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Aldolase-Like Imine Formation in the Mechanism of Action of Phosphonoacetaldehyde Hydrolase

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Phosphonoacetaldehyde hydrolase (EC 3.11.1.1), the bacterial enzyme that catalyses the reaction $HCO–CH_2–PO(OH)_2+H_2O \rightarrow HCO–CH_3+P_i$, is inactivated by borohydride if either phosphonoacetaldehyde or acetaldehyde is present. This supports the suggestion that the substrate forms an imine with an amino group of the enzyme. Such imine formation would labilize the C–P bond in the same way that aldolase and related enzymes labilize C–C and C–H bonds (Scheme 1*a*).

La Nauze *et al.* (1970) isolated and characterized phosphonoacetaldehyde hydrolase (EC 3.11.1.1), which hydrolyses 2-oxoethylphosphonic acid (phosphonoacetaldehyde, $CHO–CH_2–PO_3H_2$) to form acetaldehyde and orthophosphate. This reaction is the second step in the pathway by which *Bacillus cereus* metabolizes 2-aminoethylphosphonic acid. La Nauze & Rosenberg (1968) had previously suggested that the mechanism of hydrolysis involved labilization of the carbon–phosphorus bond by the electron-withdrawing properties of the carbonyl group (cf. Clark *et al.*, 1964). Many of the carbon–carbon and carbon–hydrogen bonds split by enzymes are in analogous positions (α–β) with respect to carbonyl groups. In these reactions enzyme–substrate binding often involves imine formation between the side chain of a lysine residue of the enzyme and the carbonyl group of the substrate. This facilitates bond scission because the protonated imine is more strongly electron-withdrawing than the carbonyl group, and the imine is easily protonated. Examples include acetoacetate decarboxylase (Fridovich & Westheimer, 1962; Westheimer, 1963), various aldolases [see Lai & Horecker (1972) and the references they cite] and 5-aminolaevulinate synthase (Nandi & Shemin, 1968); for reviews see Waley (1967), Jencks (1969), Gray (1971) and Feeney *et al.* (1975). Since imine formation could similarly labilize the carbon–phosphorus bond of $CHO–CH_2–PO_3H_2$, the same mechanism could operate in the phosphonohydrolase (Scheme 1*a*). The observations by La Nauze *et al.* (1970) that cyanide (cf. Westheimer, 1963), sulphide and sulphite all inhibited the enzyme, even when added in amounts much less than that of the substrate, could be explained if they reacted with the imine. The present paper presents evidence that imine formation occurs between the enzyme and its substrate.

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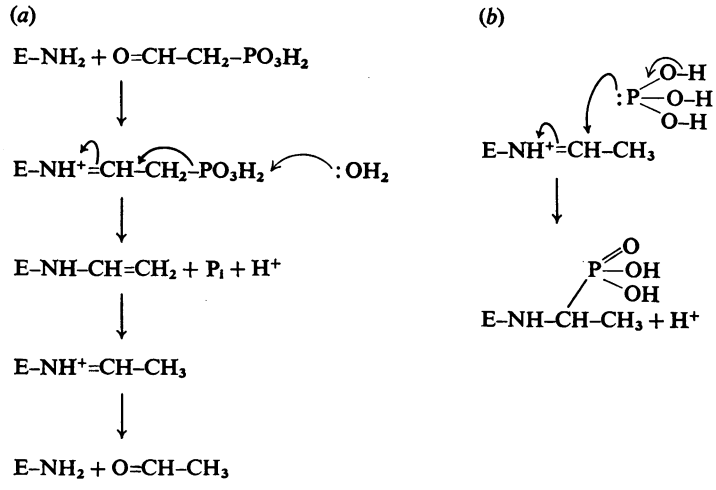
Materials and Methods

Phosphonoacetaldehyde hydrolase was isolated from *Bacillus cereus* by the method of La Nauze *et al.* (1970). Its substrate, phosphonoacetaldehyde, was used as the dilithium salt, prepared as follows. 2-Acetoxy-2-chloroethylphosphonyl dichloride was made from vinyl acetate, PCl_5 and SO_2 by the method of Lutsenko & Kirilov (1960). The $POCl_3$ and $SOCl_2$ formed were evaporated off, and without prior distillation the product was hydrolysed (see Isbell *et al.*, 1969) by cautious addition of water with cooling; the acetic acid and HCl formed and excess of water were removed by rotary evaporation. The product was dissolved in water and adjusted to pH 9 with LiOH solution. The product was precipitated by addition of acetone, and washed with acetone to remove LiCl. It gave a single spot on electrophoresis at pH 6.5 and 3.5 when stained for carbonyl group or for capacity to bind ferric ions (see Dixon & Sparkes, 1974). At pH 6.5 it possessed a mobility 1.2 times that of aspartic acid; neutral amino acids were used as indicators of electro-osmosis. Titration of 100 mg in 10 ml of water with HCl showed a pK of 6.3 and an equiv.wt. of 144 (formula wt. = 136).

Enzyme activity was assayed by P_i release as described by La Nauze *et al.* (1970). Treatments were performed in 1 ml samples of 20 μg of enzyme in a buffer of 0.1 M-triethanolamine/10 mM-$MgCl_2$, adjusted to pH 8.5 with HCl and various additions (Table 1). After 30 min at 30°C the samples were cooled in ice and enough solid $(NH_4)_2SO_4$ was added to saturate the solution after addition of 0.1 mg of ox serum albumin. The contents of each tube were filtered by suction through Millipore filters (pore size



Scheme 1. *Postulated reactions*

(*a*) Enzymic mechanism; (*b*) inactivation of the enzyme by phosphite. No attempt is made to draw hydroxyl groups in the correct ionic forms, because those that are ionized may be held by positive charges of the enzyme and so may remain electron-withdrawing.

Table 1. *Results of various treatments of phosphonoacetaldehyde hydrolase*

The treatments were for 30min at pH8.5 and 30°C, after which the enzyme was precipitated with $(NH_4)_2SO_4$, washed, redissolved and assayed.

Treatment	Percentage activity remaining
None	[100]
10mM-Li_2O_3P-CH_2-CHO	98
10mM-KBH_4	111
10mM-Li_2O_3P-CH_2-CHO+ KBH_4 (10, 1 or 0.1mM)	2–3
20mM-P_i+10mM-KBH_4	105
20mM-CH_3-CHO+10mM-KBH_4	14

0.45μm) and the filters were washed with three 2ml portions of ice-cold buffer saturated with $(NH_4)_2SO_4$. Each filter was then placed in a small tube and the precipitated protein was resuspended in 2.2ml of the buffer before 0.2ml of 10mM-Li_2O_3P-CH_2-CHO was added to start the assay of enzyme activity.

Results

Table 1 shows that borohydride inactivates the enzyme if the substrate is present; acetaldehyde can replace phosphonoacetaldehyde but is less effective. Further experiments showed that the inactivation in the presence of substrate was complete in 2min at 0°C, and that similar results were obtained if the samples were prepared for enzyme assay after the treatments by dialysis rather than precipitation.

Discussion

The fact that sulphite, sulphide and cyanide all inhibited the enzyme, even when present in amounts too small to bind a significant fraction of the substrate (La Nauze *et al.*, 1970), suggested that they might be adding to an imine formed between enzyme and substrate. Fridovich & Westheimer (1962) had shown that cyanide similarly inhibited acetoacetate decarboxylase. Such imine formation would labilize the C–P bond by the mechanism shown in Scheme 1(*a*) (see the introduction and Westheimer, 1963), because the $-NH^+=$ group is more electron-attracting than the O= group. We therefore investigated the effect of borohydride, and the results (Table 1) supported the postulated mechanism. More complete evidence could be obtained if the residue modified by treatment with borohydride and substrate were characterized.

The imine hypothesis may also explain the effect of phosphonate (phosphite), which inactivated the enzyme if phosphonoacetaldehyde or acetaldehyde was present (La Nauze *et al.*, 1970). Imines react with H_3PO_3 to form 1-aminoalkylphosphonic acids (Irani & Moedritzer, 1963); the reaction needs heating and may require the phosphonic acid to be in the $P(OH)_3$ form rather than as the predominant $H\text{-}PO(OH)_2$ tautomer. In the breakage of the C–P bond by the mechanism of Scheme 1(*a*) the C–P bond should be in a plane at right-angles to the N–C–C plane (cf. Dunathan, 1966). Thus phosphonate bound at the phosphate-binding site would be in almost the correct position for attacking the second (N-bound)

carbon atom (Scheme 1*b*). This positioning may explain the reaction of phosphonate with the enzyme, although the binding could conceivably favour formation of the $P(OH)_3$ tautomer as well. The hypothesis that phosphonate reacts as in Scheme 1(*b*) does not, however, easily explain the findings by La Nauze *et al.* (1970) that partial hydrolysis with acid, trypsin or pepsin released, in the form of phosphonate, ^{32}P label that had been added as phosphonate.

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References

Clark, V. M., Hutchinson, D. W., Kirby, A. J. & Warren, S. G. (1964) *Angew. Chem. Int. Ed. Engl.* **3**, 678–685

Dixon, H. B. F. & Sparkes, M. J. (1974) *Biochem. J.* **141**, 715–719

Dunathan, H. C. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **55**, 712–716

Feeney, R. E., Blankenhorn, G. & Dixon, H. B. F. (1975) *Adv. Protein Chem.* **29**, 135–203

Fridovich, I. & Westheimer, F. H. (1962) *J. Am. Chem. Soc.* **84**, 3208–3209

Gray, C. J. (1971) *Enzyme-Catalysed Reactions*, pp. 295–303, Van Nostrand–Reinhold, London

Irani, R. R. & Moedritzer, K. (1963) *Fr. Patent* 1 342 412 [*Chem. Abstr.* (1964) **60**, 8062]

Isbell, A. F., Englert, L. F. & Rosenberg, H. (1969) *J. Org. Chem.* **34**, 755–756

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 120–127, McGraw–Hill, New York

Lai, C. Y. & Horecker, B. L. (1972) *Essays Biochem.* **8**, 149–178

La Nauze, J. M. & Rosenberg, H. (1968) *Biochim. Biophys. Acta* **165**, 438–447

La Nauze, J. M., Rosenberg, H. & Shaw, D. C. (1970) *Biochim. Biophys. Acta* **212**, 332–350

Lutsenko, I. F. & Kirilov, M. (1960) *Dokl. Akad. Nauk SSSR* **132**, 842–845

Nandi, D. L. & Shemin, D. (1968) *J. Biol. Chem.* **243**, 1236–1242

Waley, S. G. (1967) *Q. Rev. Chem. Soc.* **21**, 379–404

Westheimer, F. H. (1963) *Proc. Chem. Soc.* 253–261