A Bioinspired Dicopper(II) Catalyst for the Transesterification of Dimethyl Phosphate

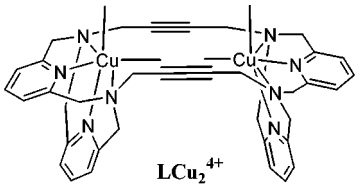
Ulrike Kühn, Sabine Warzeska, Hans Pritzkow, and Roland Krämer*

Anorganisch-Chemisches Institut
Im Neuenheimer Feld 270, 69120 Heidelberg, Germany
Received January 19, 2001
Revised Manuscript Received June 21, 2001

In enzymatic processing of nucleic acids usually divalent metal ions are required as cofactors. For hydrolytic cleavage of DNA phosphodiester bonds by the exonuclease subunit (Klenow fragment) of *E. coli* DNA polymerase I a dimetal mechanism (Scheme 1, R^1 = 3′-desoxyribosyl, R^2 = 5′-desoxyribosyl, R^3 = H) was proposed.[1] In vitro the enzyme is active with M_1, M_2 = Mg^{2+}, Zn^{2+}, $Co^{2+,}$ or Mn^{2+}.

The metal ions provide double Lewis acid activation of the bridging phosphate, M_2 generates a coordinated hydroxide nucleophile, and M_1 stabilizes the alcoholate leaving group. The trigonal-bipyramidal transition state (or intermediate) of the nucleophilic substitution reaction is efficiently stabilized (Scheme 1, right). Subsequently this (or a closely related) mechanism has been proposed, mostly on the basis of crystallographic evidence, for other phosphoryl transfer enzymes in which two active site metal ions are about 4 Å apart. It was observed in phosphate monoester cleavage by alkaline phosphatase,[2] involving a transesterification step, was proposed for restriction endonucleases,[3] for DNA polymerases[4] (Scheme 1, $R^1 = P_2O_6^{3-}$, R^3 = 3′-desoxyribosyl), and was discussed as a possible mechanism of RNA cleavage by ribozymes.[5] The probably wide importance of this dimetal mechanism in biological processing of nucleic acids and also its critical discussion[6] led us to investigate a low molecular weight mimic.

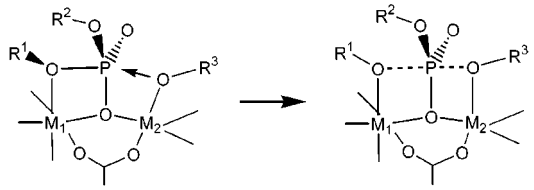
Only recently first structural model complexes (M = Cu^{2+}, Ni^{2+}) for the unusual 1,1-bridging fashion (monodentate) of a phosphodiester were reported.[7] While many important studies using simple coordination compounds gave insight into various modes for two-metal activation of phosphoryl transfer,[8] convincing evidence for the mechanism of Scheme 1 has not yet been presented. Our previous investigations of the dinuclear octaazamacrocyclic copper(II) complex $\mathbf{LCu_2}^{4+}$ [9] indicate that this system provides two available coordination sites per metal in appropriate orientation to support the reaction mechanism shown in Scheme 1. In aqueous solution the complex incorporates even at low pH



$\mathbf{LCu_2}^{4+}$

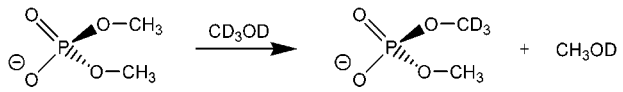
a single bridging hydroxide coligand that prevents bridging coordination of a phosphate ester substrate. However, from methanolic solution we have isolated and crystallographically characterized complexes in which nitrate and carbonate coligands

Scheme 1. Mechanism of Phosphodiester Hydrolysis by the Exonuclease Subunit of DNA Polymerase I



bind to the dimetallic site in the same 1,1-μ,η^3-fashion as the phosphodiester in Scheme 1. The nitrate complex in which **L** adopts an anti conformation lacks a free site for solvent binding in proximity to the oxyanion.

The activity of $\mathbf{LCu_2}$ for the transesterification of the simple phosphodiester dimethyl phosphate (DMP) was followed by ^{1}H NMR spectroscopy in d_4-methanol. Reaction solutions contained 2 mM $\mathbf{LCu_2(NO_3)_4}$ and 50 mM sodium dimethyl phosphate. Significant broadening of the $(CH_3O)_2PO_2^-$ doublet (coupling with ^{31}P nucleus) is attributed to coordination to paramagnetic Cu(II), with rapid exchange of the phosphodiester ligand on the NMR time scale. In D_2O solution the bridging site is blocked by OD^- and sharp P−OCH_3 signals are observed.



Transesterification of DMP is followed by release of CH_3OD (Figure 1). The identity of $(CH_3O)(CD_3O)PO_2^-$ and $(CD_3O)_2PO_2^-$ is confirmed by mass spectrometry (LDI-MS, ESI-MS). After quantitative conversion to $(CD_3O)_2PO_2^-$ (3 days at 55 °C), Cu^{2+} was masked by addition of aqueous Na_2CO_3 and a single ^{31}P NMR peak at 3.9 ppm (br, ^{31}P−^{2}H coupling not resolved) vs external H_3PO_4 is observed. This is consistent with a diester product ($(CH_3O)_2PO_2^-$ 4.0, $(CH_3O)PO_3^{2-}$ 7.1, $(CH_3O)_3PO$ 1.9 ppm in the same medium). These observations support a nucleophilic substitution mechanism at phosphorus with a CH_3O^- leaving group and CD_3O^- as incoming nucleophile. Experimental observations rule out an alternative pathway with nucleophilic attack of CD_3O^- at a carbon atom of DMP which would produce the monoester $(CH_3O)PO_3^{2-}$ (not observed by mass spectrometry) and dimethyl ether-d_3 (expected at 3.2 ppm but not observed in ^{1}H NMR).

For reaction times <10 days at 25 °C an approximately linear increase of methanol concentration (determined by integration of ^{1}H NMR signals) with time is observed, corresponding to 7 turnovers after 9 days without loss of catalyst activity. Since the cleavage rate is nearly the same for 50 and 20 mM DMP, the $\mathbf{LCu_2}$ catalyst is saturated with substrate and we can readily calculate $k_{cat} = 9(\pm 3) \times 10^{-6}$ s^{-1}, the cleavage rate constant of DMP substrate bound to $\mathbf{LCu_2}$. At 55 °C $k_{cat} = 1.2 \times 10^{-4}$ s^{-1}.

In view of the hydrolytic stability of DMP—data for transesterification are not available but the estimated rate constant for uncatalyzed P−O bond cleavage by hydroxide at pH 7 in water at 25 °C is in the order of 10^{-18} s^{-1} [8a]—it is clear that $\mathbf{LCu_2}$ very efficiently promotes the transesterification. In control experiments, copper(II) nitrate, (2,2′-bipyridine)$Cu(NO_3)_2$, and free **L** did not cleave DMP, and no trace of methanol was detectable after 4

(1) Beese, L. S.; Steitz, T. A. *EMBO J.* **1991**, *10*, 25−33.
(2) Kim, E. E.; Wyckoff, H. W. *J. Mol. Biol.* **1991**, *218*, 449−464.
(3) Kovall, R. A.; Matthews, B. W. *Curr. Opin. Chem. Biol.* **1999**, *3*, 578−583.
(4) Steitz, T. A. *J. Biol. Chem.*, **1999**, *247*, 17395−17398. Steitz, T. A. *Nature* **1998**, *391*, 231−232.
(5) Steitz, T. A.; Steitz, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6498−6502.
(6) Cowan, J. A. *Chem. Rev.* **1998**, *98*, 1067−1087.
(7) He, C.; Lippard, S. J. *J. Am. Chem. Soc.* **2000**, *122*, 184−185, He, C.; Gomez, V.; Spingler, B.; Lippard, S. J. *Inorg. Chem.* **2000**, *39*, 4188−4189.
(8) Recent reviews: (a) Williams, N. H.; Takasaki, B.; Chin, J. *Acc. Chem. Res.* **1999**, *32*, 485−493. (b) Blasko, A.; Bruice, T. C. *Acc. Chem. Res.* **1999**, *32*, 475−484. (c) Calama, M. C.; Timmermann, P.; Reinhoudt, D. N. *Chem. Soc. Rev.* **2000**, *29*, 75−86. (d) Krämer, R.; Gajda, T. *Persp. Bioinorg. Chem.* **1999**, 4, 209−240. Reviews focusing mainly on mononuclear Cu(II) complexes: (e) Hegg, E. L.; Burstyn, J. N. *Coord. Chem. Rev.* **1998**, *173*, 133−165. (f) Bashkin, J. K.; Jenkins, L. A. *Comments Inorg. Chem.* **1994**, *16*, 77−93.
(9) Warzeska, S.; R. Krämer *Chem. Commun.* **1996**, 499−500.

10.1021/ja015562c CCC: $20.00
Published on Web 07/27/2001

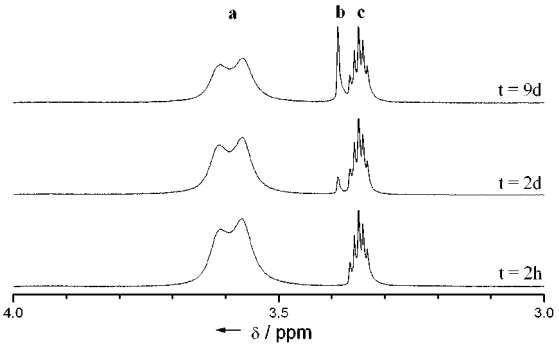
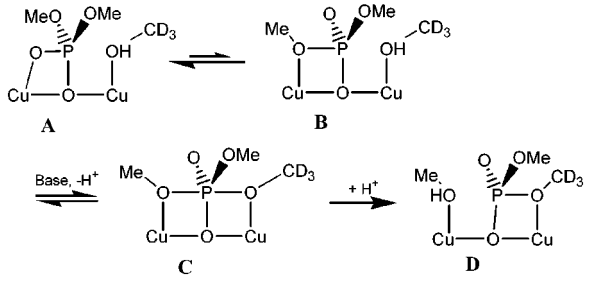


Figure 1. ^{1}H NMR spectra of reaction solutions containing 50 mM DMP and 2 mM **(L)Cu$_2$(NO$_3$)$_4$** in D_3COD at 25 °C: (a) broad signal of $(CH_3O)_2PO_2^-$; (b) released CH_3OD at 3.39 ppm; and (c) residual CD_2-HOD pentet of deuterated solvent.

Scheme 2. Proposed Mechanism for the Transesterification of $(CH_3O)_2PO_2-$ Catalyzed by LCu_2



weeks. Cleavage of simple dialkyl phosphates by M^{2+} complexes has not been observed before. A few examples for the hydrolysis of dimethyl phosphate by strongly Lewis acidic tri- and tetravalent metal ions have been reported in stoichiometric reactions and often at high temperature or low pH. Examples include Co(III) ($t_{1/2}$(DMP) = 40 days, 60 °C, pH 5.9),[10] Ce(IV) ($t_{1/2}$(DMP) = 22 min, 60 °C, pH 1.8),[11] Mo(IV) ($t_{1/2}$(DMP) = 18 d, 70 °C, pH 4.0),[12] and intramolecular alkyl phosphonate cleavage by La(III).[8b]

We propose a reaction mechanism outlined in Scheme 2. In an equilibrium of species **A** and **B** the "unreactive isomer" **A** should dominate since P−O$^-$ is a much better donor than P−OCH$_3$. In the Klenow fragment the coordination of the ether-type oxygen P−O−R (Scheme 1) is forced by interaction of protein with the DNA chain. In simple metal complexes phosphodiester coordination modes **A** and **B** have not yet been structurally characterized.

The transesterification rate decreases with increasing steric bulk of phosphodiester substituents: sodium dibenzyl phosphate at 25 °C, $k_{cat} = 9(\pm 3) \times 10^{-7}$ s^{-1}; lithium methyl-*p*-nitrophenyl phosphate at 55 °C, release of both *p*-nitrophenolate, $k_{cat} = 2.2 \times 10^{-5}$ s^{-1}, and methanolate, $k_{cat} = 1.5(\pm 0.5) \times 10^{-5}$ s^{-1}; and sodium bis(*p*-nitrophenyl)-phosphate at 55 °C, $k_{cat} = 2.9(\pm 0.7) \times 10^{-6}$ s^{-1}. In all cases reaction was monitored by ^{1}H NMR signals of released alcohols, and expected methanolysis products were identified by ESI-MS. Exchange of poor leaving group OCH_3 and good leaving group nitrophenolate at similar rates is a unique observation. Molecular models indicate that coordination of bulky leaving groups in species **B** is disfavored by interference with N−CH$_2$−py and N−CH$_2$−C≡C methylene groups of the ligand. For alternative mechanisms which do not include such or related steric discrimination, nitrophenolate cleavage should be about 10^6 times faster, as observed for Co^{III} promoted reaction.[10] In view of the determined trends in reactivity *both* OR substituents of phosphodiester appear to contribute to overall steric crowding and destabilization of **B** and **C**.

(10) Kim, J. H.; J. Chin *J. Am. Chem. Soc.* **1992**, *114*, 9792.
(11) Moss, R. A.; K. G. Ragunathan *Chem. Commun.* **1998**, 1871−1872.
(12) Kuo, L. Y.; Barnes, L. A. *Inorg. Chem.* **1999**, *38*, 814−817.

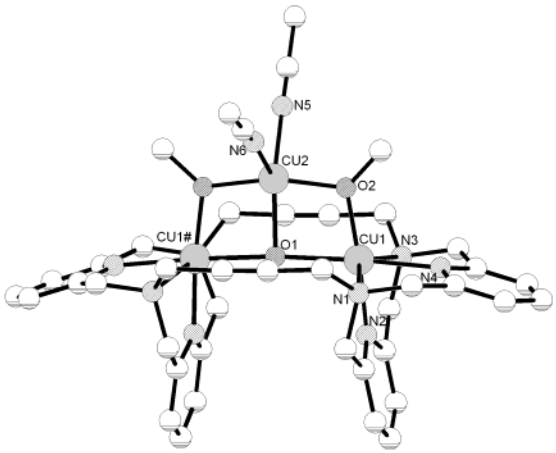


Figure 2. Crystal structure of **[LCu$_3$(μ-OH)(μ-CH$_3$O)$_2$(CH$_3$CN)$_2$]$^{3+}$** cation: Cu- - -Cu 4.328(1) Å, Cu(2)−O(2) 1.902(2) Å, Cu(2)−O(1) 1.984(3) Å; O(2)−Cu(2)−O(2#) 166°.[15]

Addition of strong acid $HO_2P(Op\text{-}C_6H_4NO_2)_2$ (1 equiv per **LCu$_2$**) to a standard assay (Figure 1) lowers the reaction rate more than 5-fold (the corresponding Na^+ salt does not affect the rate). Rate increases 1.2-fold on addition of 0.5 equiv of $NaOCD_3$, but **LCu$_2$** is inactivated by 1 equiv of $NaOCD_3$, most likely due to replacement of DMP by bridging CD_3O^- as indicated by the sharp ^{1}H NMR signal of DMP. Considerable reactivity even without addition of base is explained by the much higher basicity expected for the anionic DMP in the organic CD_3OD medium[13] compared to aqueous solution (pK_a(HDMP) = 0.8). These observations are compatible with participation of a Cu-coordinated CD_3O^- nucleophile. For attack of external methoxide a much stronger effect of proton concentration on rate would be expected, as observed in metal promoted methanolysis of amides.[14]

Finally, the crystal structure of the trinuclear complex **[LCu$_3$(μ_3-OH)(μ-CH$_3$O)$_2$)(CH$_3$CN)$_2$](ClO$_4$)$_3$** (Figure 2), obtained from **L** and 3 equiv of Cu(II) salt, supports the idea that **LCu$_2$** stabilizes the transition state of transesterification (species **C**, Scheme 1).

LCu$_2$ "incorporates" a $[Cu(\mu\text{-}OH)(\mu\text{-}OMe)_2(CH_3CN)_2]^-$ complex anion that is electronically not related to a phosphorane-type transition state but supports our steric considerations. Bonding parameters in the T-shaped Cu(2)(OMe)$_2$O(1)H fragment are related to those of the P(OMe)$_2$O moiety (P−OMe 1.87 Å, P−O 1.52 Å) in $P(OMe)_3O_2^{2-}$, the ab initio calculated structure of an energy-rich intermediate of dimethyl phosphate methanolysis.[16]

In conclusion **LCu$_2$** is the first nonenzymatic catalyst for the transesterification of simple alkyl phosphodiesters under mild conditions. It may operate by a mechanism proposed for hydrolytic or alcoholytic phosphoryl transfer in various enzymes. Studies which further support this assumption and limit other possibilities are in progress.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

Supporting Information Available: X-ray structural information and X-ray crystallographic file (CIF) of **[LCu$_3$(μ_3-OH)(μ-CH$_3$O)$_2$(CH$_3$CN)$_2$](ClO$_4$)$_3$**, **[(L)Cu$_2$(μ-CO$_3$)(CH$_3$OH)](BF$_4$)$_2$**, and **[(L)Cu$_2$(μ-NO$_3$)(NO$_3$)](NO$_3$)$_2$** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA015562C

(13) Galster, H. *pH-Messung*: VCH: Weinheim, Germany, 1990; pp 57 and 58.
(14) Neverov, A. A.; Montoya-Pelaez, P. J.; Brown, R. S. *J. Am. Chem. Soc.* **2001**, *123*, 210−217.
(15) Crystallographic data: orthorhombic; *Pnma*; green, a = 18.8716(10) Å, b = 18.4126(9) Å, c = 14.8895(8) Å, T = 173 K; Z = 4; R' = 0.0386; GOF = 1.005.
(16) Uchimaru, T.; Tsutzki, S.; Storer, J. W.; Tanabe, K.; Taira, K. *J. Org. Chem.* **1994**, *59*, 1835−1843.