results by Gelin and Karplus[14a] and structural results by Baldwin and Chothia[14b] support a proposal that the affinity of the sixth ligand for the heme is critically dependent on the tertiary heme structure that originates from the position of the proximal imidazole. This imidazole and the F helix to which it is bound undergo major structural movements on going from deoxy to ligated Hb; these changes for the α and β subunits include a 1-Å translation of the F helix across the prophyrin face and a movement of the imidazole from a position that is asymmetric with respect to the porphyrin nitrogens to one that is more symmetric. The fact that the initial spectral events of CO photodissociation to 3 ns for Hb, Hbα, and Hbβ appear to be identical and yet significantly different from those of either unstrained protoheme **1** or MbCO[5c] suggests that tertiary heme structure may be of a major factor affecting photodissociation. The geometry of the heme in HbCO is very much different from that of the linear imidazole–Fe–CO geometry found in **1**-CO. In addition to the bent FeCO geometry,[15] HbCO has about 60 interactions between the globin and the heme of 4 Å or less.[16] The predissociative geometry of the heme, particularly the position and strain on the proximal imidazole, may be very important in determining the relative position of certain critical Fe d–d and/or charge-transfer states[17] that govern the process of photodissociation.

Acknowledgment. We are grateful to the Research Corp. and to the National Science Foundation (Grant PMC 7816199 to L.J.N.) for support of this research.

Registry No. 1-CO, 69468-00-4.

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Oxygen Chiral Phosphodiesters. 7. Stereochemical Course of a Reaction Catalyzed by Staphylococcal Nuclease

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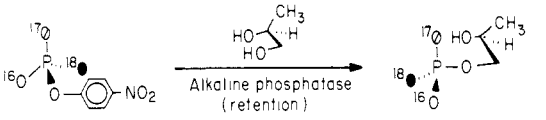
Of the phosphodiesterases that have been characterized with respect to the stereochemical course of the displacement reaction at phosphorus,[1,2] high-resolution X-ray structural data are available only for ribonuclease A.[3] This structural information, coupled with chemical data, has allowed the catalytic roles assumed by the active-site amino acid functional groups to be described. In contrast, the mechanisms of the metal ion dependent phosphodiesterases cannot be described in comparable detail. For this reason, we have determined the stereochemical course of a hydrolysis reaction catalyzed by staphylococcal nuclease. This enzyme requires calcium ions for both DNase and RNase activities. Extensive research reported by the laboratories of Anfinsen and of Cotton has provided a detailed structural description of the enzyme, with the complete amino acid sequence[4] and a 1.5-Å X-ray structure of an enzyme-inhibitor complex[5] being available. Dunn, DiBello, and Anfinsen have described a detailed kinetic study of the hydrolysis of thymidine 3′-phosphate 5′-(4-nitrophenyl phosphate) (NPpTp); these investigators also reported that attempts to trap a covalent adduct between the enzyme and substrate were unsuccessful.[6] More recently, Cotton, Hazen, and Legg have proposed a mechanism for the hydrolysis reaction catalyzed by the nuclease that is based upon the geometric relationship between the calcium ion, thymidine 3′,5′-bisphosphate (pdTp), and active-site residues found in the 1.5-Å X-ray structure.[5] The carboxylate of glutamate 43 was suggested to act as a general basic catalyst in the attack of a water molecule on the 5′-phosphorus atom of a nucleotide ester bound in the active site. In this mechanism, the calcium was postulated to assist in properly positioning the carboxylate group via an intervening water molecule and to neutralize the phosphate ester charge by a direct ionic interaction. This proposal implies that the hydrolysis reaction should proceed with inversion of configuration at phosphorus. In accord with, but not proving, this mechanism, we have found that the nuclease catalyzes the hydrolysis of one of the diastereomers of thymidine 5′-(4-nitrophenyl [^{17}O,^{18}O]phosphate) ([^{17}O,^{18}O]-NPpT) 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.

Scheme I



The R_p diastereomer of [^{17}O,^{18}O]-NPpT[2c,7,8] was hydrolyzed in $H_2{}^{16}O$ at pH 8.8 and 42 °C in the presence of 10 mM Ca^{2+} by using the nuclease (Worthington) as catalyst. The progress of the reaction was followed by HPLC, and after the reaction was approximately 80% complete (16 h), the [^{16}O,^{17}O,^{18}O]-pNP was

†NIH Research Career Development Awardee (CA-00499), 1978–1983; Fellow of the Alfred P. Sloan Foundation, 1981–1983.

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(7) The isotopic composition of the ^{17}O-enriched position is 16% ^{16}O, 52% ^{17}O, and 32% ^{18}O; the isotopic content of the ^{18}O-enriched position is 99% ^{18}O.
(8) The K_m for NPpT is 2.2 mM and that for NPpTp is 10 μM; the V_m for each of these substrates is approximately 0.6 μmol (mg min)$^{-1}$: Cuatrecasas, P.; Wilchek, M.; Anfinsen, C. B. *Biochemistry* **1969**, *8*, 2277. The V_m for the hydrolysis is DNA can be calculated to be approximately 150 μmol of internucleotide bonds (min mg)$^{-1}$ (assuming that the hypochromicity resulting from hydrolysis of internucleotide bonds causes an increase of 40% in the absorbance at 260 nm).

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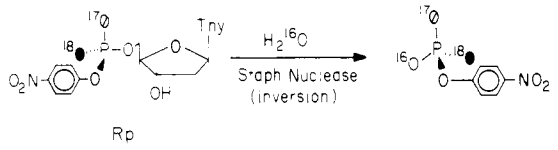
Scheme II



isolated by chromatography of the reaction mixture on DEAE-Sephadex A-25.[9] No chemical hydrolysis could be detected under the conditions of the enzymatic hydrolysis reaction.

In the stereochemical studies of enzymatic reactions reported previously by this laboratory, the reaction products were oxygen chiral nucleotides. Configurational analysis of these materials could be accomplished readily by measuring the ^{18}O perturbations on the ^{31}P NMR resonances of nucleotide derivatives obtained either by complexation with $Co(NH_3)_4(H_2O)_2^{3+}$ (diastereomers of [α-^{16}O,^{18}O]dADP[10]) or by enzymatic or chemical cyclization followed by methylation with diazomethane (various [^{16}O,^{17}O,^{18}O]-chiral 3′- and 5′-mononucleotides[2b,g,i]). In order to accomplish the configurational analysis of the sample of [^{16}O,^{17}O,^{18}O]-pNP generated in the present study, we used Knowles' procedure, in which the chiral phosphoryl group is transferred to chiral 1,2-propanediol with alkaline phosphatase as catalyst[11,12] and the resulting mixture of 1- and 2-phosphopropanediols is chemically cyclized.[13] Following methylation with diazomethane, measurement of the ^{18}O perturbations on the ^{31}P NMR resonances of the resulting cyclic triesters allows the configurations of the 1-phosphopropanediol and, therefore, of the [^{16}O,^{17}O,^{18}O]-pNP to be assigned.[14]

Starting with 320 μmol of [^{16}O,^{17}O,^{18}O]-pNP, we obtained 35 μmol of a mixture of 1- and 2-phosphopropanediols, in which the ratio of primary to secondary ester was 4.5:1, as judged by ^1H NMR at 270 MHz.[15] Following chemical cyclization and methylation,[16] the ^{31}P NMR spectrum of the mixture of syn and anti cyclic triesters shown in Figure 1 was obtained. Examination of this spectrum and the knowledge that the chemical cyclization of 1-phosphopropanediol proceeds with inversion of configuration[13] permits the configuration of the chiral phosphoryl group in the diastereomeric phosphomonoester to be assigned as S_p. The established stereochemical course of the alkaline phosphatase-catalyzed phosphoryl transfer (retention[11]) allows the configuration of the enantiomeric phosphomonoester derived from the nuclease-catalyzed reaction be assigned as S_p (Scheme I). This finding leads to the conclusion that the hydrolysis reaction catalyzed by staphylococcal nuclease proceeds with *inversion* of configuration at phosphorus (Scheme II).

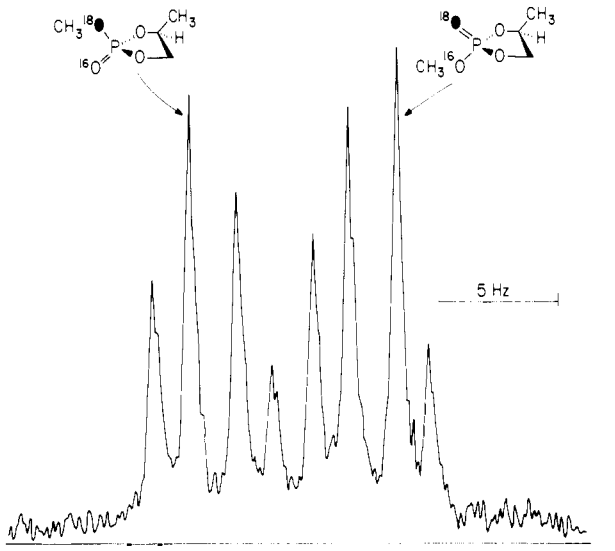


Figure 1. Proton-decoupled ^{31}P NMR spectrum at 81 MHz of the syn and anti methyl esters of isotopically labeled 2-hydroxy-2-oxo-4-methyl-1,3,2-dioxaphospholane derived from chemical cyclization of a mixture of 1- and 2-phosphopropanediols. The spectrum was obtained with a 512-Hz sweep width and a 6-s acquisition time; 7612 transients were obtained prior to application of a 0.1-Hz line broadening and Fourier transformation. The approximate chemical shift of the center of the set of resonances is +19.7 ppm (downfield, relative to an external capillary of 85% H_3PO_4). The four most downfield resonances are associated with syn methyl esters, and the four most upfield resonances are associated with the anti methyl esters.[14]

Given the structure of the nuclease–pdTp–Ca^{2+} complex,[5] the simplest and most likely explanation for this stereochemical result is that the direct attack of water on the phosphorus (to displace thymidine, the poorer leaving group) is general base catalyzed by glutamate 43.

However, the 5′-phosphate of pdTp is presumably bound to the enzyme as a dianion, whereas the 5′-phosphate of a substrate must bind as a monoanion. Since the structural consequences of this difference in electrostatics are unknown, the orientation of glu-

(9) The reaction mixture (40 mL) contained the R_p diastereomer of [^{17}O, ^{18}O]-NPpT (10 mM), borate buffer (pH 8.8, 0.1 M), $CaCl_2$ (10 mM), and enzyme (4.9 mg). The reaction was monitored by diluting aliquots of the reaction mixture and analyzing the composition of materials absorbing at 254 nm by HPLC using a C_{18} reverse-phase column and isocratic elution with a mixture of 80% 0.1 M sodium phosphate, pH 6.5, 5 mM tetrabutylammonium hydroxide, and 20% acetonitrile. When the reaction was judged to be about 80% complete (16 h), the reaction mixture was applied to a column of DEAE-Sephadex A-25 (HCO_3^-). The product was eluted with a linear gradient of triethylammonium bicarbonate, pH 7.5. The fractions containing pNP were pooled and concentrated to give 320 μmol of product (based on ϵ_{310} = 9.19 mM^{-1}). The product was dissolved in 5 mL of water, percolated through a column of Chelex-100 (Na^+), and lyophilized.

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(15) Alkaline phosphatase (Sigma, type III, 50 units) was dialyzed overnight against a buffer containing 0.15 M $KHCO_3$, 0.15 M K_2CO_3, 0.75 mM $Mg(OAc)_2$, and 75 μM $Zn(OAc)_2$. The reaction mixture (4 mL in an 8-mm NMR tube) contained 2 mL of (*S*)-1,2-propanediol (100% enantiomeric excess), 2 mL of a buffer composed of 0.15 M $KHCO_3$, 0.15 M K_2CO_3, 1.5 mM $Mg(OAc)_2$, and 0.15 mM $Zn(OAc)_2$ and prepared in 37.5% D_2O, dialyzed enzyme, and the Chelex treated [^{16}O,^{17}O,^{18}O]-pNP. The progress of the reaction was followed by ^{31}P NMR at 32 MHz. After 60% of the chiral monoester had been consumed, the reaction was stopped by the addition of 2 mL of Dowex-50 (H^+). The resin was removed by centrifugation and washed several times with deionized water. The solution was adjusted to pH 8 with concentrated NH_3, and the solution was applied to a column of Dowex 1-X-8 (HCO_3^-). The mixture of 1- and 2-phosphopropanediols was eluted with a linear gradient of triethylammonium bicarbonate. Product-containing fractions were combined and concentrated to give 35 μmol of a 4.5:1 mixture of 1- and 2-phosphopropanediols contaminated with about 1 μmol of inorganic phosphate. The labeled esters were converted to their cyclohexylammonium salts by ion exchange, and after evaporation of the solvent, the residue was washed twice with small amounts of acetone.

(16) The bis(tri-*n*-octylammonium) salt of the phosphopropanediols (30 μmol) was dried by repeated evaporation of dry dioxane. The residue was dissolved in 128 μL of methylene chloride, and 51 μmol of diisopropylethylamine was added; a few 4-Å molecular sieves were added, and the solution was allowed to stand for 6 h. A solution of 100 μmol of (diphenylphosphoryl)imidazole in 400 μL of methylene chloride was similarly dried. A 105-μL aliquot of the solution of (diphenylphosphoryl)imidazole was added to the solution of phosphopropanediols, and the cyclization reaction was allowed to proceed for 15 h. The reaction was quenched by the addition of an aqueous solution of 0.1 M triethylammonium bicarbonate. The aqueous layer obtained after washing eight times with methylene chloride was lyophilized. The residue was suspended in 0.5 mL of dry acetonitrile and sonicated for 1 h. An ethereal solution of diazomethane was added until the mixture clarified and the pale yellow color persisted. Following evaporation of the solvent, the residue was dissolved in 2.5 mL of dry acetonitrile (containing 0.6 mL of acetonitrile-d_3), sonicated briefly, and filtered into a 10-mm NMR tube. The cyclization reaction and transfer to the NMR tube were performed in a glovebag under a dry argon atmosphere; the methylation reaction was performed with precaution taken to exclude moisture.

tamate 43, the calcium ion, and the substrate as well as the roles of the glutamate and calcium ion in catalysis may not be precisely those inferred from the X-ray studies. Despite unsuccessful attempts to trap a covalent intermediate[6,17] a mechanism involving formation of an intermediate cannot be eliminated on the basis of our stereochemical result. Nucleophilic attack of glutamate 43 on the phosphorus of NPpT would result in formation of an acyl 4-nitrophenyl phosphate intermediate, with hydrolysis of the acyl phosphate ester intermediate yielding pNP. The stereochemical course of the overall hydrolysis reaction could be either retention or inversion, depending upon whether hydrolysis of the intermediate involves P–O or C–O bond cleavage, respectively. Carboxylate groups are known to be effective nucleophilic catalysts in the intramolecular hydrolysis of phosphate esters.[18] In addition, the hydrolysis of 5′-adenosyl benzoyl phosphate[19] and the methanolysis of phenyl acetyl phosphate[20] both occur with C–O bond cleavage; however, the effect of metal ion coordination on the position of bond cleavage is unknown.

The importance of this mechanism can be evaluated either by performing a single turnover experiment, in which the origin of the oxygen atom incorporated into the product can be established, or by determining whether catalysis is accompanied by the incorporation of solvent isotope into enzyme carboxylate groups.[21] Such experiments are feasible with the nuclease, since it can be isolated in gram quantities; the results of these and other experiments designed to further probe the mechanism of the reaction catalyzed by this enzyme will be reported in the future.

Since most phosphohydrolases cannot be purified in amounts compatible with such mechanistic investigation, the disconcerting possibility that an inversion of configuration may not be sufficient evidence to rule out the participation of a covalent intermediate in these reactions cannot be easily discarded. The potential occurrence of acyl phosphate ester intermediates in hydrolysis reactions does provide, however, a plausible hypothesis to explain the lack of stereochemical uniformity that has been observed for two types of phosphohydrolase reactions.[2,22]

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Registry No. Labeled *syn*-4-methyl-1,3,2-dioxaphospholane methyl ester, 66943-87-1; labeled *anti*-4-methyl-1,3,2-dioxaphospholane methyl ester, 78780-75-3.

(17) We have observed that incubation of nuclease with 7 mM NPpT, 0.5 M hydroxylamine, and 10 mM $CaCl_2$ in 0.1 M borate buffer, pH 8.8, causes no inactivation of the enzyme, as judged by assaying aliquots of the incubation mixture with heat-denatured calf thymus DNA as substrate. This inability to intercept an acyl phosphate intermediate does not exclude its participation, since the intermediate need not be accessible to the solvent. For example, water coordinated to the calcium ion could serve as the nucleophile, and this coordination site may be unable to accommodate hydroxylamine.

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Nucleophilic Attack on Olefins Initiated by Dimethyl(methylthio)sulfonium Fluoroborate (DMTSF). Azasulfenylation

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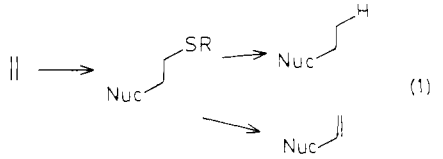
While the concept of elaboration of olefins with electrophiles is well accepted, the converse, i.e., elaboration by nucleophiles, is not, except for Michael-type systems. Reversal of olefin reactivity by use of transition metals has been a particularly exciting area of study.[1] The general utility of organosulfur substituents in terms of subsequent elimination, reduction, or substitution reactions led us to consider an RS^+ equivalent that would simultaneously invert the electronic characteristics of an olefin and permit the introduction of a wide variety of nucleophiles. Such reactions would constitute the equivalent of nucleophilic addition or substutition (see eq 1). We report that dimethyl(methyl-



(1)

thio)sulfonium fluoroborate (DMTSF, **1**)[2,3] is such a conjunctive reagent. In conjunction with nitrogen nucleophiles, an extraordinary level of regiocontrol can be exercised in an azasulfenylation reaction[4] whose products not only permit an overall olefin amination (by reductive cleavage of sulfur) but also constitute an oxazoline synthesis, a cis hydroxyamination of an olefin, and an aziridine synthesis as a result of the leaving-group properties of the sulfur.

The procedure is experimentally quite simple. A solution of the olefin in methylene chloride, nitromethane, or acetonitrile is treated with 1 equiv of **1** at 0 °C to room temperature. In some instances, addition of 1% (v/v) of dimethyl sulfide helps minimize side reactions. Addition of the nitrogen nucleophile, normally at room temperature, led to smooth, albeit slow (1–4 days) substitution to give excellent yields of the desired products. The results are summarized in Table I.

Three types of nitrogen nucleophiles were examined: amines, azide, and nitrite. The stereochemistry of the addition is trans, as has been shown for both cis- and trans-disubstituted olefins (Table I, entries 1–7, 14). For example, in the case of the acetamide derived from **2**, the ^{1}H NMR spectra showed two ^{1}H signals, at δ 3.72 (tdd, J = 10.6, 7.9, 4.1 Hz) and δ 2.38 (td, J = 10.9, 3.7 Hz), a fact only consistent with the stereochemistry depicted. Further transformations (vide infra) confirm these stereochemical assignments.

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