Simulation of Enzymatic Hydrolysis of *p*-Nitrophenyl Deoxythymidine Diphosphate Initiated by Glutamate[1]

Joan A. Deiters[2a] and Robert R. Holmes*[2b]

Contribution from the Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003, and the Department of Chemistry, Vassar College, Poughkeepsie, New York 12601. *Received July 20, 1983*

Abstract: A model, based on a molecular mechanics approach, is employed for the hydrolysis of the substrate *p*-nitrophenyl deoxythymidine diphosphate, *p*-NO_2Ph-pdTp, in the enzyme Ca^{2+}–staphylococcal nuclease. The model is based on X-ray data (1.5-Å resolution) for the enzyme–substrate system Ca^{2+}–staphylococcal nuclease–pdTp, where pdTp represents deoxythymidine diphosphate. Calcium–oxygen interactions and hydrogen bonding are included in the model to mimic enzyme–substrate interaction. A hydrolysis pathway, initiated by the active site residue glutamate 43, is simulated. There is no steric blockage impeding the movement of the Glu 43 carboxylate group toward the 5' phosphorus atom. However, the pathway is one of high energy due to the enzyme constraints and the initial positioning of Glu 43. It is concluded that the likely hydrolysis pathway involves initial nucleophilic attack by a water molecule in line with the leaving thymidine group.

As an adjunct method to our X-ray structural investigations on phosphorus compounds,[3,4] we have utilized a molecular mechanics approach[5] to gain insight into likely reaction pathways. In this extension to a transition-state configuration, we have employed Allinger's well-parameterized program[6] for carbon compounds. In so doing, it was necessary to parameterize the program for four- and five-coordinated phosphorus compounds, the latter coordinated state exhibiting nonrigid character.[7] Our X-ray structural work and earlier vibrational[3,8] studies of nonrigid phosphoranes yielded suitable parameters for the extended program. Also included to accurately portray the flexibility associated with the five-coordinated state were nonbonded interactions for the ligands directly attached to phosphorus, i.e., 1–3 interactions, since the associated angle force constants were set to zero. In this way, we are able to account for the variation of properties of different ligands attached to phosphorus and yet retain the proper degree of nonrigidity.

Computer simulation of transition states based on the latter program has given added insight in formulating likely reaction pathways, both for nonenzymatic[9-12] and for enzymatic reactions[11-13] when applied to systems where ground-state geometries are available. For example, computation of the transition-state geometry for ribonuclease action on uridylyl-(3'–5')-adenosine[13] gave a structural form in agreement with a transition-state analogue obtained by low-temperature protein crystallography[14] to within experimental uncertainty. In the case of staphylococcal nuclease, differentiation between the enzymatic and nonenzymatic routes of hydrolysis of *p*-nitrophenyl deoxythymidine diphosphate was achieved,[11,12] in agreement with experimental observations.[15] In the latter studies,[11,12] the calculation used the X-ray data (2.0-[16] and 1.5-Å[17,18] resolution) of an enzyme–inhibitor complex, staphylococcal nuclease–thymidine 3',5'-bis(phosphate), pdTp, calcium ion as a starting point, and showed that there were no steric barriers along the reaction pathway for the mechanism proposed by Cotton et al.,[17] namely, attack by $H_2O(3)$ in line with the oxygen attached to the 5' carbon (see Figures 1a and 2). The simulation[11,12] of the reaction pathway calculated minimum energy conformations for the enzyme–substrate system, staphylococcal nuclease–thymidine 3'-phosphate 5'-(*p*-nitrophenyl phosphate), -*p*-NO_2Ph-pdTp, calcium ion, at various points along the hydrolysis reaction pathway.

In a recent communication, Mehdi and Gerlt[19] reported that staphylococcal nuclease catalyzes the hydrolysis of one of the diastereoisomers of thymidine 5'-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]-NPpT) in the presence of calcium ion in $H_2{}^{16}O$ to yield 4-nitrophenyl [^{16}O,^{17}O,^{18}O]phosphate([^{16}O,^{17}O,^{18}O]-pNP with *inversion* of configuration at phosphorus. This finding is in accord with a proposed mechanism by Cotton et al.[17] involving attack by H_2O in line with thymidine (Figure 1a). Mehdi and Gerlt[19] also gave consideration to the possibility of direct attack by Glu 43, as shown in Figure 1b. The experimental results[19] did not differentiate these two possible mechanisms, since attack by Glu 43 and attack by H_2O could both lead to inversion of configuration. In addition, the fact that the carboxylate group is known to be an effective nucleophile in the intramolecular hydrolysis of phosphate esters[20] suggests that attack by Glu 43 may be a possible step in the mechanism of phosphate hydrolysis in staphylococcal nuclease.

To differentiate between these two possible mechanisms, it seemed worthwhile to carry out a computer simulation of attack on the phosphorus atom by the carboxylate group of the Glu 43 side chain of staphylococcal nuclease and to compare these results with our previous study based on attack by a water molecule.[11,12]

Methods

The same set of atoms and interactions, bond parameters, force constants, and calculational methods were used for the system staphylococcal nuclease–Ca^{2+}–*p*-NO_2Ph-pdTp as previously de-

(1) Pentacoordinated Molecules. Part 54. Previous paper in the series: Vollano, J. F.; Day, R. O.; Holmes, R. R. *Organometallics* **1984**, *3*, 745.
(2) (a) Vassar College. (b) University of Massachusetts.
(3) Holmes, R. R. *ACS Monogr.* **1980**, *No. 175*, Chapter 2.
(4) Holmes, R. R. *Acc. Chem. Res.* **1979**, *12*, 257.
(5) Deiters, J. A.; Gallucci, J. C.; Clark, T. E.; Holmes, R. R. *J. Am. Chem. Soc.* **1977**, *99*, 5461.
(6) (a) Allinger, N. L.; Tribble, M. T.; Miller, M. A.; Wertz, D. H. *J. Am. Chem. Soc.* **1971**, *93*, 1637. (b) Wertz, D. H.; Allinger, N. L. *Tetrahedron* **1974**, *30*, 1579.
(7) In ref 3, Chapter 3.
(8) In ref 3, Chapter 4.
(9) Day, R. O.; Husebye, S.; Deiters, J. A.; Holmes, R. R. *J. Am. Chem. Soc.* **1980**, *102*, 4387.
(10) Gallucci, J. C.; Holmes, R. R. *J. Am. Chem. Soc.* **1980**, *102*, 4379.
(11) Deiters, J. A.; Gallucci, J. C.; Holmes, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 5457.
(12) Deiters, J. A.; Holmes, R. R. *J. Am. Chem. Soc.* **1983**, *105*, 609.
(13) (a) Holmes, R. R.; Deiters, J. A.; Gallucci, J. C. *J. Am. Chem. Soc.* **1978**, *100*, 7393. (b) Holmes, R. R. *ACS Monogr.* **1980**, *No. 176*, p 199f.
(14) Petsko, G., personal communication.
(15) Dunn, B. M.; DiBello, C.; Anfinsen, C. B. *J. Biol. Chem.* **1973**, *248*, 4769.
(16) Arnone, A.; Bier, C. J.; Cotton, F. A.; Day, V. W.; Hazen, E. E., Jr.; Richardson, D. C.; Richardson, J. S.; Yonath, A. *J. Biol. Chem.* **1971**, *246*, 2302.
(17) (a) Cotton, F. A.; Hazen, E. E., Jr.; Legg, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2551. (b) Tucker, P. W.; Hazen, E. E. Jr.; Cotton, F. A. *Mol. Cell. Biochem.* **1979**, *23*, 67.
(18) Hazen, E. E., Jr., personal communication.
(19) Mehdi, S.; Gerlt, J. A. *J. Am. Chem. Soc.* **1982**, *104*, 3223.
(20) Steffens, J. J.; Siewers, I. J.; Benkovic, S. J. *Biochemistry* **1975**, *14*, 2431.

0002-7863/84/1506-3307$01.50/0 © 1984 American Chemical Society

Table I. Minimum Energy and Distances for the System p-NO_2Ph-pdTp–Ca^{2+}–Staphylococcal Nuclease

reaction coordinate position	P–O_x bond[a] l_0, Å	P–O_x bond[a] k_s, mdyn/Å	energy, kcal/mol: attack by Glu 43[b]	energy, kcal/mol: attack by $H_2O(3)$[c,d]	P–O_x distance, Å: attack by Glu 43[b]	P–O_x distance, Å: attack by $H_2O(3)$[c,d]
initial conformation	0.00	0.00	−1.44	−1.44	5.21	4.12
point 1[e]	3.0	0.18	9.36	0.38	3.50	3.04
point 2[e]	2.5	0.41	21.03	1.41	2.95	2.57

[a] Strainless bond length (l_0) and force constant (k_s). [b] P–O_x = P–O_{41}. [c] P–O_x = P–O_{47}. [d] Results for attack by $H_2O(3)$ from ref 12. [e] Points 1 and 2 along the reaction coordinate are the same as previously defined.[12]

Table II. Distribution of Strain Energy in Minimum Energy Conformations (kcal/mol)

E	starting conformation	point 1: attack by Glu 43	point 1: attack by $H_2O(3)$	point 2: attack by Glu 43	point 2: attack by $H_2O(3)$
E(bond stretching)	0.64	5.19	0.80	10.97	1.02
E(bond bending)	2.41	5.51	2.84	9.77	2.85
E(nonbonded interactions)	−4.59	−1.45	−3.39	0.18	−2.58
E(torsion energy)	0.10	0.11	0.13	0.11	0.12
E(total)	−1.44	9.36	0.38	21.03	1.41

scribed.[11,12] The initial atomic coordinates for the system were based on data of 1.5-Å resolution for the enzyme–inhibitor system staphylococcal nuclease–Ca^{2+}–pdTp.[17,18]

In Figure 2, a portion of the system of atoms used in the calculation is shown along with the atom numbering system employed. A weak force constant between O_{41} (Glu 43) and P_{22} was introduced to pull Glu 43 toward phosphorus. Energies and distances were calculated for three points along the reaction coordinate: starting point (no P_{22}–O_{41} force constant); point 1, at which P_{22}–O_{41} is held at 3.0 Å by a small force constant of 0.18 mdyn/Å; point 2, at which P_{22}–O_{41} is held at 2.5 Å by a force constant of 0.41 mdyn/Å. The conditions for the calculation of the reaction path corresponding to attack by Glu 43 were identical with those previously described[12] except for the following: (1) A bond between Glu 43 and phosphorus (O_{41}–P_{22}) was formed in line with O_{21} (oxygen attached to 5′ carbon of pdTp). This bond replaced the H_2O–P_{22} bond (O_{47}–P_{22}) in the calculations already reported.[12] (2) The weak interaction between Glu 43 and $H_2O(1)$ was removed in order to allow Glu 43 to move toward the 5′-phosphate.

In all calculations, the following atoms were held fixed during minimizations: Ca^{2+}, three atoms of the thymine plane, three atoms of ribose, and the α and β carbons of each amino acid moiety.

The latter conditions still allow considerable flexibility to the active-site residues, e.g., bond rotations, angle bending, and bond stretching. This flexibility is amplified by the presence of many weak force constants, particularly for hydrogen bonding and ion–dipole interactions and by the freedom of the phosphorus geometry to rearrange as the transition state is formed.

Results and Disscussion

In Tables I and II, the results of the calculation for attack by Glu 43 are reported and compared to the results obtained[12] for attack for H_2O.

Apparently, under the conditions imposed by the calculation, the distance between O_{41} and P_{22} (corresponding to Glu 43 attack) is too great to be spanned without highly straining the system. The strain energy increases rapidly from −1.44 to 21.03 kcal/mol when the Glu 43–P_{22} distance decreases from 5.21 to 2.95 Å compared to a much smaller change in energy from −1.44 to 1.41 kcal/mol when $H_2O(3)$ moves in toward P_{22}. In Glu 43 attack, the P–O_{41} distance remains about 0.5 Å longer than the strainless bond length, l_0, because a decrease in P_{22}–O_{41} distance causes a great increase in strain energy.

In the calculation of the minimum energy conformation for point 1 on the Glu 43 reaction path, the phosphorus atom P_{22} and O_{41} (Glu 43) both had to move at lease 1 Å. We find that the movement of phosphorus strains the bonds between the 5′-phosphate oxygens and the argenine residues;[21] this movement also

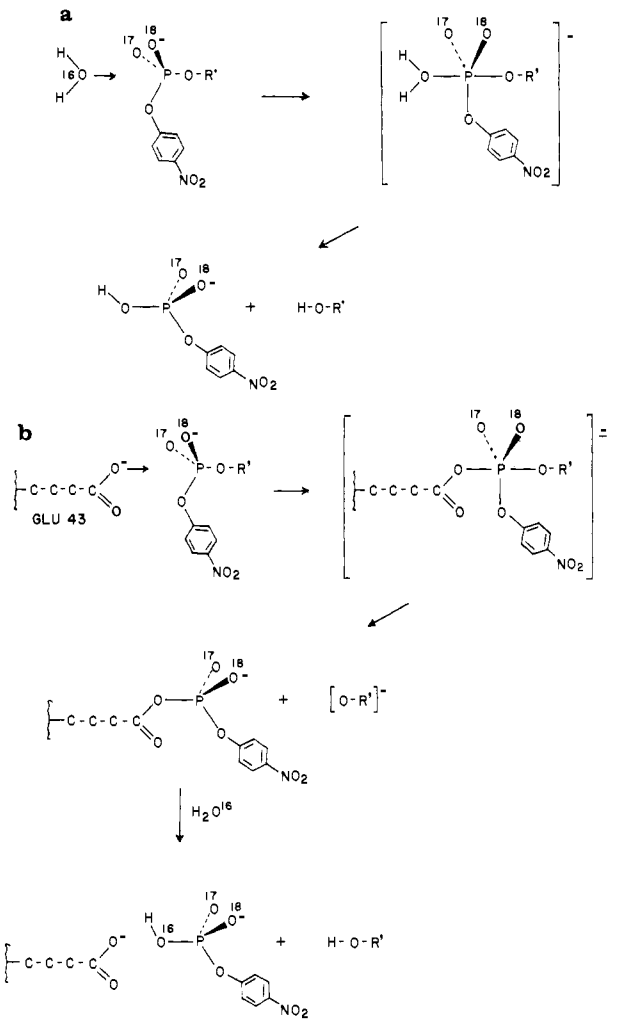
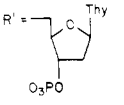


Figure 1. Hydrolysis of phosphate by H_2O and by carboxylate anion. These in-line mechanisms, which involve a trigonal-bipyramidal transition state, could lead to inversion of configuration. In enzymatic hydrolysis is O–R′ is the leaving group where



(a) Attack by H_2O. (b) When the attacking group is the carboxylate ion of glutamate 43, inversion of configuration might also occur.

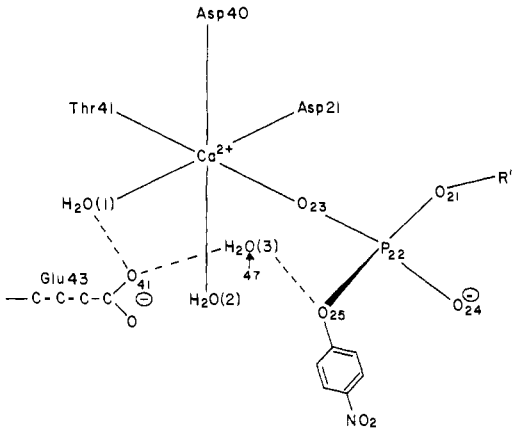


Figure 2. A portion of the system included in the staphylococcal nuclease–Ca^{2+}–*p*-NO_2Ph-pdTp calculation. Amino acid residues which were included in the calculation but not illustrated: Arg 35, Arg 87, Tyr 85, and Tyr 113. Oxygens 23 and 24 are bonded to the enzyme through Arg 35; oxygens 21 and 24 are bonded to Arg 87. R' is defined in Figure 1. The oxygen atom of $H_2O(3)$ is O_{47}.

causes the calcium coordination sphere to be strained and also causes strain in the bonds and angles between the 5'-phosphorus and ribose. Since the α and β carbons of Glu 43 were held fixed throughout the calculation, movement of O_{41} toward P_{22} strains the bonds and angles of Glu 43.

The results obtained in this calculation might be compared to those obtained in a simulation of ribonuclease action on uridylyl-(3'–5')-adenosine.[13] There it was shown (under similar restrictions) that the movement of Lys 41 could easily span a 4.8-Å distance to interact with a cyclized intermediate.

An additional calculation was performed to see if any steric interference is present hindering the movement of Glu 43 toward phosphorus. When the α and β carbons of Glu 43 were released from their fixed positions, thus allowing unrestricted movement, the energy of the system was calculated to be quite similar to the energy calculated for attack for $H_2O(3)$. The implication is that motion of Glu 43 is not impeded by steric blockage at the enzyme active site.

On the basis of these calculations, we conclude that initiating attack by Glu 43 is not a feasible mechanism for the hydrolysis by staphylococcal nuclease on *p*-NO_2Ph-pdTp. Only if there were severe modification of the active site in the presence of the latter substrate which allows much closer approach of Glu 43 to the 5'-phosphate, would this mechanism become feasible. Our previous results of ribonuclease action on uridylyl-(3'–5')-adenosine (UpA) using this model program,[13] which were supported by low-temperature protein crystallographic studies,[14] indicate this possibility highly unlikely.[22] Hence, the favored mechanism involves attack by a water molecule, $H_2O(3)$, in line with the leaving group, thymidine 3'-phosphate (Figure 2).

Acknowledgment. This investigation was supported by a grant from the National Institutes of Health (GM 21466) and is gratefully acknowledged. Appreciation is expressed to the University of Massachusetts Computing Center for generous allocation of computer time.

Registry No. NO_2Ph-pdTp, 24418-11-9; glutamate, 56-86-0; staphylococcal nuclease, 9013-53-0.

(21) The interactions between Arg 35 and Arg 87 with the 5'-phosphate oxygens are not shown in Figure 2 but were included in the calculation. For a discussion of phosphate–arginine bonding, see ref 11 and 12.

(22) A copy of our program is available from R.R.H.

Photochemistry of Flavins with Sulfur-Activated Carboxylic Acids: Identification and Reactions of the Photoproducts

Gert A. Eberlein* and Michael F. Powell†

Contribution from the Institut fur Biologie, Universitaet Konstanz, Am Giesberg, D-7250 Konstanz, West Germany. *Received May 31, 1983*

Abstract: Photoreduction of 3-methyllumiflavin by α-sulfide- or α-disulfide-substituted carboxylic acids does not give dihydroflavin–4a-sulfur adducts or result in the sulfur–carbon bond scission as claimed previously[1,2] (eq 7 and 19). Instead decarboxylation of the acid accompanied by dihydroflavin–4a-carbon adduct formation (eq 8 and 10) was shown to occur. Several other substitution products were also isolated and characterized, including an example of the little known 6-substituted flavins. Isoalloxazine also gave similar products, including the 8-methyl-substituted derivatives, when dithiodiglycolic acid was employed. A primary electron-transfer mechanism between photoexcited lumiflavin and substituted carboxylic acid with consecutive radical coupling is supported. Reaction of 4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin with formic acid and acetic anhydride gave 5-formyl-4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin and 5,8,10,11-tetramethyl-8*H*-benzo[*g*]thiazolo[3,4-*e*]pteridine-4,6-dione (eq 17). The latter compound is a modified flavin containing four rings (ring closure over the 4a and 5 positions) and was found to be stable toward photoinduced oxidation. Dihydroflavin was found to convert sulfides to sulfoxides in the presence of oxygen; two sulfoxy diastereoisomers of 4a-(((carbomethoxymethyl)sulfinyl)methyl)-4a,5-dihydrolumiflavin are described herein (eq 11). Intramolecular reduction of the 6α-disulfide bond in 6-(((carboxymethyl)dithio)methyl)-1,5-dihydrolumiflavin was observed (eq 13). Scission of the disulfide bond in 4a-(((carboxymethyl)dithio)methyl)-4a,5-dihydro-3-methyllumiflavin by various nucleophiles gave 4a,5-dihydro-3-methyllumiflavin-4a-methyl mercaptan (eq 23, 28, 34) which rapidly decomposed to eliminate thioformaldehyde as indicated by the formation of thioformaldehyde polymers of flavin 4a-adducts.

The flavin moiety is the active component of more than a hundred different flavoproteins which are able to undergo both electron- and group-transfer reactions.[3] Oxidized flavins are redox active in both the ground and excited states. The excited state

* Address correspondence to Department of Chemistry, University of California at Santa Barbara, Santa Barbara, CA 93106.
† Present address: Lawrence Berkeley Laboratories, University of California at Berkeley, Berkeley, CA 94720.

(1) Knappe, W.-R.; Hemmerich, P. *Z. Naturforsch., B* **1972**, *27B*, 1032–1034.

0002-7863/84/1506-3309$01.50/0 © 1984 American Chemical Society