Modeling the DNA Methylphosphotriester Repair Site in *Escherichia coli* Ada. Why Zinc and Four Cysteines?

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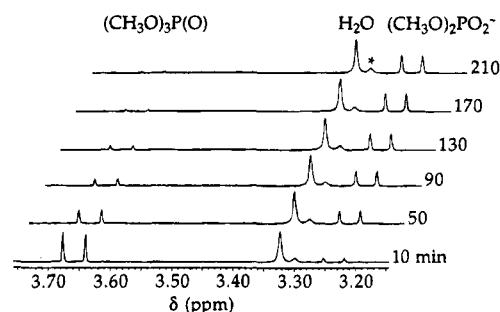Nonenzymatic alkylating agents often create DNA lesions with toxic, mutagenic, and carcinogenic consequences.[1,2] To defend against these adducts, nearly all organisms possess proteins that repair alkylated DNA.[1,2] *Escherichia coli* employs the Ada protein both to repair DNA alkylation products[3-6] and to regulate the transcription of genes which encode for DNA alkylation repair proteins.[7,8] The primary lesions repaired by Ada are O^6-methylguanine, O^4-methylthymine, and the S_p diastereomer of methylphosphotriesters.[4-6] Ada removes the offending methyl groups by direct, stoichiometric, and irreversible transfer to specific cysteine residues in the protein.[3-5] In particular, Cys_{321} is responsible for repair of the base adducts[4] and Cys_{69} repairs the methylphosphotriesters.[3] Cys_{69} is one of four cysteines tightly bound to a zinc ion[9-13] essential for protein activity.[13]

From an inorganic perspective, we are interested in the role of the $[Zn(S\text{-cysteine})_4]^{2-}$ site. Why is the active Cys_{69} residue coordinated to zinc? Why is zinc ligated by four cysteines? How does zinc modulate the nucleophilicity of the coordinated thiolate compared to that of the free thiol? We have begun to address these questions through the use of small-molecule model chemistry. In this initial study, we report the use of $[(CH_3)_4N]_2$-$[Zn(SC_6H_5)_4]$[14] to represent the $[Zn(S\text{-cys})_4]^{2-}$ site of Ada and $(CH_3O)_3P(O)$ to mimic the DNA methylphosphotriester lesion. The new compounds $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$ (MeIm = 1-methylimidazole) and $[Zn(SC_6H_5)_2(MeIm)_2]$ were prepared to model the behavior of alternative $[Zn(S\text{-cys})_3(N\text{-histidine})]^-$ and $[Zn(S\text{-cys})_2(N\text{-his})_2]$ sites. In this manner, we discovered a trend in reactivity among the zinc compounds which may provide insight into the regulation of thiol reactivity by metal coordination.

The 1:1 reaction of $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ and $(CH_3O)_3P$-(O) in DMSO-d_6 yields quantitatively $(CH_3O)_2PO_2^-$ and CH_3-SC_6H_5 as indicated by ${}^1H$ NMR spectroscopy. This reaction is similar to DNA methylphosphotriester repair by Ada in that a phosphotriester and a zinc thiolate are converted to a phos-

Figure 1. ${}^1H$ NMR spectral changes used to follow the reaction of $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ with $(CH_3O)_3P(O)$ in DMSO-d_6 under pseudo-first-order conditions with $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ in excess. The asterisk denotes a signal arising from ${}^{13}C$–H coupling in the $(CH_3)_4N^+$ counterion.

phodiester and a methyl thioether. Proton chemical shifts of the resulting $CH_3SC_6H_5$ are identical to those of a genuine sample, indicating that the thioether product is not coordinated to zinc. This result differs from that obtained for the zinc[9] and cadmium[10] forms of Ada in which, after methylphosphotriester repair, the $S\text{-MeCys}_{69}$ reaction product is coordinated to the metal. In the model chemistry, $(CH_3O)_2PO_2^-$ is at least partially coordinated, as evidenced by a broad peak ($\Delta\nu_{1/2} = 60$ Hz) in the ${}^{31}P$ NMR spectrum which is shifted downfield by 0.6 ppm relative to the narrow ($\Delta\nu_{1/2} = 5.1$ Hz) ${}^{31}P$ resonance of a genuine sample of $NH_4[(CH_3O)_2PO_2]$ at the same concentration.

Kinetic studies of the $(CH_3O)_3P(O)$ demethylation by $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ (Figure 1) reveal a second-order rate constant of $(1.6 \pm 0.3) \times 10^{-2}$ M^{-1} s^{-1} at 24.8 (± 0.3) °C as measured by ${}^1H$ NMR spectroscopy in DMSO-d_6.[15] In buffered aqueous solution at 4 °C, Ada repair of a single-stranded DNA methylphosphotriester lesion occurs with a second-order rate constant of 2.8×10^2 M^{-1} s^{-1}.[16] This large difference in absolute rate constants is not unexpected given the usual ability of proteins to operate more efficiently than biomimetic model compounds. In addition, aromatic thiolates used in the present, first-generation zinc complexes are expected to have decreased nucleophilicity relative to the aliphatic cysteine thiolates in Ada.

We were interested to explore whether a zinc thiolate with a ligand environment other than $[Zn(S\text{-cys})_4]^{2-}$ could also demethylate phosphotriesters. To address this question, the complexes $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$[17] and $[Zn(SC_6H_5)_2$-$(MeIm)_2]$[18] were synthesized and characterized by ${}^1H$ NMR,

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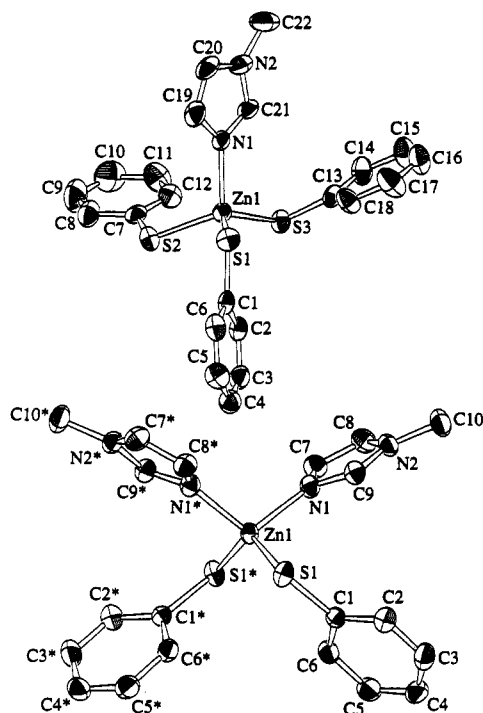(14) Dance, I. G.; Choy, A.; Scudder, M. L. *J. Am. Chem. Soc.* **1984,** *106,* 6285–6295.

(15) Experiments were carried out under pseudo-first-order conditions with $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ in excess. Conductivity and kinetic studies indicated significant ion pairing of $[Zn(SC_6H_5)_4]^{2-}$ with $(CH_3)_4N^+$ in DMSO solution. To obtain the second-order rate constant, k_2, data were analyzed with the use of a model in which $[Zn(SC_6H_5)_4]^{2-}$ reacts with $(CH_3O)_3P(O)$ but the ion-paired species $\{[(CH_3)_4N][Zn(SC_6H_5)_4]\}^-$ does not. The k_2 value was obtained as the inverse of the ordinate intercept of a plot of $[Zn_{total}]/k_{obs}$ versus $[(CH_3)_4N^+]_{free}$, where $[(CH_3)_4N^+]_{free}$ refers to the concentration of $(CH_3)_4N^+$ not involved in ion pairing, $[Zn]_{total}$ is the concentration of added zinc complex, and k_{obs} is the observed pseudo-first-order rate constant. Details will be reported elsewhere.

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(17) Anhydrous $ZnCl_2$ (27.5 mmol) was dissolved in a methanol (55 mL) solution of C_6H_5SH (83.1 mmol) and Li metal (83.1 mmol). After 3 h of stirring, MeIm (27.9 mmol) was added and stirring was continued for 2.5 h. Crystalline $(CH_3)_4NCl$ (27.8 mmol) was dissolved in the reaction solution, and n-C_4H_9OH (50 mL) was added over 25 min. The solution was stored at -20 °C overnight. Crystals (23.9 mmol, 87%) were collected by filtration, washed with n-C_4H_9OH, and dried in vacuo. ${}^1H$ NMR (DMSO-d_6): δ 3.06 (s, 12 H, $(CH_3)_4N^+$), 3.61 (s, 3 H, CH_3), 6.71 (t, 3 H, p-H), 6.84 (s, 1 H, imid H-5), 6.85 (t, 6 H, m-H), 7.11 (s, 1 H, imid H-4), 7.37 (d, 6 H, o-H), 7.60 (s, 1 H, imid H-2). Anal. Calcd for $C_{26}H_{33}N_3S_3Zn$: C, 56.87; H, 6.06; N, 7.65. Found: C, 56.74; H, 6.03; N, 7.58. Crystal data for $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$ $(C_{26}H_{33}N_3S_3Zn, M_r = 549.16)$ at 199 K: size ca. $0.22 \times 0.23 \times 0.37$ mm, space group $P2_1/n$, $a = 10.330(5)$ Å, $b = 25.758(2)$ Å, $c = 10.580(1)$ Å, $\beta = 102.00(1)°$, $V = 2754(2)$ Å^3, $Z = 4$. For 2574 unique, observed reflections with $F^2 > 3\sigma(F^2)$ and 298 variable parameters, the final discrepancy indices were $R = 0.048$ and $R_w = 0.055$.

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Figure 2. ORTEP diagrams of the [Zn(SC$_6$H$_5$)$_3$(MeIm)]$^-$ anion (top) and [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$] (bottom) showing the 50% probability thermal ellipsoids for all non-hydrogen atoms. Atoms related by a C_2 axis are designated with an asterisk. Selected interatomic distances (Å) and angles (deg) are as follows. [Zn(SC$_6$H$_5$)$_3$(MeIm)]$^-$: Zn$-$S1, 2.316-(2); Zn$-$S2, 2.321(2); Zn$-$S3, 2.314(2); Zn$-$N1, 2.072(5); S1$-$Zn$-$S2, 106.56(7); S1$-$Zn$-$S3, 124.14(7); S2$-$Zn$-$S3, 110.75(7); S1$-$Zn$-$N1, 99.3(2); S2$-$Zn$-$N1, 114.4(1); S3$-$Zn$-$N1, 103.8(2). [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$]: Zn$-$S1, 2.2916(7); Zn$-$N1, 2.038(2); S1$-$Zn$-$S1*, 128.47(4); S1$-$Zn$-$N1, 106.61(7); S1$-$Zn$-$N1*, 102.38(7); N1$-$Zn$-$N1*, 109.8(1).

elemental analysis, and single-crystal X-ray diffraction methods (Figure 2). Both (CH$_3$)$_4$N[Zn(SC$_6$H$_5$)$_3$(MeIm)] and [Zn(SC$_6$H$_5$)$_2$-(MeIm)$_2$] react with (CH$_3$O)$_3$P(O) to yield (CH$_3$O)$_2$PO$_2$$^-$ and CH$_3$SC$_6$H$_5$. It is therefore conceivable that [Zn(S-cys)$_3$(N-his)]$^-$ and [Zn(S-cys)$_2$(N-his)$_2$] sites would also be capable of meth-ylphosphotriester repair. Kinetic examination of these reactions, however, revealed that the three zinc compounds have markedly different reactivities toward (CH$_3$O)$_3$P(O) (Table 1). The tetrathiolate [Zn(SC$_6$H$_5$)$_4$]$^{2-}$ ion has a much larger rate constant than [Zn(SC$_6$H$_5$)$_3$(MeIm)]$^-$, which in turn, reacts significantly more rapidly than the neutral molecule [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$]. From these results, we conclude that, although alternative [Zn-(S-cys)$_3$(N-his)]$^-$ and [Zn(S-cys)$_2$(N-his)$_2$] sites could repair al-kylated phosphates, the [Zn(S-cys)$_4$]$^{2-}$ center will do so with

Table 1. Kinetic Rate Constants for Reactions of Benzenethiolate and its Zinc Complexes with (CH$_3$O)$_3$P(O)^a

compound	k (s^{-1})
[(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$]	$(8 \pm 2) \times 10^{-5}$
(CH$_3$)$_4$N[Zn(SC$_6$H$_5$)$_3$(MeIm)]	$(6 \pm 2) \times 10^{-6}$
[Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$]	$\leq 5 \times 10^{-8}$
(CH$_3$)$_4$N(SC$_6$H$_5$)	$(1.1 \pm 0.4) \times 10^{-4}$

^{a} Reactions were carried out under pseudo-first-order conditions with zinc or tetramethylammonium thiolate in excess at 5 mM and (CH$_3$O)$_3$P(O) at 1 mM. Experiments with [(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$] concentrations up to 20 times that of (CH$_3$O)$_3$P(O) exhibited similar pseudo-first-order behavior. Kinetics were monitored by ^{1}H NMR spectroscopy in DMSO-d_6 at 24.3 ($\pm$0.4) °C. Errors are upper and lower bounds estimated from triplicate repeats of the experiments.

the highest rate constant. The extremely slow reaction of [Zn-(SC$_6$H$_5$)$_2$(MeIm)$_2$] and (CH$_3$O)$_3$P(O) suggests, in particular, that [Zn(S-cys)$_2$(N-his)$_2$], the prototypical zinc finger motif, is a poor general nucleophile. This lack of reactivity may help to explain the preponderance of [Zn(S-cys)$_2$(N-his)$_2$] sites employed for structural purposes in many protein systems.[19–21]

To explore further the question of why Cys$_{69}$ in Ada is coordinated to zinc, we compared the reactivities of [(CH$_3$)$_4$N]$_2$-[Zn(SC$_6$H$_5$)$_4$], C$_6$H$_5$SH, and (CH$_3$)$_4$N(SC$_6$H$_5$). The last reacts with (CH$_3$O)$_3$P(O) to yield (CH$_3$O)$_2$PO$_2$$^-$ and CH$_3$SC$_6$H$_5$. A kinetic study of this reaction (Table 1) provides a rate constant similar to that of [(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$].[22] Benzenethiol, C$_6$H$_5$SH, however, does not react with (CH$_3$O)$_3$P(O) in DMSO-d_6 even after 1 month. This result clearly demonstrates that protonation and metalation can differ dramatically in their ability to regulate the nucleophilicity of a thiolate. Perhaps coordina-tion of Cys$_{69}$ to a zinc center in Ada has evolved for the purpose of sustaining the nucleophilicity of Cys$_{69}$ under physiological conditions.

In summary, [(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$], (CH$_3$)$_4$N[Zn(SC$_6$H$_5$)$_3$-(MeIm)], [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$], and (CH$_3$)$_4$N(SC$_6$H$_5$) all react with (CH$_3$O)$_3$P(O) to yield (CH$_3$O)$_2$PO$_2$$^-$ and CH$_3$SC$_6$H$_5$. Kinetic analysis reveals the following series of rate constants: [(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$] $\approx$ (CH$_3$)$_4$N(SC$_6$H$_5$) $>$ (CH$_3$)$_4$N[Zn-(SC$_6$H$_5$)$_3$(MeIm)] $>$ [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$]. We conclude that [Zn(S-cys)$_4$]$^{2-}$ is the optimum zinc center for DNA methylphos-photriester repair and that the thiolate center in the [Zn(S-cys)$_2$-(N-his)$_2$] zinc finger motif has been deactivated, making it better suited for a structural rather than a functional role. Our model chemistry further suggests that coordination of Cys$_{69}$ to the {Zn-(S-cys)$_3$}$^-$ moiety in Ada maintains its nucleophilicity and hence its ability to function in DNA methylphosphotriester repair.

(18) Preparation of [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$] was by modification of a literature procedure for {Zn[S-2,3,5,6-(CH$_3$)$_4$C$_6$H]$_2$(MeIm)$_2$}. (Corwin, D. T., Jr.; Koch, S. A. *Inorg. Chem.* **1988**, *27*, 493−496.) Anhydrous ZnCl$_2$ (13.6 mmol) was added to an ethanol (250 mL) solution of C$_6$H$_5$SH (27.7 mmol) and Li metal (31.1 mmol). After 2 h of stirring, MeIm (37.6 mmol) was added and stirring was continued for 15 min. Ethanol was removed in vacuo to yield a white, dry solid. Addition of toluene (250 mL) and refluxing for 2 h resulted in a cloudy solution, which was filtered while hot. Slow cooling afforded colorless crystals, which were collected by filtration and dried in vacuo. The filtrate was stored at −20 °C overnight to yield a second crop of crystals, which were collected by filtration and dried in vacuo. Total yield was 11.1 mmol, 82%. ^{1}H NMR (DMSO-d_6): δ 3.66 (s, 6 H, CH$_3$), 6.80 (t, 2 H, p-H), 6.87 (s, 2 H, imid H-5), 6.92 (t, 4 H, m-H), 7.20 (s, 2 H, imid H-4), 7.29 (d, 4 H, o-H), 7.73 (s, 2 H, imid H-2). Anal. Calcd for C$_{20}$H$_{22}$N$_4$S$_2$Zn: C, 53.63; H, 4.95; N, 12.51. Found: C, 54.02; H, 4.91; N, 12.56. Crystal data for [Zn(SC$_6$H$_5$)$_2$(MeIm)$_2$] (C$_{20}$H$_{22}$N$_4$S$_2$Zn, M_r = 447.95) at 199 K: size ca. 0.25 × 0.28 × 0. 32 mm, space group $C2/c$, a = 12.372-(2) Å, b = 18.703(2) Å, c = 9.329(2) Å, β = 100.60(2)°, V = 2121.7(6) Å^3, Z = 4. For 1894 unique, observed reflections with $F^2 > 3\sigma(F^2)$ and 123 variable parameters, the final discrepancy indices were R = 0.036 and R_w = 0.040.

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(22) Our experiments do not address the formal possibility that ben-zenethiolate dissociated from [(CH$_3$)$_4$N]$_2$[Zn(SC$_6$H$_5$)$_4$] might be the species reactive toward (CH$_3$O)$_3$P(O). If such were the case, it would not alter our conclusions.