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Stereochemistry of Phospho Group Transfer Catalyzed by a Mutant Alkaline Phosphatase

John E. Butler-Ransohoff,[‡] Debra A. Kendall,[‡] Sally Freeman,[§] Jeremy R. Knowles,[§] and E. T. Kaiser*,[‡]

Laboratory of Bioorganic Chemistry and Biochemistry, Rockefeller University, New York, New York 10021, and Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

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ABSTRACT: The stereochemical course of the phospho group transfer catalyzed by mutant (S102C) alkaline phosphatase from *Escherichia coli* was investigated by using ^{31}P nuclear magnetic resonance spectroscopy. Transphosphorylation from 4-nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]phosphate to (*S*)-propane-1,2-diol occurs with overall retention of configuration at phosphorus. This result is consistent with the view that the hydrolysis of substrates by this mutant enzyme proceeds by way of a covalent phosphoenzyme intermediate in the same manner as the wild-type alkaline phosphatase.

Bacterial alkaline phosphatase is present in the periplasmic space of *Escherichia coli* (*E. coli*) and catalyzes the nonspecific hydrolysis of a wide range of phosphate monoesters (Reid & Wilson, 1971). The mechanism of the enzymatic reaction has been thoroughly studied, and the formation of a phosphorylated enzyme intermediate is now widely accepted as an essential step in the reaction pathway. At low pH, the rate-limiting step of the enzymatic reaction is the dephosphorylation of the covalent intermediate (Reid & Wilson, 1971; Coleman & Gettins, 1983), the accumulation of which has allowed its identification as the phosphate monoester of Ser-102 (Schwartz et al., 1963). At high pH, however, the rate-limiting step of the reaction is the release of noncovalently bound product phosphate from the active site of the enzyme. Even at high pH the overall stereochemical course of the reaction catalyzed by bacterial alkaline phosphatase is known to be retention of configuration at phosphorus (Jones et al., 1978), and this finding is consistent with the necessary formation of the phosphoenzyme intermediate.

We have reported the use of site-directed mutagenesis to replace the active-site hydroxyl group with a thiol by changing Ser-102 to Cys. The resulting thiol enzyme [S102C, previously referred to as thiol alkaline phosphatase (TAP)] was found to catalyze the hydrolysis of a variety of phosphate monoesters (Ghosh et al., 1986), though the V_{max} observed for the mutant enzyme was found to be dependent upon the pK_a of the substrate's leaving group. This observation suggests that, unlike the behavior of the wild-type enzyme (Hall & Williams, 1986), the rate-determining step of the reaction catalyzed by the mutant S102C phosphatase is the phosphorylation of the active-site thiol group rather than either dephosphorylation or product release. Further evidence was provided by the fact that the V_{max} values of the S102C alkaline phosphatase are independent of the concentration of tris(hydroxymethyl)-aminomethane (Tris) in the reaction buffer. Tris acts as an alternate phospho group acceptor from the wild-type phosphoenzyme, and higher concentrations of Tris increase the rate

[‡] Rockefeller University.
[§] Harvard University.

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of the wild-type-catalyzed reaction as expected if dephosphorylation is rate-limiting (Engstrom, 1962). With the mutant enzyme, for which phosphorylation is rate-controlling, the lack of any dependence of V_{max} upon the concentration of Tris is expected.

Most of the characteristics of the mutant S102C alkaline phosphatase suggest that the number and sequence of catalytic steps have been conserved. However, the possibility that the mutant enzyme does not use the same catalytic mechanism as the wild type had to be considered since the active-site thiol group failed to react with a host of thiol-modifying reagents (Ghosh et al., 1986). Potentially irreversible inhibitors such as 5,5′-dithiobis(2-nitrobenzoic acid), 6,6′-dithiodinicotinic acid, 4,4′-dipyridyl disulfide, 4-(chloromercuri)benzoate, 4-(hydroxymercuri)benzoate, Hg^{2+} salts, iodoacetate, and iodoacetamide did not react with the active-site thiol group of the mutant enzyme. The results with the first five reagents can be rationalized on the basis of the stringent steric requirements of the active site of alkaline phosphatase: None of these reagents seems to fit in the active site, on the basis of a computer-graphics analysis using the published crystal structure data (Sowadski et al., 1985). This argument cannot apply, however, to the last three reagents, and the lack of any effect by these materials seems best explained by assuming a tight coordinative interaction between the thiol group and one of the active-site zinc atoms.

No phosphoenzyme could be detected by precipitating S102C alkaline phosphatase in the presence of $^{32}P_i$ at low pH (Ghosh et al., 1986), although this method works well with the wild-type enzyme (Levine et al., 1969). Furthermore, attempts to detect the covalent intermediate by presteady-state kinetic measurements have failed to furnish any evidence for the accumulation of an intermediate. No "burst" was detected over a wide range of pH values when S102C alkaline phosphatase and high concentrations of its best substrate, 2,4-dinitrophenyl phosphate, were mixed. The wild-type enzyme, in contrast, shows a stoichiometric burst at low pH values, where dephosphorylation of the covalent intermediate is rate-limiting (Ko & Kezdy, 1967).

Finally, the results of partition experiments (J. E. Butler-Ransohoff and E. T. Kaiser, unpublished results) seem to argue against the formation of a covalent intermediate. If an intermediate is generated that can react with different acceptors, its existence may be confirmed by the constancy of the product ratios when several different substrates are used. Thus, partition experiments of the wild-type phosphoenzyme between water and Tris give a constant product ratio that is independent of the substrate leaving group, as is required by a mechanism that proceeds through a common reaction intermediate (Barrett et al., 1969). In contrast, the S102C alkaline phosphatase shows a variable product ratio that depends upon the nature of the substrate leaving group. While such variability does not disprove the existence of a common phosphoenzyme (since the substrate leaving group could still be enzyme-bound during the phospho-transfer step), it is clear that the path of catalysis followed by the mutant enzyme could be different from that of the wild-type enzyme. Indeed, all of the above could suggest that the thiol group does not participate directly in the catalytic act and that we must consider the possibility that S102C alkaline phosphatase uses a carbonic anhydrase type mechanism in which water (or Tris) ligated to Zn^{2+} is the primary nucleophile.

At this point, it seemed that only the stereochemical course of the phospho group transfer could give unambiguous information as to which of the two possible mechanisms is followed by the mutant enzyme. We have therefore determined the stereochemical course of the transphosphorylation from 4-nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]phosphate to (*S*)-propane-1,2-diol catalyzed by the S102C enzyme. The stereochemistry of the product has been analyzed by ^{31}P NMR spectroscopy as described earlier (Buchwald & Knowles, 1980).

Experimental Procedures

Materials. Chiral 4-nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]phosphate was synthesized as described by Freeman et al. (1987). Expression and isolation of mutant S102C alkaline phosphatase was as described previously (Ghosh et al., 1986). (*S*)-Propane-1,2-diol was purchased from Sigma. Diphenylphosphorylimidazole was synthesized according to the method of Dabkowski et al. (1968). Other materials were of analytical grade or were recrystallized and redistilled from bench-grade products. Water was double distilled from an all-glass apparatus.

Methods. Exploratory transphosphorylation experiments were first conducted to optimize the reaction conditions and to assess the purity of the isolated product. The progress of the reaction was followed by measuring the *p*-nitrophenol concentration at pH 8.0 ($\epsilon_{410} = 1.62 \times 10^4$ M^{-1} cm^{-1}). The free phosphate concentration was determined according to the method of Ames (1966). The fraction of phosphate transferred to propanediol was calculated from the difference in the concentrations of *p*-nitrophenol and free phosphate, and these values were confirmed by integration of the ^1H-decoupled ^{31}P NMR signals in the reaction mixtures. Organic phosphate was detected and quantified by ashing portions (100 μL) with $Mg(NO_3)_2$ (50 μL, 10% w/v) in ethanol as described by Ames (1966). All chromatographic separations were performed at 4 °C, and all evaporations of buffers and solvents were done under vacuum (1 Torr) at room temperature.

Transphosphorylation. The reaction mixture (21 mL) contained 50% (v/v) (*S*)-propane-1,2-diol, *p*-nitrophenyl (R_P)-phosphate bis(trimethylammonium) salt (75 mM), NaCl (375 mM), $NaHCO_3$ (112 mM), and Na_2CO_3 (112 mM), at pH 9.5. The enzyme concentration was 2.7 μM on the basis of a subunit molecular weight of 47 000 and $E^{1\%}_{278} = 0.72$ (Plocke & Vallee, 1962). The reaction mixture was left at room temperature for 100 h. At the end of this period, 64% of the chiral *p*-nitrophenyl phosphate had been consumed; 7.8% had been transferred onto (*S*)-propanediol.

The reaction mixture was quenched by passing through a column (2.5 × 5 cm) of AG50-X4 (H^+ form). The eluate was quickly neutralized with triethylamine and diluted with water to a final volume of 4 L. This solution was loaded onto a column (2.5 × 20 cm) of AG2-X8 (HCO_3^- form) previously equilibrated with a 50 mM triethylammonium bicarbonate buffer, pH 6.9. The phosphopropanediols were eluted from the column by using a linear gradient (2 + 2 L; 50–250 mM) of triethylammonium bicarbonate buffer, pH 6.9. Fractions (8 mL) were collected at a flow rate of 1.1 mL/min. The fractions containing a mixture of 1- and 2-phosphopropanediol (eluting between 122 and 135 mM) were pooled and evaporated to dryness. The residue was dissolved in isopropyl alcohol (10 mL) and the solvent then removed by evaporation. This procedure was repeated twice in order to eliminate traces of triethylammonium bicarbonate.

The triethylammonium salt of phosphopropanediol was converted to the disodium salt by consecutive passage through two columns (20 mL each) of AG50-X4 (H^+ and Na^+ form). The eluate was concentrated, dissolved in D_2O (1 mL), and concentrated again. After this procedure was repeated twice,

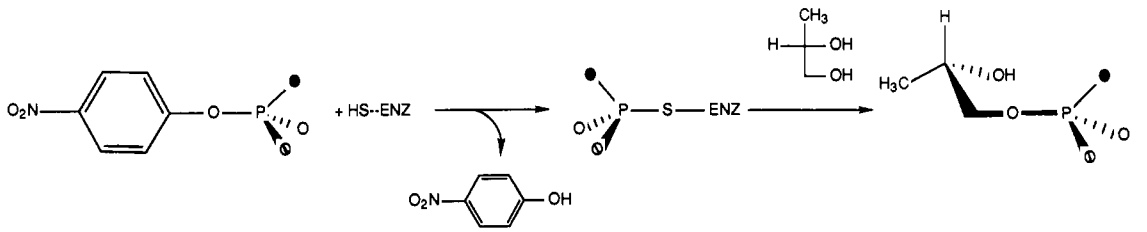

FIGURE 1: Double-displacement solvolysis by (*S*)-propane-1,2-diol of chiral 4-nitrophenyl phosphate catalyzed by mutant S102C alkaline phosphatase if the mechanism requires a phosphoenzyme intermediate. It is assumed that each step occurs with inversion at phosphorus. (O = ^{16}O; ⊘ = ^{17}O; ● = ^{18}O.)

the ^1H NMR spectrum of the residue was recorded in D_2O. No contaminants could be detected by ^1H NMR; only signals from the two phosphopropanediols were observed. The methyl group signals were assigned as described by Abbott et al. (1979): 1-phosphoisomer δ = 1.04 (d, J = 6.3 Hz); 2-phosphoisomer δ = 1.10 (d, J = 6.3 Hz). The chemical shift is in reference to (trimethylsilyl)propanesulfonic acid. Integration of these signals gave a ratio of 1-/2-phosphoisomer of 12:1. [The wild-type-catalyzed phospho group transfer occurs with a regioselectivity of 9:1 (Jones et al., 1978).] Total phosphate determination showed the sample to contain phosphoric esters equivalent to 6.5% of the starting chiral phosphate.

The sample of phosphopropanediols was converted to the bis(tri-*n*-butylammonium) salt by passage through a column (2.5 × 5 cm) of AG50-X4 (pyridinium form) and adding tri-*n*-butylamine (73 mg, 206 μmol) to the eluate. After the sample was evaporated to dryness, dry dioxane (5 mL) was added and then removed by evaporation. This procedure was then repeated. The crystalline salt was dissolved in dry CH_2Cl_2 (400 μL) and the solution stirred with molecular sieves (4 Å) for 5 h. Diisopropylethylamine (30 μL) and freshly prepared diphenylphosphorylimidazole (1 equiv, 150 μL of a CH_2Cl_2 solution containing 200 mg/mL) were added, and the mixture was stirred for 72 h at room temperature. Triethylammonium bicarbonate (50 mM) buffer (5 mL) was added, and the aqueous layer was washed with CH_2Cl_2 (3 × 5 mL). The aqueous phase was neutralized to pH 6 and diluted to the same conductivity as 10 mM triethylammonium bicarbonate buffer, pH 6.9. The solution was loaded onto a column (1.5 × 15 cm) of AG1-X8 (HCO_3^- form) previously equilibrated with the above buffer. The column was eluted with a linear gradient (10–250 mM, 250 + 250 mL) of the same buffer. The fractions eluting between 60 and 75 mM triethylammonium bicarbonate buffer contained the cyclized phosphate diester (68 μmol, 66% yield), which were pooled and then evaporated to dryness. The residue was dissolved in isopropyl alcohol (5 mL) and the solvent removed by evaporation. This procedure was repeated twice; the oil thus obtained was dissolved in acetonitrile-d_3 (4 mL) and sonicated with excess dry ethereal diazomethane. After evaporation to half the original volume, the product was transferred to a 10-mm tube, and the ^{31}P NMR spectrum was recorded immediately. The product is a mixture of the syn and anti cyclic phosphate triester (approximately 1:1).

Results and Discussion

Two possible mechanisms have been considered for the mutant alkaline phosphatase catalyzed hydrolysis of phosphate monoesters (Figure 1). On the basis that enzyme-catalyzed substitution steps at phosphorus are "in-line" displacements (Knowles, 1980), the two mechanisms have different stereochemical outcomes. If the reaction indeed proceeds through the formation of a covalent intermediate and the phospho group is subsequently transferred to water or to an alternate nucleophilic acceptor, then the configuration at phosphorus would show overall retention as the consequence of two successive inversions. On the other hand, the configuration at phosphorus would be inverted if the mutant-catalyzed solvolysis occurred in a single substitution step.

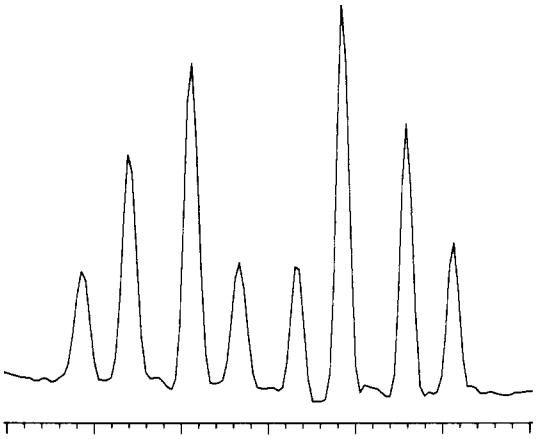

FIGURE 2: ^{31}P NMR spectrum of the cyclic phosphate triesters derived from the phospho-(*S*)-propane-1,2-diols. These were obtained by transphosphorylation catalyzed by mutant S102C alkaline phosphatase from *E. coli*, using 4-nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]phosphate as substrate. The spectrum was taken in a Nicolet NT360 spectrometer at 146.2 MHz with an internal deuterium field lock (spectral width, 1000 Hz; acquisition time, 4.10 s; pulse width, 21 μs; number of transients, 1000). The sample (34 mM) was dissolved in CD_3CN and the spectrum measured at room temperature. The scale is 1 Hz/division.

Our experiment demonstrates that the transphosphorylation catalyzed by the S102C mutant proceeds with retention at phosphorus: The product 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol was found to be 81 ± 5% R_P (Figure 2). Integration of the spectral data and calculations based on the isotopic composition of the phosphate group transferred that allow for the measured contamination of the product by the 2-phosphoisomer lead to the comparison of the observed and predicted peak intensities given in Table I. Within the error limits characteristic of this method, these data show that transphosphorylation from 4-nitrophenyl phosphate to propane-1,2-diol occurs with retention of configuration at phosphorus. This result shows that the transferred phosphorus atom undergoes an even number of displacements: The most probable pathway involves two inversions and a single phosphoenzyme intermediate.[1]

The observation that the catalytic efficiency of the S102C enzyme is not affected by the Tris concentration but is sensitive to the nature of the phenolic group is consistent with the hypothesis that the Ser to Cys mutation has changed the rate-determining step from release of product phosphate to

[1] The possibility of a pathway involving pseudorotation should formally be considered but is as yet unprecedented in enzymology.

Table I: Peak Integrals for the ^{31}P NMR Spectra (Figure 2) Obtained from the Stereochemical Analysis of the Mutant Enzyme Catalyzed Transphosphorylation from 4-Nitrophenyl (R_P)-[^{16}O,^{17}O,^{18}O]Phosphate to (*S*)-Propane-1,2-diol

	peak no.							
	1	2	3	4	5	6	7	8
observed	15.5	29.4	39.7	15.5	14.3	41.0	27.9	16.8
predicted[a] for *R*	13.6	25.8	45.0	15.6	13.6	45.0	25.8	15.6
predicted[a] for *S*	13.6	45.0	25.8	15.6	13.6	25.8	45.0	15.6

[a] Calculated on the basis of the known isotopic composition of 4-nitrophenyl phosphate and a 1-/2-phosphopropanediol ratio of 12:1 and by assuming 100% enantiomeric purity of the acceptor (*S*)-propane-1,2-diol.

phosphorylation of the enzyme. This seems to be the case over the whole pH range, and it explains why no kinetic burst could be observed. The unexpected dependence of the partition ratios on the chemical nature of the substrate leaving group probably reflects a situation in which the leaving group remains in the active site long enough to influence the selective binding of the alternate nucleophilic acceptor. Finally, the stereochemical course described in this paper provides strong evidence that the chemical path of the catalytic mechanism has been conserved in this mutant.

The Ser to Cys mutation in bacterial alkaline phosphatase has consequences that are analogous to the Glu to Asp mutation in triose phosphate isomerase (Raines et al., 1986): While the overall chemical mechanism is retained, each of these mutations changes the relative free energy of the transition states and alters the rate-limiting step of the catalyzed reaction (Knowles, 1987).

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