# Investigation of the *Bacillus cereus* Phosphonoacetaldehyde Hydrolase. Evidence for a Schiff Base Mechanism and Sequence Analysis of an Active-Site Peptide Containing the Catalytic Lysine Residue†

David B. Olsen,‡ Timothy W. Hepburn,‡ Malcolm Moos,§ Patrick S. Mariano,‡ and Debra Dunaway-Mariano*,‡

*Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, and Food and Drug Administration, Bethesda, Maryland 20892*

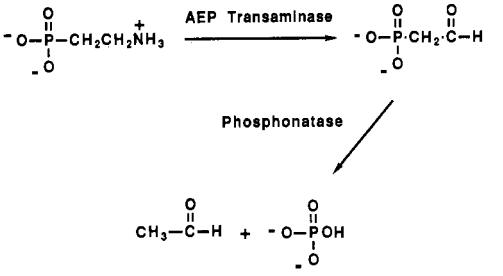
*Received August 31, 1987; Revised Manuscript Received November 9, 1987*

ABSTRACT: Reaction of *Bacillus cereus* phosphonoacetaldehyde hydrolase (phosphonatase) with phosphonoacetaldehyde or acetaldehyde in the presence of $NaBH_4$ resulted in complete loss of enzymatic activity. Treatment of phosphonatase with $NaBH_4$ in the absence of substrate or product had no effect on catalysis. Inactivation of phosphonatase with [$^3$H]$NaBH_4$ and phosphonoacetaldehyde, $NaBH_4$ and [$^{14}$C]acetaldehyde, or $NaBH_4$ and [2-$^3$H]phosphonoacetaldehyde produced in each instance radiolabeled enzyme. The nature of the covalent modification was investigated by digesting the radiolabeled enzyme preparations with trypsin and by separating the tryptic peptides with HPLC. Analysis of the peptide fractions revealed that incorporation of the $^3$H- or $^{14}$C-radiolabel into the protein was reasonably selective for an amino acid residue found in a peptide fragment observed in each of the three trypsin digests. Sequence analysis of the $^3$H-labeled peptide fragment isolated from the digest of the [2-$^3$H]phosphonoacetaldehyde/$NaBH_4$-treated enzyme identified $N^{\epsilon}$-ethyllysine as the radiolabeled amino acid. The ability of the phosphonatase competitive inhibitor ($K_i$ = 230 ± 20 $\mu$M) acetonylphosphonate to protect the enzyme from phosphonoacetaldehyde/$NaBH_4$-induced inactivation suggested that the reactive lysine residue is located in the enzyme active site. Comparison of the relative effectiveness of phosphonoacetaldehyde and acetaldehyde as phosphonatase inactivators showed that the *N*-ethyllysine imine that is reduced by the $NaBH_4$ is derived from the corresponding *N*-(phosphonoethyl) imine. On the basis of these findings, a catalytic mechanism for phosphonatase is proposed in which phosphonoacetaldehyde is activated for P–C bond cleavage by formation of a Schiff base with an active-site lysine. Accordingly, an *N*-ethyllysine enamine rather than the high-energy acetaldehyde enolate anion is displaced from the phosphorus.

Phosphorus is found in biological systems principally in the forms of inorganic phosphate and pyrophosphate and as organic phosphate esters, anhydrides, and phosphoanhydrides. The biosynthesis and biodegradation of these compounds involve phosphoryl-transfer reactions that are catalyzed by enzymes specialized in inducing P–O bond formation and P–O bond cleavage. In recent years phosphonates have been recognized as a new class of phosphorus-containing natural products [for review on this topic see Mastalerz (1984) and Hilderbrand (1983)]. These compounds, in which the phosphorus atom is bonded directly to a carbon atom, have been found in a variety of organisms. In some of these organisms, rather elaborate phosphonopeptides and phosphonolipids are synthesized, while in others ingested phosphonates serve as a source of carbon and/or phosphorus.

Enzymes that catalyze phosphoryl-transfer reactions of inorganic or organic phosphates appear to do so by an associative process wherein an oxyanion (or functional equivalent) attacks the phosphoryl phosphorus atom, displacing a second oxyanion (or equivalent) as the leaving group. The phosphotransferase either binds the oxyanion nucleophile directly from solution or generates the oxyanion by deprotonation of the bound acid form of the nucleophile. Likewise, the oxyanion leaving group may be stabilized by the enzyme through hydrogen-bonding interactions with an active-site residue or through coordination to a metal ion cofactor. Because the p$K_a$ values of carbon acids are so large, we expect the catalytic mechanism of enzymes catalyzing P–C bond formation and P–C bond cleavage in phosphonate biosynthetic and biodegradative pathways to differ significantly from those catalyzing phosphoryl-transfer reactions of phosphates. Not surprisingly, phosphonate analogues of phosphate substrates are inert to the action of phosphoryl-transfer enzymes (Engel, 1976).

Scheme I: Pathway for AEP Degradation in *B. cereus* and *P. aeruginosa*




To date, little is known about the mechanism of enzyme-catalyzed P–C bond cleavage (Wackett et al., 1987; Cordeiro et al., 1986; Frost et al., 1987; Cassaigne et al., 1976; Daughton et al., 1979; Dumora, et al., 1983; La Nauze & Rosenberg, 1968; La Nauze et al., 1970, 1977), and even less information about the mechanism of enzyme-catalyzed P–C bond formation (Seto et al., 1982; Warren, 1968; Horiguchi & Rosenberg, 1975) is available. Ongoing studies in our laboratory are

† This work was supported by grants from the NIH (GM 28688 and GM 29016). D.D.-M. acknowledges support by a NIH Career Development Award (ES-00111) and by an Alfred P. Sloan Foundation Award.

* Author to whom correspondence should be addressed.
‡ University of Maryland.
§ Food and Drug Administration.

0006-2960/88/0427-2229$01.50/0 

focused on the enzymology of P–C bond formation and cleavage in the biosynthesis and biodegradation of the most predominant and ubiquitous naturally occurring phosphonate, (2-aminoethyl)phosphonate (AEP).[1] AEP biodegradation in *Bacillus cereus* and *Pseudomonas aeruginosa* has been most thoroughly examined (Dumora et al., 1983; La Nauze & Rosenberg, 1968; La Nauze et al., 1970, 1977). In these bacterial systems, AEP is ultimately converted to acetaldehyde and orthophosphate. As indicated in Scheme I, the first step of AEP biodegradation in these systems is the enzymic transamination of AEP to produce phosphonoacetaldehyde. The transaminase of *P. aeruginosa* has been purified and shown to use pyruvate as the amino group acceptor (Dumora et al., 1983). La Nauze et al. (1970, 1977) have purified and characterized the phosphonoacetaldehyde hydrolase (phosphonatase) from *B. cereus*.

In this study we have extended the work of La Nauze et al. (1970, 1977) to examine the catalytic mechanism of the *B. cereus* phosphonatase. Here we report evidence for the intermediacy of a Schiff base between an active-site lysine and phosphonoacetaldehyde. In addition, the sequence of the lysine-containing active-site peptide is reported. These results and their interpretation in terms of a mechanism for enzyme-catalyzed P–C bond cleavage are discussed below.

## Materials and Methods

### *Materials*

*Cell-Free Preparations of Bacillus cereus (AI-2).* The (AI-2) variant of *B. cereus* was a kind gift from Dr. La Nauze of the University of Melbourne. The bacteria were cultured on agar plates prepared from AG media (La Nauze et al., 1970) containing AEP as the sole phosphorus source. Inocula were prepared by growth on liquid AG media before overnight growth on PPYG media (La Nauze et al., 1970). The cells were centrifuged and resuspended in a phosphate-free medium as previously described (La Nauze et al., 1970). The harvested cells (ca. 160 g) were resuspended in 550 mL of 50 mM triethanolamine (pH 7.5) containing 10 mM $MgCl_2$ and 0.1 mM dithiothreitol. The bacteria were lysed by using a French pressure cell press at 19 000 psi, and the resulting homogenate was centrifuged for 20 min at 10 000 rpm. [$^3H$]*NaBH*$_4$ (SA = 528 $\mu$Ci/$\mu$mol), [$^3H$]*H*$_2$*O* (SA = 18 $\mu$Ci/$\mu$mol), and [$^{14}C$]*acetaldehyde* (SA = 9.5 $\mu$Ci/$\mu$mol) were purchased from New England Nuclear. [*2-*$^3H$]*Phosphonoacetaldehyde* was prepared by adding 10 $\mu$L of 47 mM phosphonoacetaldehyde (Isabell et al., 1969) (pH 9) to 100 $\mu$L of [$^3$H]$H_2O$ (18 $\mu$Ci/$\mu$mol) containing 20 mM $MgCl_2$. The solution was incubated at 25 °C for 24 h. During this period complete exchange had occurred without degradation of the phosphonoacetaldehyde.

*N*$^\epsilon$*-Ethyl-*L*-lysine.* The synthesis of this compound was adapted from the procedure used by Means et al. (1968) for the synthesis of *N*-ethylbutylamine. *N*$^\alpha$-(Carbobenzyloxy)-L-lysine (0.4 g, 1.43 mmol) and 430 $\mu$L of acetaldehyde were added to 70 mL of boric acid buffer (200 mM, pH 8.9) at 0 °C. Solid sodium borohydride (0.08 g) was then added over a 2-min period, and the resulting solution was stirred for 1 h at 0 °C and then evaporated to dryness in vacuo at 25 °C, redissolved in 50 mL of $H_2O$, and made acidic with the addition 6 N HCl (pH 2.0). The borate buffer was removed by rotary evaporation with 3 × 50 mL portions of methanol. The *N*$^\alpha$-CBZ-*N*$^\epsilon$-ethyl-L-lysine was dried over $P_2O_5$ and converted to its methyl ester with thionyl chloride and methanol (Bezas & Zervas, 1961). The reaction mixture was concentrated to 20 mL, and 3 × 30 mL portions of benzene were added and evaporated to remove excess thionyl chloride and solvent. The methyl ester was extracted from an aqueous solution with 3 × 50 mL portions of chloroform, and ca. 100 mg of it was applied to a silica gel preparative TLC plate. The TLC was developed by using acetone as the mobile phase. This procedure removed starting material and the small amount of *N*$^\alpha$-CBZ-*N*$^\epsilon$,*N*$^\epsilon$-diethyl-L-lysine produced by the reaction. The purified product (46 mg) was converted to *N*$^\epsilon$-ethyl-L-lysine by hydrolysis in 1 mL of 0.1 N HCl at 70 °C for 3 h (unoptimized yield 10%). The byproducts were removed by rotary evaporation: $^1$H NMR ($D_2O$) $\delta$ 1.21 (t, $J$ = 7.2 Hz, $NCCH_3$, 3 H), 1.49 (m, $CCH_2C$, 2 H), 1.67 (m, $CCH_2C$, 2 H) 1.89 (m, $CCH_2C$, 2 H), 3.00 (m, $NCH_2$, 4 H), 3.99 (t, $J$ = 6.1 Hz, NCHC, 1 H).

*N*$^\epsilon$*-(2-Phosphonoethyl)-*L*-lysine.* *N*$^\alpha$-(Benzyloxycarbonyl)-L-lysine (83 mg, 0.296 mmol) was dissolved in 15 mL of $H_2O$ containing 223 mg (1.64 mmol) of phosphonoacetaldehyde dilithium salt. This mixture was cooled to 0 °C, and 18.5 mg (0.489 mmol) of $NaBH_4$ was added over a 2-min period. The reaction was stirred for 1 h at 0 °C and then applied to a Dowex 1 (carbonate) anion-exchange column (2.2 × 45 cm). The amino acid derivative was eluted from the column by using a 1-L linear gradient of 0.0–0.7 M ammonium carbonate. The chromatography was monitored at 255 nm, and the product-containing fractions were concentrated in vacuo to yield 97 mg (75% yield) of the ammonium salt.

*N*$^\epsilon$-(Phosphonoethyl)-L-lysine was generated by heating 97 mg of CBZ-*N*$^\epsilon$-(2-phosphonoethyl)-L-lysine in 5 mL of 1 N HCl at 70 °C for 12 h. The solution was cooled to 25 °C and extracted with 5 mL of ether. The aqueous phase was concentrated by rotary evaporation and triturated with ethanol to remove the ammonium chloride. The residue was dried in vacuo to give 70 mg (98%) of the dihydrochloride: $^{31}$P NMR ($D_2O$, pH 7.3) 17.7 ppm; $^1$H NMR ($D_2O$, pH 7.3) $\delta$ 1.43 (m, $CCH_2C$, 2 H), 1.65 (m, $CCH_2C$, 2 H), 1.88 (m, $CCH_2C$, 2 H), 2.15 (m, $CCH_2P$, 2 H), 2.99 (t, $J$ = 7.7 Hz, $NCH_2C$, 2 H), 3.16 (m, $NCH_2P$, 2 H), 4.02 (t, $J$ = 6.2 Hz, NCHC, 1 H); $^{13}$C NMR ($D_2O$, pH 7.3) $\delta$ 174.7 (C=O), 55.5 (NCH), 49.5 (N=$CH_2$), 45.6 ($NCH_2$), 31.9 ($CH_2$), 27.7 ($CH_2$), 27.3 (d, $J$ = 133.0 Hz, $CH_2P$), 24.1 ($CH_2$).

*Acetonylphosphonate.* Acetonylphosphonic dichloride was prepared according to the method of Lutsenko and Kirilov (1960). To a solution of phosphorus pentachloride (41.75 g, 0.2 mol) in carbon tetrachloride (300 mL) at −25 °C isopropenyl acetate (11.0 mL, 0.1 mol) was added dropwise over 20 min. The solution was stirred for 2 h at −25 °C, and then sulfur dioxide was bubbled through until the white precipitate was dissolved. The solvent and resulting phosphoryl chloride were removed in vacuo. The residue was heated to 100 °C for 1 h under vacuum to remove acetyl chloride. Without purification, the mixture was dissolved in THF and cooled to 0 °C, and 5 mL of water were added. After 1 h at 25 °C, the THF was removed by rotary evaporation, and the residue was redissolved in 100 mL of $H_2O$. The solution was made alkaline with LiOH (pH 9.0), decolorized with activated charcoal, and then concentrated to 15 mL. The lithium salt

[1] Abbreviations: AEP, (2-aminoethyl)phosphonate; THF, tetrahydrofuran; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NADH, dihydronicotinamide adenine dinucleotide; FPLC, fast protein liquid chromatography; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid; DEAE, diethylaminoethyl; TLC, thin-layer chromatography; SA, specific activity; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; $P_i$, inorganic phosphate.

of the product was precipitated with absolute ethanol, isolated by centrifugation, dissolved in $H_2O$, and then concentrated to dryness in vacuo to yield 6.05 g (40.3%) of white solid: $^{31}P$ NMR ($D_2O$, pH 10.1) 10.10 ppm (t, $J$ = 20.2 Hz); $^1H$ NMR ($D_2O$, pH 10) $\delta$ 2.23 (s, $COCH_3$, 3 H), 2.85 (d, $J$ = 20.2 Hz, $COCH_2P$, 2 H); $^{13}C$ NMR ($D_2O$, pH 10) $\delta$ 216.1 (d, CCP, $J$ = 4.6 Hz), 51.6 (d, CP, $J$ = 102.5 Hz), 33.3 (s, $CH_3$).

## Methods

*Purification of Phosphonatase.* Phosphonatase was prepared by using a modification of the method reported by La Nauze et al. (1970). All operations were carried out at 4 °C. Six hundred milliliters of cell-free extract (see Materials) was treated with 120 mL of 0.45% protamine sulfate solution to precipitate nucleic acids. The enzyme was precipitated from the supernatant as the 40–70% $(NH_4)_2SO_4$ protein cut. The enzyme was dialyzed against buffer containing 50 mM triethanolamine (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M NaCl and then loaded onto a DEAE-Sephadex A-50-120 column (5 × 25 cm). Chromatography was carried out by using 2 L of a linear gradient NaCl (0.15–0.40 M). The phosphonatase activity [detected by using the yeast alcohol dehydrogenase–NADH spectrophotometric assay (La Nauze et al., 1970)] eluted at ca. 0.3 M NaCl. The phosphonatase-containing fractions were combined, concentrated with an Amicon protein concentrator, and then chromatographed on a Sephadex G-150 column (3.2 × 100 cm) with triethanolamine (50 mM, pH 7.5)–$MgCl_2$ (10 mM)–dithiothreitol (0.1 mM) buffer as eluant. The phosphonatase-containing fractions were pooled and then concentrated to ca. 10 mL. The protein solution was then chromatographed by FPLC (Pharmacia LCC-500) with a Pharmacia Mono Q HR 5/5 anion-exchange column and 20 mL of a linear gradient of 0.0–0.4 M NaCl in buffer [50 mM triethanolamine (pH 7.5), 10 mM $MgCl_2$, and 0.1 M dithiothreitol] (1.0 mL/min flow rate). The enzyme eluted at a NaCl concentration of 0.25 M and was judged on the basis of SDS gel electrophoresis to be greater than 90% pure (SA = 6 units/mg; yield = 10 mg). The enzyme (1 mg/mL) was stored at −80 °C in the triethanolamine–$Mg^{2+}$–DTT buffer.

*Acetonylphosphonate Inhibition.* The $K_i$ of acetonylphosphonate was determined by measuring the initial velocity of phosphonatase-catalyzed hydrolysis of phosphonoacetaldehyde as a function of substrate concentration (17–150 $\mu$M) and acetonylphosphonate inhibitor concentration (0 and 0.5 mM). Reaction solutions contained 5 mM $MgCl_2$ and 30 mM HEPES (pH 6.9). The $K_i$ value was calculated from the initial velocity data by using the rate equation for competitive inhibition

$$v = VA/[K_a(1 + I/K_i) + A] \quad (1)$$

where $v$ is the initial velocity, $V$ is the maximal velocity, $A$ is the concentration of the substrate, $K_a$ is the Michaelis constant for the substrate, and $I$ is the concentration of the inhibitor.

*Inactivation of Phosphonatase with $NaBH_4$.* In these experiments 25 $\mu$M phosphonatase in 20 $\mu$L of 0.1 M Taps (pH 8.5) and 5 mM $MgCl_2$ was reacted at 0 °C for 30 s with acetaldehyde, phosphonoacetaldehyde, acetonylphosphonate, or phosphonoacetaldehyde plus acetonylphosphonate in the presence of 75 $\mu$M $NaBH_4$. Aliquots of the reaction mixtures were assayed for phosphonatase activity with 1-mL assay solutions. Control reactions containing enzyme, buffer, $MgCl_2$, and $NaBH_4$ were run under identical conditions.

*Preparation of Radiolabeled Phosphonatase.* (*a*) *Phosphonoacetaldehyde plus [$^3H$]$NaBH_4$.* Tritiated enzyme was prepared by the simultaneous additions of 2.5 $\mu$L of 40 mM [$^3H$]$NaBH_4$ (SA = 2.5 × $10^{13}$ cpm/mol) and 5 $\mu$L of 1 mM phosphonoacetaldehyde (both at 0 °C) to 92.5 $\mu$L of an ice-cooled solution of HEPES (0.1 M, pH 7), $MgCl_2$ (8 mM), and phosphonatase (15.3 nmol). After 30 s, the reaction was diluted with 1 mL of 50 mM triethanolamine (pH 7.5) and dialyzed against 3 × 4 L portions of 50 mM triethanolamine (pH 7.5, 4 °C).

(*b*) [$^{14}C$]*Acetaldehyde plus $NaBH_4$.* $^{14}C$-Labeled phosphonatase was prepared by the addition of 7.5 $\mu$L of 40 mM $NaBH_4$ (0 °C) to an ice-cooled solution containing HEPES (0.1 M, pH 7), $MgCl_2$ (8 mM), [$^{14}C$]acetaldehyde (10 mM, SA = 2.3 × $10^{13}$ cpm/mol), and 93.5 nmol of enzyme. After 30 s, the reaction was terminated, and the labeled enzyme was dialyzed as described above.

(*c*) [2-$^3H$]*Phosphonoacetaldehyde plus $NaBH_4$.* One hundred ten microliters of 4.3 mM [2-$^3H$]phosphonoacetaldehyde (0 °C) (see above) was added simultaneously with 5 $\mu$L of 40 mM $NaBH_4$ to an ice-cooled solution containing HEPES (0.1 M, pH 7), $MgCl_2$ (8 mM), and 219 nmol of enzyme. The reaction was terminated after 30 s, and the labeled enzyme was dialyzed as described above.

*Digestion of Radiolabeled Enzyme.* The lyophilyzed enzyme was oxidized with performic acid (Hirs, 1956), lyophilized, and then dissolved in 6 M urea–50 mM $NH_4HCO_3$ buffer (pH 8.2). After 2 h of incubation at 37 °C, the solution was diluted to 2 M urea with 50 mM $NH_4HCO_3$. Three additions of trypsin (2% w/w) were made over a 24-h period (37 °C). The digested enzyme was lyophilyzed and then dissolved in 0.05% TFA.

*HPLC Fractionation and Sequence Determination of the Radiolabeled Tryptic Peptide.* Aliquots of the digested enzyme were subjected to reverse-phase high-performance liquid chromatography (Perkin-Elmer Series 3B chromatography system). The column used for separation of the tryptic peptides generated from the [2-$^3H$]phosphonoacetaldehyde-labeled enzyme was an Alltech 250 × 4.6 mm Econosphere 300 reverse-phase $C_{18}$ column. The column used for the separation of the tryptic peptides generated from the [$^{14}C$]acetaldehyde- and [$^3H$]$NaBH_4$-labeled enzymes was an Alltech 250 × 46 mm Ultrasphere-ODS reverse-phase $C_{18}$ column. Solvents A (0.05% aqueous TFA) and B (0.05% TFA in acetonitrile) were used. The column was eluted isocratically for 5 min with 0.1% solvent A at 0.8 mL/min. At this point the following linear gradients were employed: T-1, 65 min (0–35% solvent B); T-2 40 min (35–60% solvent B); T-3 15 min (60–90% solvent B). The elution profile was monitored at 220 nm. Eight-hundred-microliter fractions were collected and assayed for radioactivity. After a final purification step (Applied Biosystems Model 130A chromatograph; $C_8$ reverse-phase cartridge, 0.1% TFA–acetonitrile gradient), the amino acid sequence of the radiolabeled peptide was determined by using an Applied Biosystems 470A sequence and a 120A online PTH analyzer.

## Results and Discussion

Enzymic P–C bond cleavage in phosphonoacetaldehyde is analogous to the C–C and C–H bond cleavage reactions catalyzed by $\beta$-keto acid decarboxylases and aldolases. Previous studies of the mechanism of action of the decarboxylases and aldolases have shown that imine formation between substrate and an active-site lysine is often a prelude to C–H or C–C bond cleavage [for a general discussion of these reactions see Walsh (1979)]. In other cases the enolate anion formed by C–H or C–C bond cleavage is stabilized by coordination to an enzyme-bound $Mg^{2+}$ ion.

The initial studies of phosphonatase carried out by La Nauze et al. (1970) demonstrated that catalysis by this enzyme

Table I: Inactivation of Phosphonatase with $NaBH_4$[a]

| expt | reaction components | inactivation (%) |
|---|---|---|
| 1 | enzyme, $NaBH_4$ | 0 |
| 2 | enzyme, 10 mM acetaldehyde, $NaBH_4$ | 100 |
| 3 | enzyme, 10 mM phosphonoacetaldehyde, $NaBH_4$ | 100 |
| 4 | enzyme, 2 mM acetaldehyde, $NaBH_4$ | 25 |
| 5 | enzyme, 2 mM phosphonoacetaldehyde, $NaBH_4$ | 100 |
| 6 | enzyme, 250 $\mu$M acetaldehyde, $NaBH_4$ | 5 |
| 7 | enzyme, 250 $\mu$M phosphonoacetaldehyde, $NaBH_4$ | 100 |
| 8 | enzyme, 180 $\mu$M phosphonoacetaldehyde, $NaBH_4$ | 100 |
| 9 | enzyme, 10 mM acetonylphosphonate, $NaBH_4$ | 0 |
| 10 | enzyme, 180 $\mu$M phosphonoacetaldehyde, 10 mM acetonylphosphonate, $NaBH_4$ | 45 |

[a] Reactions were carried out for 30 s at 0 °C. Reactions contained 25 $\mu$M phosphonatase, 75 $\mu$M $NaBH_4$, 5 mM $Mg^{2+}$, and 100 mM TAPS (pH 8.5).

is dependent upon the presence of a bound $Mg^{2+}$ ion. However, further investigation showed that the metal ion does not coordinate to the substrate and that its probable cofactor role is a structural one in which it stabilizes the active homodimer form of the enzyme. To test the possibility that the phosphonoacetaldehyde may be activated for P–C bond cleavage by imine formation with an active-site lysine, La Nauze et al. (1977) treated the enzyme with $KBH_4$ in the presence and absence of 10 mM phosphonoacetaldehyde or 10 mM acetaldehyde. Inactivation of the enzyme, presumably via reduction of the enzyme–substrate and enzyme–product adduct, was observed. On the basis of this result La Nauze et al. (1977) proposed that the phosphonatase-catalyzed reaction proceeds by way of a Schiff base intermediate.

Our own studies of the mechanism of action of phosphonatase have been initially focused on determining if the catalyzed hydrolysis of phosphonoacetaldehyde does proceed via a Schiff base intermediate as implicated by the observed $KBH_4$ inactivation. First, we repeated the original inactivation experiment under less forcing conditions (0 °C, 30 s, 75 $\mu$M $NaBH_4$) than were used by La Nauze et al. (1977) (30 °C, 30 min, 10 mM $KBH_4$). As indicated by the data displayed in Table I, even under these rather mild conditions inactivation of the enzyme occurs when it is treated with $NaBH_4$ in the presence of 10 mM phosphonoacetaldehyde or 10 mM acetaldehyde. Treatment of the enzyme with $NaBH_4$ alone did not result in inactivation.

We next set out to determine whether or not the chemical modification of the enzyme by substrate or product in combination with $NaBH_4$ was specific for a single lysine and, if so, to determine the structure of the modified lysine. Three approaches to this problem were tried. First, the enzyme was treated with [$^3$H]$NaBH_4$ and phosphonoacetaldehyde. Trypsin-catalyzed hydrolysis of the modified protein gave tryptic peptides that were separated by reverse-phase HPLC. The tritium content of the separated peptides was determined by liquid scintillation counting. A number of the peptide fractions were found to contain tritium. However, the two fractions that eluted with 21% and 27% $CH_3CN$ contained by far the greatest amount of radiolabel. When the reaction time was increased from 30 s to 3 min, the amount of tritium found distributed among the peptide fractions was significantly increased. Treatment of the enzyme alone with [$^3$H]$NaBH_4$ also lead to tritium incorporation. These findings suggested that the [$^3$H]$NaBH_4$ reacts directly with the protein in addition to reducing the Schiff base.[2] Repetition of the [$^3$H]-

[2] The reduction of proteins with $NaBH_4$ is well documented (Crestfeld et al., 1960; Seon, 1967).

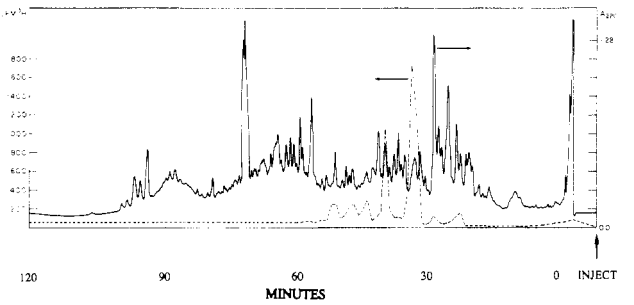



FIGURE 1: Reverse-phase HPLC elution profile of the tryptic peptides generated from [2-$^3$H]phosphonoacetaldehyde/$NaBH_4$-treated phosphonatase. Peptide elution was carried out by using a step gradient of 0.05% TFA in water and 0.05% TFA in $CH_3CN$ as described under Methods. (—) $A_{220}$; (···) cpm.

Chart I: Amino Acid Sequence of the (A) Major (20% $CH_3CN$) [$^3$H]Peptide and (B) Minor (23% $CH_3CN$) Peptide Isolated from the Tryptic Digest of the [2-$^3$H]Phosphonoacetaldehyde/$NaBH_4$-Treated Phosphonatase

(A)-Leu-[$^3$H]ethyl-Lys-Ile-Asp-His-Val-Arg
(B)-Tyr-([$^3$H]?)-Asn-Ala-Leu-Val-Tyr-Arg

$NaBH_4$/phosphonoacetaldehyde labeling experiment consistently produced a 27% $CH_3CN$ radiolabeled peptide with high specific activity. Although we suspected that the 27% $CH_3CN$ [$^3$H]peptide contained the reduced Schiff base, we could not be sure that it was the only [$^3$H]peptide that did. Thus, other radiolabeling methodologies were evaluated.

Reaction of phosphonatase with [$^{14}$C]acetaldehyde and $NaBH_4$ followed by trypsin digestion and HPLC separation produced two $^{14}$C-containing fractions. The elution position of the first fraction (27% $CH_3CN$) coincided with the elution position of the [$^3$H]peptide generated from the [$^3$H]-$NaBH_4$/phosphonoacetaldehyde-treated enzyme. The other $^{14}$C-labeled peptide eluted with 39% $CH_3CN$. The longer retention time of this peptide suggests that it might be accounted for by incomplete digestion of the specifically labeled protein. On the other hand, acetaldehyde is very reactive with primary amines such as lysine, and the formed Schiff bases are easily reduced by $NaBH_4$. Therefore, we could not rule out the possibility that modification of a lysine residue, other than the one responsible for catalysis, gives rise to the 39% $CH_3CN$ [$^{14}$C]peptide.

In order to increase the specificity of the labeling reagent, the potentially less reactive phosphonoacetaldehyde was used to incorporate the radiolabel. The HPLC profile (obtained by using an HPLC column different from the ones used for fractionation of the tryptic digests described above) of the tryptic digest generated from the [2-$^3$H]phosphonoacetaldehyde/$NaBH_4$-treated enzyme is shown in Figure 1. As can be seen from the profile, a single fraction (eluted with 20% $CH_3CN$) contained a majority of the tritium. A second fraction eluted with 23% $CH_3CN$ contained 30% of the radioactivity. The two $^3$H-containing fractions were further purified on a HPLC column. In each case the radioactivity coincided with a single peptide, which in turn was subjected to sequence analysis. The sequences obtained are shown in Chart I.

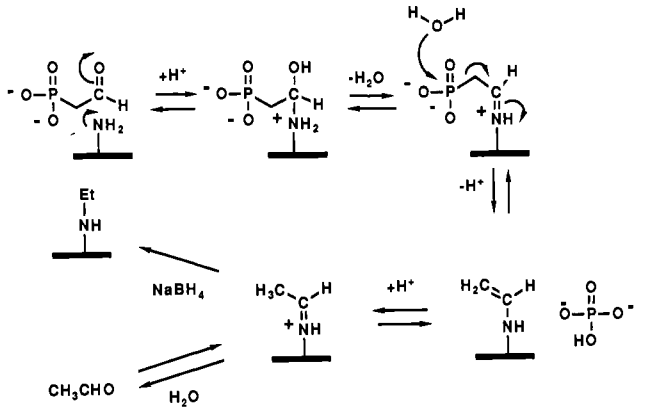
The radiolabeled amino acid of the major (or 20% $CH_3CN$) [$^3$H]peptide was identified by comparison to synthetic standards (i.e., $N^{\epsilon}$-ethyllysine and $N^{\epsilon}$-(phosphonoethyl)lysine) as $N$-ethyllysine. The sequence of the minor (or 23% $CH_3CN$) [$^3$H]peptide (Figure 1) was found to be different from that of the major [$^3$H]peptide. In addition, the $^3$H-labeled amino acid generated from the minor [$^3$H]peptide did not coelute with any of the amino acid standards including the $N^{\epsilon}$-ethyllysine

and $N^{\epsilon}$-(phosphonoethyl)lysine. Although we presently cannot account for the presence of radiolabel found in the 23% $CH_3CN$ [$^3$H]peptide fraction, we can conclude that a single lysine of phosphonatase is N-ethylated by reaction of the enzyme with phosphonoacetaldehyde and $NaBH_4$. Since the tryptic peptides of the [$^3$H]$NaBH_4$/phosphonoacetaldehyde- and [$^{14}$C]acetaldehyde/$NaBH_4$-treated phosphonatase were separated on a different type of $C_{18}$ HPLC column than was used to separate the tryptic peptides of the [2-$^3$H]-phosphonoacetaldehyde/$NaBH_4$-treated enzyme (see Methods), the elution positions of the radiolabeled peptides are not directly comparable. We assume, however, that the 27% $CH_3CN$ [$^3$H]peptide and the 27% $CH_3$ [$^{14}$C]peptide obtained in the first two experiments are equivalent in sequence not only with each other but also with the 20% $CH_3CN$ [$^3$H]peptide generated from the [2-$^3$H]phosphonoacetaldehyde/$NaBH_4$-treated enzyme. Thus, both phosphonoacetaldehyde and acetaldehyde lead to an *N*-ethyllysine imine that is trapped by reduction by the $NaBH_4$.

Having found that a single phosphonatase lysine does indeed react with substrate to form a Schiff base, we next addressed the question of whether this lysine is located in the enzyme's active site. One approach to this problem was to determine whether or not an inert structural analogue of phosphonoacetaldehyde could protect the enzyme from $NaBH_4$-induced inactivation by competing with phosphonoacetaldehyde for the substrate binding site. First, a number of commercial and synthetic analogues of phosphonoacetaldehyde were tested as potential tight binding inhibitors. The only compound that was found to have a $K_i$ value less than 1 mM is acetonylphosphonate ($K_i$ = 230 ± 20 $\mu$M). Acetonylphosphonate competes with phosphonoacetaldehyde for the substrate binding site on the enzyme but it does not appear to serve as a substrate for the enzyme. Even the use of very high levels of enzyme in conjunction with a long incubation time did not lead to detectable levels of acetone and $P_i$ in the incubation mixtures. Under the conditions used in the protection experiment [10 mM acetonylphosphonate, 0.18 mM phosphonoacetaldehyde ($K_m$ = 40 $\mu$M)], ca. 89% of the enzyme would be complexed with acetonylphosphonate and ca. 11% with phosphonoacetaldehyde. We observed that 65% of the enzyme was protected by the acetonylphosphonate from the $NaBH_4$-induced inactivation process (Table I). This finding suggests that the lysine modified by phosphonoacetaldehyde and $NaBH_4$ is in the active site of the enzyme.[3]

The intermediacy of the Schiff base formed between the phosphonatase lysine residue and phosphonoacetaldehyde during catalysis is also implicated by the finding that an *N*-ethyllysine residue rather than an *N*-(phosphonoethyl)lysine residue is isolated from the active-site tryptic peptide of $NaBH_4$/phosphonoacetaldehyde-treated enzyme. This finding demonstrates that an *N*-ethyllysine imine is formed from phosphonoacetaldehyde and enzyme. This imine could be formed via dephosphonylation of the *N*-(phosphonoethyl)lysine imine or, alternatively, from the acetaldehyde generated as a result of dephosphonylation of the phosphonoacetaldehyde. These two routes can be distinguished by comparing the relative efficiency of phosphonoacetaldehyde- and acetaldehyde-induced inactivation of phosphonatase with $NaBH_4$. As indicated by the data shown in Table I, reduction in the concentration of the acetaldehyde used in the inactivation reaction mixture from 10 mM to 2 mM or 250 $\mu$M dramatically reduces the amount of enzyme that is inactivated. In contrast, 180 $\mu$M phosphonoacetaldehyde causes complete inactivation of the enzyme. Importantly, acetaldehyde and phosphonoacetaldehyde undergo minimal reduction (<10%) by the $NaBH_4$ under the conditions of the phosphonatase inactivation experiment. Thus, the observed greater efficiency of the phosphonoacetaldehyde as an inactivator serves to rule out the inactivation pathway involving *N*-ethyllysine imine formation from acetaldehyde generated by dephosphonylation of phosphonoacetaldehyde.

Scheme II: Phosphonatase Reaction Pathways Involved in $NaBH_4$/Phosphonoacetaldehyde-Induced Inactivation and in Phosphophonoacetaldehyde Hydrolysis




Since the phosphonoacetaldehyde/$NaBH_4$-induced inactivation of phosphonatase (Scheme II) proceeds via reduction of the *N*-ethyllysine imine produced by dephosphonylation of the *N*-(phosphonoethyl)lysine imine, it can be argued that the conversion of phosphonoacetaldehyde to acetaldehyde and orthophosphate proceeds through these same imine intermediates. Failure to trap the *N*-(phosphonoethyl)lysine imine with the $NaBH_4$ treatment indicates that this imine dephosphonylates faster than it undergoes reduction. This result is not surprising. The acetoacetate/$NaBH_4$ inactivation of acetoacetate decarboxylase involves reduction of the *N*-isopropyllysine imine formed by decarboxylation of the *N*-(isopropylcarboxy)lysine imine (Warren et al., 1966). Apparently, charged substituents such as the $PO_3^{2-}$ or $CO_2^-$ group retard reduction of the imine. Repulsion between the negatively charged substituent and the borohydride would also account for the relatively slow rate of acetonyl phosphonate/$NaBH_4$-induced inactivation of phosphonoacetaldehyde.[3]

The *N*-ethyllysine imine appears to undergo reduction faster than hydrolysis. As a product inhibitor, acetaldehyde has (as does $P_i$) a $K_i$ value that is equal to or greater than 100 mM (La Nauze et al., 1970). Thus, it is not surprising that the amount of *N*-ethyllysine imine found at steady state in the phosphonatase-catalyzed hydrolysis of phosphonoacetaldehyde would be more than that found in the corresponding equilibrium mixture of enzyme and acetaldehyde. Since each of the phosphonoacetaldehyde analogues tested (including the alcohol) that do not form a Schiff base with the enzyme have $K_i$ values $\gg$1 mM (unpublished results), we attribute the low $K_m$ value (40 $\mu$M) of phosphonoacetaldehyde to the high steady-state level of the Schiff base(s).

[3] Although under the conditions of this experiment acetonylphosphonate itself does not cause inactivation of the enzyme in the presence of $NaBH_4$, after significantly longer incubation periods, inactivation is observed (unpublished results). This result indicates that acetonylphosphonate, like phosphonoacetaldehyde, forms a Schiff base with the active-site lysine residue. Since the amount of protein protected by the acetonylphosphonate roughly corresponds to that predicted on the basis of the relative concentrations of acetonylphosphonate and phosphonoacetaldehyde in the reaction mixture and on the acetonylphosphonate $K_i$ and phosphonoacetaldehyde $K_m$, the lysine that forms the Schiff base must be in the enzyme active site.

In conclusion, the Schiff base mechanism utilized by phosphonatase to bring about P–C bond cleavage in phosphonoacetaldehyde is analogous to the Schiff base mechanisms used by a number of aldolases and β-keto decarboxylases. The catalytic efficiency (1000 $min^{-1}$) and high degree of substrate specificity of phosphonatase are evidence that phosphonatase is a specialized enzyme and not simply a *B. cereus* β-keto decarboxylase or aldolase that has a fortuitous phosphonoacetaldehyde hydrolase activity.[4] Exactly why *B. cereus* is equipped with a gene-encoding phosphonatase is presently unclear, as is the mechanism by which the gene is turned on during $P_i$ starvation. Finally, the results presented above suggest one general method by which enzymes can catalyze P–C bond cleavage. In the case of the phosphonatase reaction, the formation of the protonated imine activates this process by making the leaving group a stable enamine and thus avoiding the generation of a high-energy acetaldehyde enolate.

**Registry No.** Lys, 56-87-1; phosphonoacetaldehyde hydrolase, 37289-42-2; $N^{\epsilon}$-ethyl-L-lysine, 1635-05-8; $N^{\alpha}$-Cbz-L-lysine, 2212-75-1; acetaldehyde, 75-07-0; $N^{\alpha}$-Cbz-$N^{\epsilon}$-L-lysine, 112792-93-5; $N^{\alpha}$-Cbz-$N^{\epsilon}$-L-lysine, 112792-94-6; $N^{\epsilon}$-(2-phosphonoethyl)-L-lysine·2HCl, 112792-97-9; phosphonoacetaldehyde·2Li, 112792-95-7; Cbz-$N^{\epsilon}$-(2-phosphonoethyl)-L-lysine·$NH_3$, 112792-96-8; acetonylphosphonate, 6913-02-6; phosphorus pentachloride, 10026-13-8; isopropenyl acetate, 108-22-5; acetonylphosphonyl chloride, 5849-63-8; acetonylphosphonate·2Li, 112792-98-0.

## References


Bezas, B., & Zervas, L. (1961) *J. Am. Chem. Soc. 83*, 719.

Cassaigne, A., Lacoste, A. M., & Neuzil, E. (1976) *C. R. Seances Acad. Sci., Ser. D 282*, 1637.

Cordeiro, M. L., Poapliano, D. L., & Frost, J. W. (1986) *J. Am. Chem. Soc. 108*, 332.

Crestfield, A. M., Skupin, J., Moore, S., & Stein, W. H. (1960) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 19*, 341.

Daughton, C. G., Cook, A. M., & Alexander, M. (1979) *FEMS Microbiol. Lett. 5*, 91.

Dumora, C., LaCoste, A. M., & Cassaigne, A. (1983) *Eur. J. Biochem. 133*, 119.

Engel, R. (1977) *Chem. Rev. 77*, 349.

Frost, J. W., Loo, S., Cordeiro, M. L., & Li, D. (1987) *J. Am. Chem. Soc. 109*, 2166.

Hilderbrand, R. L. (Ed.) (1983) *The Role of Phosphonates in Living Systems*, CRC Press, Boca Raton, FL.

Hirs, C. H. W. (1956) *J. Biol. Chem. 219*, 611.

Horiguchi, M., & Rosenberg, H. (1975) *Biochim. Biophys. Acta 404*, 333.

Isabell, A. F., Englert, L. F., & Rosenberg, H. (1969) *J. Org. Chem. 34*, 755.

La Nauze, J. M., & Rosenberg, H. (1968) *Biochim. Biophys. Acta 165*, 438.

La Nauze, J. M., Rosenberg, H., & Shaw, D. C. (1970) *Biochim. Biophys. Acta 212*, 332.

La Nauze, J. M., Coggins, J. R., & Dixon, H. B. F. (1977) *Biochem. J. 165*, 409.

Lutsenko, I. F., & Kirilov, M. (1960) *Dokl. Akad. Nauk. SSSR 132*, 842.

Mastalerz, P. (1984) in *Natural Products Chemistry* (Zalewski, R. I., & Skolik, J. J., Eds.) Elsevier, Amsterdam.

Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192.

Seon, B. K. (1967) *J. Biochem. (Tokyo) 61*, 606.

Wackett, L. P., Spencer, S. L., Venditti, C. P., & Walsh, C. T. (1987) *J. Bacteriol. 169*, 710.

Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, p 669, W. H. Freeman, New York.

Warren, S., Zeiner, B., & Westheimer, F. H. (1966) *Biochemistry 5*, 817.

Warren, W. A. (1968) *Biochim. Biophys. Acta 156*, 340.


[4] Phosphonatase is present in wild type *Bacillus cereus* as well as in the higher yielding AI-2 mutant that served as the major source of enzyme in this study. A number of phosphonates and phosphate esters have been tested as potential phosphonatase substrates and found to be unreactive (unpublished results). In addition, phosphonatase does not catalyze the decarboxylation of malonic semialdehyde or acetoacetate.