Functional Evidence That the 3′-5′ Exonuclease Domain of *Escherichia coli* DNA Polymerase I Employs a Divalent Metal Ion in Leaving Group Stabilization

Jessica F. Curley,[†] Catherine M. Joyce,[‡] and Joseph A. Piccirilli*,[†,§]

Departments of Biochemistry and Molecular Biology and Chemistry, Howard Hughes Medical Institute University of Chicago, Chicago, Illinois 60637 Department of Molecular Biophysics and Biochemistry Yale University, New Haven, Connecticut 06520

Received June 18, 1997

Phosphoryl transfer reactions are ubiquitous in biology, and the macromolecules that catalyze them often require divalent metal ions for tertiary structure or catalytic activity or both. Determining the role of the metal ions in catalysis is a difficult challenge in mechanistic enzymology, even with the aid of crystallographic studies. Sulfur substitution of the oxygens on the reactive phosphoryl group provides a means to test metal ion−phosphoryl oxygen interactions because some divalent metals such as Mn^{2+} readily accept sulfur as a ligand, whereas others such as Mg^{2+} do not. Thus, a shift in metal specificity from Mg^{2+} toward Mn^{2+} following sulfur substitution provides strong evidence for a direct metal ion interaction with the substituted oxygen atom.[1] Metal−ligand specificity was first used two decades ago to determine the screw sense specificity of enzymes that use metal complexes of adenine nucleotides.[1d] However, only recently has it been exploited to reveal the catalytic principle of leaving group stabilization in enzymatic phosphoester transfer reactions.[2] Although this approach has become popular in recent years, particularly as a tool for identifying metal ligands on protein and RNA enzymes,[3] some of the conclusions derived from these analyses have been controversial (refs 3f and 4−7). It is therefore necessary to establish the efficacy of the approach by investigating an enzyme for which independent structural evidence exists in favor of a catalytic role for metal ions.[8] Thus, we applied the metal specificity switch approach to test the role of a metal ion in leaving group stabilization at the structurally defined 3′-5′ exonuclease site of the large proteolytic fragment (Klenow fragment) of *Escherichia coli* DNA polymerase I (Kf).[8]

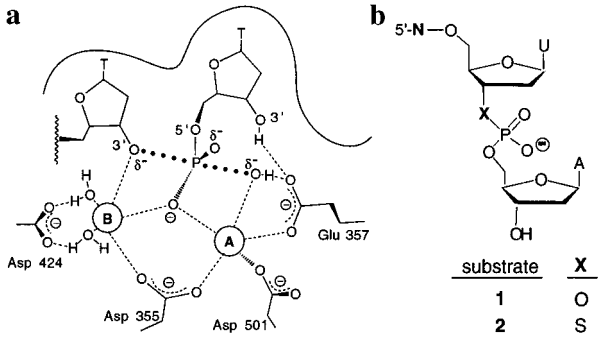

Figure 1. (a) The proposed transition state for phosphoryl transfer in the 3′-5′ exonuclease site of Kf. (b) The single-stranded oligonucleotide substrates used in this work. Compound **1** contains all normal phosphodiester linkages, and **2** contains a single 3′-*S*-phosphorothiolate diester linkage[2,17] (**N** = 5′-dCCCUC). Substrates contained 2′-deoxyuridine because of the availability of the 3′-*S*-thiophosphoramidite.[2]

In the Kf exonuclease reaction, the 3′ terminal phosphodiester linkage of a DNA oligonucleotide is cleaved by attack of water or hydroxide ion, yielding dNMP and a shortened oligonucleotide ending with a 3′ hydroxyl. The most prominent structural feature of the exonuclease site is a binuclear metal center that is proposed to mediate phosphoryl transfer (Figure 1a). In enzyme−product (dNMP) complexes, a pentacoordinate metal (A) shares a ligand, Asp-355, with an octahedral metal (B).[8b,c] Superposition of wild-type structures bound with product onto mutant enzyme structures (lacking metal ion B) bound with oligonucleotide substrate[8b,c,9] places the 3′ oxygen atom (the leaving group) of the substrate within the inner coordination sphere of metal ion B (2.4 Å).[8b] Therefore, metal ion B is proposed to interact directly with the 3′ oxygen atom in the transition state, presumably stabilizing the developing negative charge on the oxyanion leaving group. Although the two-metal-ion mechanism of Kf is thought to be a general strategy by which many protein enzymes and ribozymes catalyze phosphoryl transfer,[8a,10] there is no direct biochemical evidence that the 3′-5′ exonuclease employs a metal ion in this role.

To investigate the proposed interaction, we employed a substrate **2** containing a 3′-*S*-phosphorothiolate diester linkage (Figure 1b), in which the 3′ bridging oxygen atom of the first phosphorothiolate linkage encountered by the exonuclease was replaced by a sulfur atom. We measured rates for cleavage of **1** and **2** as 5′-radiolabeled, single-stranded substrates with Kf in excess over substrate and with either Mg^{2+} or Mn^{2+} as the only divalent cation. The rate of disappearance of substrate (7 mer) represents the rate of cleavage of the first phosphodiester linkage. Plots of k_{obs} were linear with Kf concentration (see the Supporting Information); the slopes of these lines therefore represent second-order rate constants for reactions of enzyme and substrate and are shown as k_{cat}/K_M values in Table 1.

The phosphorothiolate linkage of **2** reacted extremely slowly in Mg^{2+} compared to the normal phosphodiester linkage in **1**, requiring ∼200-fold higher concentrations of Kf to enhance cleavage above background. The thio effect, defined as the ratio of specificity constants $(k_{cat}/K_M)_1^{Mg^{2+}}/(k_{cat}/K_M)_2^{Mg^{2+}}$, was 50 000 (Table 1) and indicates that in Mg^{2+} the enzyme is highly specific for the normal phosphodiester linkage. When Mn^{2+} replaced Mg^{2+}, k_{cat}/K_M for **1** increased by a factor of nearly 740 $[(k_{cat}/K_M)_1^{Mn^{2+}}/(k_{cat}/K_M)_1^{Mg^{2+}}]$. Multiple turnover experi-

† Department of Chemistry, University of Chicago.
‡ Yale University.
§ Department of Biochemistry and Molecular Biology, University of Chicago.

(1) (a) Sigel, R. K. O.; Song, B.; Sigel, H. *J. Am. Chem. Soc.* **1997**, *119*, 744−755. (b) Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262−5271. (c) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991**, *30*, 4844−4854. (d) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1978**, *253*, 4823−4825.

(2) Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85−88.

(3) (a) Chen, Y.; Li, X.; Gegenheimer, P. *Biochemistry* **1997**, *36*, 2425−2438. (b) Sarnovsky, R. J.; May, E. W.; Craig, N. L. *EMBO J.* **1996**, *15*, 6348−6361. (c) Warnecke, J. M.; Furste, J. P.; Hardt, W.-D.; Erdmann, V. A.; Hartmann, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8924−8928. (d) Harris, M. E.; Pace, N. R. *RNA* **1995**, *1*, 210−218. (e) Christian, E. L.; Yarus, M. *Biochemistry* **1993**, *32*, 4475−4480. (f) Dahm, S. C.; Uhlenbeck, O. C. *Biochemistry* **1991**, *30*, 9464−9469. (g) Burgers, P. M. J.; Eckstein, F. *J. Biol. Chem.* **1979**, *254*, 6889−6893.

(4) Kuimelis, R. G.; McLaughlin, L. W. *Biochemistry* **1996**, *35*, 5308−5317. Kuimelis, R. G.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1995**, *117*, 11019−11020.

(5) Zhou, D.-M.; Usman, N.; Wincott, F. E.; Matulic-Adamic, J.; Orita, M.; Zhang, L.-H.; Komiyama, M.; Kumar, P. K. R.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 5862−5866.

(6) Pontius, B. W.; Lott, W. B.; von Hippel, P. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2290−2294.

(7) Zhou, D.-M.; Kumar, P. K. R.; Zhang, L.-H.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 8969−8970.

(8) (a) Steitz, T. A.; Steitz, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6498−6502. (b) Beese, L. S.; Steitz, T. A. *EMBO J.* **1991**, *10*, 25−33. (c) Derbyshire, V.; Freemont, P. S.; Sanderson, M. R.; Beese, L.; Friedman, J. M.; Joyce, C. M.; Steitz, T. A. *Science* **1988**, *240*, 199−201.

(9) Freemont, P. S.; Friedman, J. M.; Beese, L. S.; Sanderson, M. R.; Steitz, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8924−8928.

(10) Venclovas, C.; Siksnys, V. *Nat. Struct. Biol.* **1995**, *2*, 838−841. Grindley, N. D. F.; Leschziner, A. E. *Cell* **1995**, *83*, 1063−1066. Joyce, C. M.; Steitz, T. A. *Annu. Rev. Biochem.* **1994**, *63*, 777−822.

S0002-7863(97)02014-3 CCC: $14.00

Table 1. Rate Constants for Cleavage of DNA Substrates with Mg^{2+} or Mn^{2+} [a]

substrate	k_{cat}/K_M (M^{-1} s^{-1})	
	Mg^{2+}	Mn^{2+}
1	6.4×10^3	4.7×10^6
2	0.13[b]	7.7×10^3
thio effect (**1**/**2**)	50 000	610
relative thio effect	80	1

[a] Reactions at 37 °C with 50 mM Tris-HCl (pH 7.6), 8 mM metal dichloride, 10 mg/mL bovine serum albumin (BSA), and 5 mM DTT; reaction profiles were obtained to ensure metal and BSA concentrations were optimal. [b] Because the reaction of **2** with Mg^{2+} is so slow, this rate has been corrected by subtracting the rates of background degradation obtained from control reactions without Kf.

ments with substrate in excess of enzyme revealed that this is largely an effect on K_M (data not shown). Kf-catalyzed cleavage of **2** increased even more dramatically when Mn^{2+} replaced Mg^{2+} (~60 000-fold) and was readily observed at nanomolar enzyme concentrations. The thio effect $[(k_{cat}/K_M)_{\mathbf{1}}^{Mn^{2+}}/(k_{cat}/K_M)_{\mathbf{2}}^{Mn^{2+}}]$ was 610, reduced from 50 000 in Mg^{2+} and indicates that the specificity of the enzyme for the normal substrate vs the modified substrate is reduced in Mn^{2+} compared to Mg^{2+}.[11] In nonenzymatic reactions, the ability of divalent metal ions to catalyze cleavage of a phosphorothiolate linkage correlates with the thiophilicity of the metal ion.[12] Considering that Mn^{2+} coordinates to sulfur more effectively than Mg^{2+} in complexes with ATPβS[1b] or AMPS,[1a] the ~80-fold rescue of the thio effect by Mn^{2+} in the exonuclease reaction strongly supports the proposal that metal ion B interacts directly with the leaving group.[13,14] Additionally, the thio effects were similar (within error) at all pH values tested (Figure 2), showing that the Mn^{2+} rescue is not fortuitous at a given pH.

To our knowledge, our results provide the first functional evidence that protein enzymes use metal ions to stabilize oxyanion leaving groups. The corroboration of the biochemical analysis presented here with the crystallographic results[8,9] establishes the metal specificity approach as a viable means of exploring the role of metal ions in biological catalysis. Furthermore, this study reinforces conclusions that often must be deduced in the absence of independent structural data. Metal specificity experiments have implicated a metal ion in leaving group stabilization for self-splicing by the *Tetrahymena* ribozyme[2,15] and for nuclear premessenger RNA splicing by the spliceosome.[16] Thus, it appears that this catalytic strategy may be used by protein, RNA, and ribonucleoprotein enzymes.

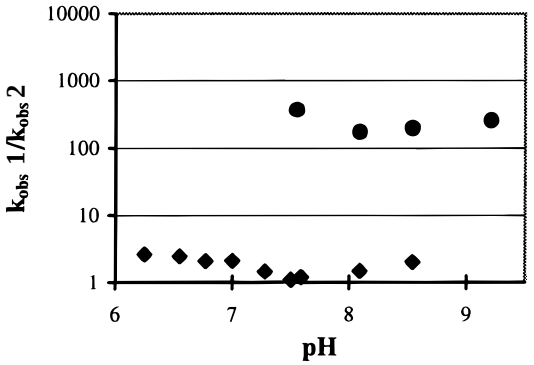

Figure 2. The pH dependence of the thio effect for a given metal under k_{cat}/K_M conditions (● Mg^{2+}, ♦ Mn^{2+}).

Acknowledgment. We thank L. Munishkina and S. Sun for oligonucleotide synthesis, members of the Piccirilli lab for advice and discussions, and Drs. D. Lynn, T. Pan, E. Sontheimer, and the reviewers for comments on the manuscript. We also thank Chad Brautigam and Tom Steitz for permission to cite unpublished results. C.M.J. acknowledges support from NIH Grant GM-28550 and thanks X. C. Sun for help with enzyme purification. JFC was supported in part by a GAANN fellowship and by the Molecular and Cell Biology (2T32GM07183) NIH training grant; J.A.P. is an Assistant Investigator of the Howard Hughes Medical Institute.

Supporting Information Available: Experimental details of enzyme isolation, product identification, and kinetic assays (4 pages). See any current masthead page for ordering and Internet access instructions.

JA972014J

(11) (a) We also examined cleavage rates of a pair of longer oligonucleotide substrates, *dTGCGTCCGGCGTCCU$_X$A, where X represents a 3′O or 3′S phosphate ester linkage. The k_{cat}/K_M values for these substrates were significantly greater than those for **1** and **2**, presumably due to the tighter binding and decreased K_M of the longer substrates[11b] compared to those of **1** and **2**. However, we still observed a rescue of the thio effect by Mn^{2+}. (b) Derbyshire, V.; Grindley, N. D.; Joyce, C. M. *EMBO J.* **1991**, *10*, 17−24.

(12) Kuimelis, R. G.; McLaughlin, L. W. *Nucleic Acids Res.* **1995**, *23*, 4753−4760.

(13) The use of metal specificity experiments to infer a Mg^{2+}−oxygen interaction assumes that Mg^{2+} occupies the same binding site as Mn^{2+}. The results of competition experiments were consistent with this assumption. At different concentrations of Mn^{2+} (0.1 or 0.2 mM), increasing Mg^{2+} (0−12 mM) inhibited the reaction of the 3′S substrate relative to the unmodified substrate. See also ref 14.

(14) (a) Two important conditions for this interpretation are (1) the thio effects are due to differences in chemical interactions of the metal ions with sulfur and oxygen rather than structural perturbations that could arise from different binding modes for each metal ion and substrate and (2) the chemical step is being monitored in the kinetic assays. Crystallographic data reveal very little structural perturbation of the ES complex when Mg^{2+} replaces Mn^{2+} in site B or when sulfur replaces oxygen (C. A. Brautigam, S. Sun, J.A.P., T. A. Steitz, manuscript in preparation), even though the S−P bond is longer than the O−P bond by ~0.45 Å.[14b] These results suggest that both substrates react via the same catalytic mechanism with either metal ion. Although it is not possible to rule out that a conformational change limits the exonuclease reaction rate, the reaction of **1** in Mg^{2+} displays both a log linear dependence on pH (with slope = 1, data not shown) and a rate diminuation when the nonbridging oxygen is substituted with sulfur (J.F.C., J.A.P., unpublished results).[1c] These data suggest that chemistry is rate limiting for k_{cat}/K_M. Substrate binding is not likely to be rate limiting because the values reported in Table 1 are far below that for diffusion-controlled binding, ~10^8 M^{-1} s^{-1}.[14c] In addition, a longer oligonucleotide[11] reacted in the presence of Mn^{2+} with $k_{cat}/K_M = 4 \times 10^8$ M^{-1} s^{-1}, close to the diffusion controlled limit. This dramatic increase in rate would not be observed if the reaction of **1** were already limited by diffusion controlled binding. (b) Griep, M. A.; Reems, J. A.; Franden, M. A.; McHenry, C. S. *Biochemistry* **1990**, *29*, 9006−9014. Frey, P. A.; Sammons, R. D. *Science* **1985**, *228*, 541−545. (c) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman & Co.: New York, 1985; p 152.

(15) Weinstein, L. B.; Jones, B. C. N. M.; Cosstick, R.; Cech, T. R. *Nature* **1997**, *388*, 805−808.

(16) Sontheimer, E. J.; Sun, S.; Piccirilli, J. A. *Nature* **1997**, *388*, 801−805.

(17) Cosstick, R.; Vyle, J. S. *Nucleic Acids Res.* **1990**, *18*, 829−835.