# 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 $CH_3SC_6H_5$ 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 phosphodiester 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*-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.

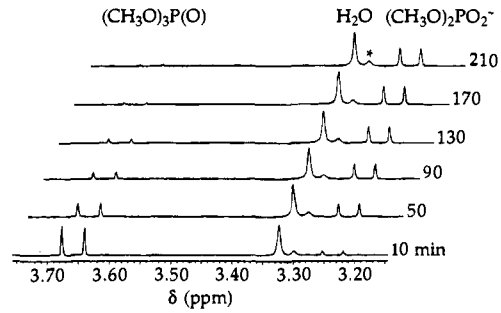


**Figure 1.** $^1H$ NMR spectral changes used to follow the reaction of $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ with $(CH_3O)_3P(O)$ in DMSO-$d_6$ under pseudo-first-order conditions with $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ in excess. The asterisk denotes a signal arising from $^{13}C-H$ coupling in the $(CH_3)_4N^+$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

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 $2.8 \times 10^2$ $M^{-1}$ $s^{-1}$.[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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(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-$d_6$): $\delta$ 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 × 0.23 × 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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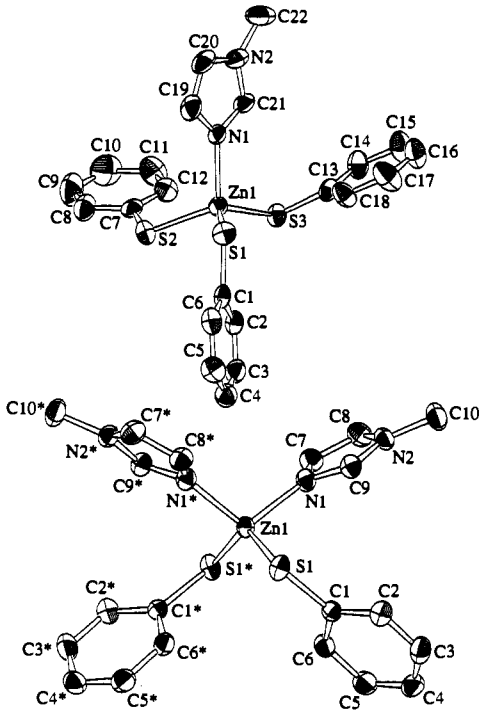


**Figure 2.** ORTEP diagrams of the $[Zn(SC_6H_5)_3(MeIm)]^-$ anion (top) and $[Zn(SC_6H_5)_2(MeIm)_2]$ (bottom) showing the 50% probability thermal ellipsoids for all non-hydrogen atoms. Atoms related by a $C_2$ axis are designated with an asterisk. Selected interatomic distances (Å) and angles (deg) are as follows. $[Zn(SC_6H_5)_3(MeIm)]^-$: 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). $[Zn(SC_6H_5)_2(MeIm)_2]$: 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 $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$ and $[Zn(SC_6H_5)_2(MeIm)_2]$ react with $(CH_3O)_3P(O)$ to yield $(CH_3O)_2PO_2^-$ and $CH_3SC_6H_5$. It is therefore conceivable that $[Zn(S\text{-cys})_3(N\text{-his})]^-$ and $[Zn(S\text{-cys})_2(N\text{-his})_2]$ sites would also be capable of methylphosphotriester repair. Kinetic examination of these reactions, however, revealed that the three zinc compounds have markedly different reactivities toward $(CH_3O)_3P(O)$ (Table 1). The tetrathiolate $[Zn(SC_6H_5)_4]^{2-}$ ion has a much larger rate constant than $[Zn(SC_6H_5)_3(MeIm)]^-$, which in turn, reacts significantly more rapidly than the neutral molecule $[Zn(SC_6H_5)_2(MeIm)_2]$. From these results, we conclude that, although alternative $[Zn(S\text{-cys})_3(N\text{-his})]^-$ and $[Zn(S\text{-cys})_2(N\text{-his})_2]$ sites could repair alkylated phosphates, the $[Zn(S\text{-cys})_4]^{2-}$ center will do so with the highest rate constant. The extremely slow reaction of $[Zn(SC_6H_5)_2(MeIm)_2]$ and $(CH_3O)_3P(O)$ suggests, in particular, that $[Zn(S\text{-cys})_2(N\text{-his})_2]$, the prototypical zinc finger motif, is a poor general nucleophile. This lack of reactivity may help to explain the preponderance of $[Zn(S\text{-cys})_2(N\text{-his})_2]$ sites employed for structural purposes in many protein systems.[19-21]

**Table 1.** Kinetic Rate Constants for Reactions of Benzenethiolate and its Zinc Complexes with $(CH_3O)_3P(O)$[a]

| compound | $k$ ($s^{-1}$) |
|---|---|
| $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ | $(8 \pm 2) \times 10^{-5}$ |
| $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$ | $(6 \pm 2) \times 10^{-6}$ |
| $[Zn(SC_6H_5)_2(MeIm)_2]$ | $\leq 5 \times 10^{-8}$ |
| $(CH_3)_4N(SC_6H_5)$ | $(1.1 \pm 0.4) \times 10^{-4}$ |

[a] Reactions were carried out under pseudo-first-order conditions with zinc or tetramethylammonium thiolate in excess at 5 mM and $(CH_3O)_3P(O)$ at 1 mM. Experiments with $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ concentrations up to 20 times that of $(CH_3O)_3P(O)$ exhibited similar pseudo-first-order behavior. Kinetics were monitored by $^1H$ NMR spectroscopy in DMSO-$d_6$ at 24.3 (±0.4) °C. Errors are upper and lower bounds estimated from triplicate repeats of the experiments.

To explore further the question of why $Cys_{69}$ in Ada is coordinated to zinc, we compared the reactivities of $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$, $C_6H_5SH$, and $(CH_3)_4N(SC_6H_5)$. The last reacts with $(CH_3O)_3P(O)$ to yield $(CH_3O)_2PO_2^-$ and $CH_3SC_6H_5$. A kinetic study of this reaction (Table 1) provides a rate constant similar to that of $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$.[22] Benzenethiol, $C_6H_5SH$, however, does not react with $(CH_3O)_3P(O)$ 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 coordination 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, $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$, $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$, $[Zn(SC_6H_5)_2(MeIm)_2]$, and $(CH_3)_4N(SC_6H_5)$ all react with $(CH_3O)_3P(O)$ to yield $(CH_3O)_2PO_2^-$ and $CH_3SC_6H_5$. Kinetic analysis reveals the following series of rate constants: $[(CH_3)_4N]_2[Zn(SC_6H_5)_4] \approx (CH_3)_4N(SC_6H_5) > (CH_3)_4N[Zn(SC_6H_5)_3(MeIm)] > [Zn(SC_6H_5)_2(MeIm)_2]$. We conclude that $[Zn(S\text{-cys})_4]^{2-}$ is the optimum zinc center for DNA methylphosphotriester repair and that the thiolate center in the $[Zn(S\text{-cys})_2(N\text{-his})_2]$ 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 $\{Zn(S\text{-cys})_3\}^-$ moiety in Ada maintains its nucleophilicity and hence its ability to function in DNA methylphosphotriester repair.

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**Supporting Information Available:** Tables of crystallographic data including atomic positional and thermal parameters for $(CH_3)_4N[Zn(SC_6H_5)_3(MeIm)]$ and $[Zn(SC_6H_5)_2(MeIm)_2]$ (12 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(18) Preparation of $[Zn(SC_6H_5)_2(MeIm)_2]$ was by modification of a literature procedure for $\{Zn[S\text{-}2,3,5,6\text{-}(CH_3)_4C_6H]_2(MeIm)_2\}$. (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 $C_6H_5SH$ (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%. $^1H$ NMR (DMSO-$d_6$): $\delta$ 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 $C_{20}H_{22}N_4S_2Zn$: C, 53.63; H, 4.95; N, 12.51. Found: C, 54.02; H, 4.91; N, 12.56. Crystal data for $[Zn(SC_6H_5)_2(MeIm)_2]$ ($C_{20}H_{22}N_4S_2Zn$, $M_r = 447.95$) 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, $\beta = 100.60(2)°$, $V = 2121.7(6)$ 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 benzenethiolate dissociated from $[(CH_3)_4N]_2[Zn(SC_6H_5)_4]$ might be the species reactive toward $(CH_3O)_3P(O)$. If such were the case, it would not alter our conclusions.