR_i]$, and two plots were constructed, with different assumptions for the species R_i. In the first case, only the high-affinity Cys was taken into account, so the abscissa is simply $[HSCH_2CONH_2]/[(CH_3)_2AsSCH_2CONH_2]$. In the second case, R_i is assumed to include two other accessible Cys sites per enzyme (low-affinity sites), which have a similar affinity for $(CH_3)_2As(III)$ as does $HSCH_2CONH_2$. Two protein sites were assumed because there are three Cys residues in the enzyme which are partially modified by NEM (Everett et al., 1990). In both cases, the binding constant was extracted from the slopes of the straight lines, and $K = 27$ and $K = 92$ were obtained for the first and the second case, respectively.

ODMR Transitions and ZFS. The ODMR transition frequencies and the values of D and E for the free enzyme and the enzyme–arsenical complex at 1.2 K are presented in Table I together with the full-width at half-maximum of the transitions extrapolated to zero sweep rate. The ZFS observed for the free enzyme, $D = 2.99$ GHz and $E = 1.34$ GHz, are similar to those for the unperturbed Trp found for the methylase–arsenic complex. The heavy-atom-perturbed Trp shows only a slight apparent reduction in both D and E parameters. The $|D| + |E|$ signal, which is normally absent in unperturbed Trp but is often observed in heavy-atom-perturbed Trp (Zang et al., 1988), is present but very weak for the perturbed Trp. Its frequency could not be determined accurately. We also observed an increase in the ODMR line widths for the heavy-atom-perturbed Trp (Table I).

Triplet-State Lifetime and Sublevel Kinetics. The triplet-state sublevel kinetics were measured for both perturbed and unperturbed Trp residues in the methylase–arsenical complex. The average lifetime of the lowest triplet state of the individual Trp residues was measured by selective exci-

Table II: Total Triplet-State Decay Constants and Relative Radiative Rate Constants of Trp Residues in the Arsenical–Methylase Complex

sample	k_x (s^{-1})	k_y (s^{-1})	k_z (s^{-1})	k_{calc}[a] (s^{-1})	k_{av}[b] (s^{-1})	$k_x^r:k_y^r:k_z^r$
methylase–As(III) complex						
λ_{exc} = 295 mm, λ_{em} = 412.2 nm	0.296	0.099	0.066	0.154	0.172	
λ_{exc} = 305 nm, λ_{em} = 420.5 nm	37.5	1.56	1.80	13.6	14.6	1:0.03:0.01
Trp[c]	0.240	0.12	0.038	0.132	0.136	1:<0.1:<0.1
YYW–CH_3Hg^{II}[d]	6.2	6.9	72.1	30.1	28.8	~0.0:~0.0:1.0

[a] $k_{calc} = (k_x + k_y + k_z)/3$. [b] Measurement made at 77 K. [c] From Zuclich et al. (1974). [d] From Zang et al. (1988). The sample is a point-mutated bacteriophage T4 lysozyme containing only Trp-158.

tation and monitoring of the individual 0,0 bands. The results are summarized in Table II. The lifetime of the heavy-atom-perturbed Trp is much shorter than that of the unperturbed Trp. The phosphorescence decay measurements at 77 K were fit to two components for unperturbed Trp. The major long-lived component decay originates from the Trp triplet state, while the minor (<14%) component of ~1.0 s was attributed to tyrosine emission. For the heavy-atom-perturbed Trp, on the other hand, the decay at 77 K showed three components, the major one (68%) of 68 ms being assigned to the perturbed Trp residue (Table II), a minor long component (5%) of about 2.5 s assigned to the residual emission of unperturbed Trp along with contaminants, and a remaining third component of about 0.35 s. It is possible that the 0.35-s component originates from As(III)-perturbed Trp in a somewhat different configuration with respect to the heavy-atom perturber. The large contribution of this component makes it unlikely that it originates from a contaminant. The triplet-state decay of methylase reacted with $(CH_3)_2AsSCH_2CONH_2$ also showed a dominating short-lifetime component of 70 ms (65%), which is assigned to As(III)-perturbed Trp. The individual decay rate constants for all three sublevels of perturbed and unperturbed Trp are listed in Table II, together with those of free Trp and of a methylmercury-perturbed Trp measured in a point-mutated bacteriophage T4 lysozyme. The triplet lifetime obtained at 77 K is compared with the calculated average sublevel lifetime, with good agreement. The sublevel decay rate constants for perturbed Trp which were obtained from the MSR experiment (data not given) are in reasonable agreement with those obtained by the MIDP and fast-passage methods (Table II). The relative radiative rate constants for perturbed Trp, calculated from these and the observed preexponential factors, are presented in Table II.

The phosphorescence, triplet-state lifetime, and ZFS of the enzyme remained virtually unchanged when it was bound to sinefungin and DNA. The phosphorescence of the ternary complex showed a 0,0 band at 412.5 nm with a lifetime of 5.9 s (85%) and ZFS values of 2.98 GHz for D and 1.34 GHz for E. These values when compared with those of the free enzyme in Table I confirm that the Trp residues are not perturbed upon forming the ternary complex. Also, no interaction between Trp residues and the adenine moiety of sinefungin (in the methylase–sinefungin complex) was observed, although any such interaction might be difficult to detect in the absence of a heavy atom. The phosphorescence spectrum, triplet lifetime, and ZFS remained the same as those of the free enzyme, within experimental error.

Enzyme Activity Assay. The activity of methylase, with the addition of 1 mol equiv of the arsenical, was not affected. It showed the same rate of DNA methylation as that of the free enzyme, transferring during a 1-h period 5.8 nM methyl groups to DNA per minute. The phosphorescence spectrum of this enzyme–arsenical complex at 77 K showed a 0,0 band at ca. 420 nm, indicating that the enzyme was modified. On the other hand, by adding a 20-fold molar excess of arsenical to protein and allowing a longer reaction time, the activity of the enzyme was lost, giving a cpm reading in the range of the ones observed at zero time.

Discussion

The binding of an arsenical, $(CH_3)_2AsSR$, to *Eco*RI methylase by thiol exchange causes a large perturbation of a Trp residue, as demonstrated by the wavelength shift of the phosphorescence and the decrease in the overall triplet-state lifetime. The external HAE generally enhances the rate of intersystem crossing, resulting in the quenching of fluorescence and shortening of the triplet lifetime. The extent of the HAE depends on the perturber and its orientation and distance with respect to the chromophore. It increases with increasing atomic number of the heavy atom (McGlynn et al., 1969). Although arsenic is not particularly heavy (Z = 33), it causes a substantial perturbation of Trp, suggesting that it lies very close to a Trp residue.

The unmodified enzyme shows a uniform phosphorescence spectrum with no optical resolution of the two Trp residues at positions 185 and 225 (Newman et al., 1981). This suggests either that both Trp residues experience the same microenvironment or that one of the Trp emissions is very weak with respect to the other. By monitoring the variation of ODMR frequency (ν) with wavelength (λ) through the 0,0 band, discontinuities of the plot of ν vs λ are sometimes observed in proteins containing multiple Trp sites (von Schütz et al., 1974). Such discontinuities have been interpreted previously as evidence for the resolution by ODMR of Trp residues in an unresolved phosphorescence spectrum. For the methylase, such a plot (data not shown) contains no discontinuity, suggesting that either both Trp residues in the uncomplexed enzyme experience a similar microenvironment or that only one Trp is noticeably radiative. Upon binding of the arsenical, the major part of the phosphorescence spectrum of Trp shifts about 8 nm to the red relative to that of unperturbed Trp, as can be seen by its 0,0 band at 420.5 nm and its major vibronic band at 448.5 nm. The large red-shift in phosphorescence could result from stabilization of the triplet state by a more polarizable local environment (Hershberger et al., 1980). The shoulder at 412.7 nm in the phosphorescence spectrum of the complex enzyme (Figure 2B) results from unperturbed Trp. However, it is possible that only one of the two Trp residues of the uncomplexed enzyme is radiative. Thus, the remaining emission at 412.7 nm in the arsenical–methylase complex could originate either from a distinct unperturbed Trp site, from residual unreacted methylase, or partially from both.

The ZFS values obtained for both Trp residues by slow-passage measurements show that D and E are changed very little by binding of the arsenical. The $|D| + |E|$ transition, which is usually observed for heavy-atom-perturbed Trp, is very weak in the case of the As(III)-perturbed Trp. This is largely due to T_x being the only significantly radiative sublevel. The slow-passage ODMR line widths of the As(III)-perturbed Trp are much broader than those of normal Trp, which may originate from the sensitivity of the line widths to perturbations in the Trp environment. It has been demonstrated before

(Williamson & Kwiram, 1988) that intramolecular spin–orbital coupling plays a very small role in the ODMR line-broadening process or in the magnitude of the ZFS of Trp, and that local electric fields play a more decisive role. Since the ZFS for the arsenic-perturbed Trp remains virtually unchanged with respect to the unperturbed Trp, it is reasonable to assume that the spin–orbit coupling contribution to the line width is negligible. Site heterogeneity, on the other hand, may be a major contributor to this process. Binding of the arsenical may occur in several protein conformations, causing the Trp to experience slightly different microenvironments, thus giving broader ODMR transitions.

The triplet-state sublevel kinetics were measured for each Trp residue in the As–methylase complex. The normal Trp has, as expected, small values of k_x, k_y, and k_z, close to those of free Trp. The calculated average lifetime is in fairly good agreement with the lifetime obtained at 77 K, where spin–lattice relaxation averages out the individual sublevel properties. For the heavy-atom-perturbed Trp, there is a reduction of the individual sublevel lifetimes. Although all sublevel decay constants are affected, k_x is preferentially increased. The relative radiative decay rate constants presented in Table II show that T_x also is the most radiative sublevel. The kinetics are compared with those of free Trp (Zuclich et al., 1974) and with a methylmercury-perturbed Trp in T4 lysozyme (Zang et al., 1988). In the lysozyme–CH_3Hg complex, the enzyme had two Trp → Tyr mutations, leaving only Trp-158, which is perturbed by the Hg atom. In this system, specific sublevel kinetics were obtained, and it was found that the T_z sublevel is the most affected. The selectivity was attributed to the Hg atom lying close to the out-of-plane z axis of Trp-158, thus enhancing the T_z radiative character as well as drastically increasing its total decay rate constant. These results were interpreted according to a model of Weinzierl and Friedrich (1981) to explain the effects of an external heavy-atom perturbation on the triplet-state sublevel dynamics of an aromatic chromophore. This model is based on overlap integrals between the π orbitals of the probe molecule and the p orbitals of the heavy-atom perturber. According to this model, an out-of-plane approach of the heavy atom along the center of the chromophore induces radiative character selectively into the out-of-plane sublevel (as observed in the T4 lysozyme–CH_3Hg complex), whereas both T_x and T_z or T_y and T_z radiation are enhanced upon placing the heavy atom in the xz and yz planes, respectively. In the methylase–arsenical complex, T_x is the most radiative sublevel, suggesting that the As atom is located toward the edge of the indole ring, within the xz plane.

The reaction of cacodylic acid with organic thiols, such as ME, Cys, DTT, and glutathione, has been observed previously (Jacobson et al., 1972). It was reported that the disappearance of the SH group is accompanied by the disappearance of the cacodylic acid function, indicating that cacodylic acid reacts spontaneously with thiol reagents. Pentavalent organic arsenic compounds do not affect enzyme activity (Gordon & Quastel, 1948; Rosenthal, 1932), but became active poisons of sulfhydryl enzymes when reduced to the +3 oxidation state. In a recent report (Boyle et al., 1988), the poisoning of the enzyme UDP-galactosyltransferase from human pleural effusion was attributed to cacodylate buffer. This inhibitory effect was observed only in the presence of ME, but the mechanism of the inhibition was not discussed, except for a suggestion that a reduced form of cacodylate may inhibit the enzyme by affecting an essential thiol component. This is in accord with our observations that the +5 arsenic in cacodylate shows no apparent reactivity with *Eco*RI methylase. Upon addition of a sulfhydryl reagent, we propose that the arsenic in cacodylic acid buffer is reduced to the +3 oxidation state following the general reaction scheme presented by Barber (1932):

$$(CH_3)_2As(O)OH + 3HSR \rightarrow (CH_3)_2AsSR + RSSR + 2H_2O \quad (2)$$

The reactive product, $(CH_3)_2AsSR$, in turn exchanges its mercaptide group with a Cys residue in the enzyme. The fact that we observed the same effects with the synthesized thioarsenical, R = CH_2CONH_2, provides further evidence for this proposed scheme. The reversibility of the sulfhydryl exchange reaction was shown by exchanging the buffer of the complexed methylase with a phosphate buffer in the presence of DTT which led to the disappearance both of the red-shifted Trp phosphorescence spectrum and of the short-lived phosphorescence component. The thiol exchange reaction taking place was presented in eq 1.

The question arises as to the identity of the high-affinity cysteine residue. The equilibrium constant, $K \geq 27$, is a measure of the relative affinity of this sulfhydryl site for the arsenical. The amino acid sequence of *Eco*RI methylase (Newman et al., 1981) reveals seven Cys residues with no disulfide bridges as well as two Trp residues at positions 185 and 225. Recent studies (Everett et al., 1990) show that three Cys residues in methylase (25, 116, and 223) are partially modified by 2 mol of NEM, rendering the enzyme inactive. However, when the enzyme was bound to sinefungin and the DNA substrate, only ca. 1 mol of NEM is incorporated per mole of enzyme, and methylase remains active. Cys-25 and to some extent Cys-116 remain accessible to NEM modification, despite the very stable complex formed between methylase–sinefungin–DNA. These results suggested that NEM modification of Cys-223 in the protein causes the loss of enzymatic activity. On the basis of these findings, our enzyme activity measurements made on the methylase–As(III) complex suggest that the high-affinity Cys residue is not Cys-223, because the enzyme remains fully active with the binding of 1 mol of thioarsenite per mole of methylase. The presence of an HAE perturbation on Trp phosphorescence confirmed the binding of As(III) to the protein. The activity of methylase is completely lost upon incubating the protein with a 20-fold molar excess of $(CH_3)_2AsSCH_2CONH_2$, indicating that the arsenical eventually binds to other Cys residue(s) in methylase, most likely including Cys-223. The HAE caused by the arsenical on methylase is greatly reduced when the enzyme is bound to sinefungin and DNA, as shown in Figure 4. Also, fluorescence quenching is inhibited (Figure 5). The cysteine which interacts with As(III) leading to an HAE in Trp is at least partially protected, indicating that upon forming this ternary complex this site is less accessible to the arsenical. The decrease in accessibility of the arsenical to the high-affinity Cys in the ternary complex may originate from a change in conformation of the enzyme upon forming the complex, removing this Cys from the proximity of Trp. Another possibility is that sinefungin and DNA may physically protect this Cys site from the arsenical. Reich and Everett (1990) using photoaffinity labeling identified the peptides involved in SAM binding to the enzyme. It was observed that a peptide of 16 amino acids, in the segment containing residues 206–221, constitutes part of the cofactor binding site. There is a possibility that the high-affinity Cys in the enzyme might be Cys-116. Although not associated with the cofactor binding site, the accessibility of this residue to the arsenical decreases when the methylase ternary complex is formed. Cys-116 is partially labeled by NEM (Everett et al., 1990), but to a

smaller extent than with thioarsenite. The binding of both Cys reagents is greatly reduced in the presence of the DNA substrate and cofactor. However, the high-affinity Cys may also be assigned to another site that is brought into close proximity to a Trp residue in the folding process of the protein. In any case, our enzyme activity measurements appear to rule out Cys-223 as the high-affinity residue.

The two Trp residues in *Eco*RI methylase are not involved in close-range interactions with brominated uracil bases upon binding sinefungin and DNA to the enzyme based on the absence of a HAE. The decamer used in our experiments is appropriate as a substrate for the enzyme. We found it to be readily methylated by methylase in the presence of SAM, and it was observed to bind as a substrate to *Eco*RI endonuclease in previous studies (Jhon et al., 1988). Close-range interactions between Trp residues and brominated uracil bases would be observable, if present, via an HAE in Trp. It was found that no perturbation of the triplet state of Trp residues of methylase occurs, even though there is a Trp (225) in proximity to the substrate and cofactor binding sites. The phosphorescence spectrum, triplet lifetime, and ZFS do not differ from those in the uncomplexed enzyme, suggesting that little change occurs in the Trp microenvironment.

ACKNOWLEDGMENTS

We are grateful to Dr. Paul Modrich for his generous gift of *Eco*RI methylase and to Dr. Norbert Reich for sharing his results with us prior to their publication. We also thank Bruce Schlyer for helpful discussions and Paul Bruins for his technical assistance.

REFERENCES

Anderson, R. R., & Maki, A. H. (1980) *J. Am. Chem. Soc. 102*, 163–167.

Barber, H. J. (1932) *J. Chem. Soc.*, 1365–1369.

Bhandari, C. S., Mahrot, U. S., & Sosani, N. C. (1971) *J. Prakt. Chem. 313*, 849–854.

Brennan, C. A., Van Cleve, M. D., & Gumport, R. I. (1986) *J. Biol. Chem. 261*, 7279–7286.

Boyle, F. A., Neil, D. C., & Peters, T. J. (1988) *Clin. Chim. Acta 172*, 291–296.

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

Gordon, J. J., & Quastel, J. H. (1948) *Biochem. J. 42*, 337–350.

Hershberger, M. V., & Maki, A. H. (1980) *Biopolymers 19*, 1329–1344.

Hershberger, M. V., Maki, A. H., & Galley, W. C. (1980) *Biochemistry 19*, 2204–2209.

Jack, W. E., Rubin, R. A., Newman, A., & Modrich, P. (1981) *Gene Amplification and Analysis* (Chirikjian, J. G., & Papase, T. S., Eds.) pp 165–179, Elsevier/North-Holland, New York.

Jacobson, K. B., Murphy, J. B., & Das Sarma, B. (1972) *FEBS Lett. 22*, 80–82.

Jhon, N.-I., Casas-Finet, J. R., Maki, A. H., & Modrich, P. (1988) *Biochim. Biophys. Acta 949*, 189–194.

Kelly, R. C., Jensen, D. E., & von Hippel, P. H. (1976) *J. Biol. Chem. 251*, 7240–7250.

Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., & Chase, J. W. (1987a) *J. Biol. Chem. 262*, 10938–10945.

Khamis, M. I., Casas-Finet, J. R., & Maki, A. H. (1987b) *J. Biol. Chem. 262*, 1725–1733.

Maki, A. H. (1984) *Biol. Magn. Reson. 6*, 187–294.

Mao, S. Y., Maki, A. H., & deHaas, G. H. (1985) *FEBS Lett. 185*, 71–75.

Mao, S. Y., Maki, A. H., & deHaas, G. H. (1986) *Biochemistry 25*, 2781–2786.

McGlynn, S. P., Azumi, H., & Kinoshita, M. (1969) *Molecular Spectroscopy of the Triplet State*, Prentice-Hall, Englewood Cliffs, NJ.

Modrich, P. (1982) *CRC Crit. Rev. Biochem. 13*, 287–323.

Newman, A. K., Rubin, R. A., Kim, S. H., & Modrich, P. (1981) *J. Biol. Chem. 256*, 2131–2139.

Razin, A., Cedar, H., & Riggs, A. D. (1984) *DNA Methylation—Biochemistry and Biological Significance*, Springer-Verlag, New York.

Reich, N. O., & Everett, E. A. (1990) *J. Biol. Chem. 265*, 8929–8934.

Reich, N. O., & Mashhoon, N. (1990) *J. Biol. Chem. 265*, 8966–8970.

Rosenthal, S. M. (1932) *Public Health Rep. 47*, 241–256.

Rubin, R. A., & Modrich, P. (1977) *J. Biol. Chem. 252*, 7265–7272.

Schmidt, J., Veeman, W. S., & van der Waals, J. H. (1969) *Chem. Phys. Lett. 4*, 341–346.

Shain, A. L., & Sharnoff, M. (1973) *J. Chem. Phys. 59*, 2335–2343.

Stocken, L. A., & Thompson, R. H. S. (1946) *Biochem. J. 40*, 529–554.

Teale, F. W., & Weber, G. (1975) *Biochem. J. 65*, 476–482.

Tsao, D. H. H., Casas-Finet, J. R., Maki, A. H., & Chase, J. W. (1989) *Biophys. J. 55*, 927–936.

von Schütz, J. U., Zuclich, J., & Maki, A. H. (1976) *J. Am. Chem. Soc. 96*, 714–719.

Weinzierl, G., & Friedrich, J. (1981) *Chem. Phys. Lett. 80*, 55–59.

Williamson, R. L., & Kwiram, A. L. (1988) *J. Chem. Phys. 88*, 6092–6106.

Winscom, C. J., & Maki, A. H. (1971) *Chem. Phys. Lett. 12*, 264–268.

Zang, L.-H., Ghosh, S., & Maki, A. H. (1988) *Biochemistry 27*, 7820–7825.

Zuclich, J., von Schütz, J. U., & Maki, A. H. (1974) *Mol. Phys. 28*, 33–47.