Allosteric regulation of phosphonoacetaldehyde hydrolase by n-butylphosphonic acid

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The effect of n-butylphosphonic acid on the activity of phosphonoacetaldehyde hydrolase from *Pseudomonas aeruginosa* was investigated: at low concentrations this compound appeared as an activator of the enzyme activity, whereas at higher concentrations it exhibited inhibitory properties. The experimental results were modelled according to an allosteric model involving two different classes of sites for n-butylphosphonic acid.

INTRODUCTION

A phosphonoacetaldehyde hydrolase (EC 3.11.1.1), trivial name phosphonatase, has been characterized in *Pseudomonas aeruginosa* (Dumora *et al.*, 1989). This enzyme, inducible by the naturally occurring 2-aminoethylphosphonic acid, catalyses the C–P bond cleavage of phosphonoacetaldehyde into acetaldehyde and orthophosphate. Phosphonoacetaldehyde is itself produced from 2-aminoethylphosphonic acid by a specific aminotransferase (Dumora *et al.*, 1983). Thus the degradation of 2-aminoethylphosphonic acid, by a two-step pathway of transamination and hydrolysis, allows this molecule to provide a nitrogen, phosphorus and carbon source for cells.

Enzymes able to cleave the C–P bond of organophosphonates (only two of which have been identified to date) have been recognized to play an important role in natural detoxification since an increasing number of herbicides, insecticides and flame retardants belongs to this class of compounds (Lacoste *et al.*, 1989). Hence the study of the mechanism of action and of regulation of these bacterial systems is of interest.

Phosphonoacetaldehyde hydrolase from *Ps. aeruginosa* has a homodimeric structure with a subunit of M_r 30000 and an isoelectric point of 5.2. The enzyme exhibits apparent Michaelis–Menten saturation kinetics with a K_m value of 200 μM and a catalytic-centre activity of 300 min^{-1} .

Phosphonatase has been isolated from both a Gram-positive bacterium, *Bacillus cereus* (La Nauze *et al.*, 1970), and a Gram-negative bacterium, *Ps. aeruginosa*. A mechanistic study of the enzyme of *B. cereus* was undertaken by La Nauze *et al.* (1977). Phosphonatase inactivation following treatment with NaBH_4 in the presence of phosphonoacetaldehyde or acetaldehyde was the first evidence for a Schiff-base formation, between an amino group residue of the enzyme and the carbonyl group of the substrate, which facilitates the labilization of the C–P bond. More recently, Olsen *et al.* (1988) identified labelled *N*-ethyllysine from the trypsin digest of $[2\text{-}^3\text{H}]\text{phosphonoacetaldehyde}/\text{NaBH}_4$ -treated enzyme, which gives evidence for a Schiff-base intermediate between a lysine residue and the substrate. In the enzyme from *Ps. aeruginosa*, NaBH_4 failed to reduce the imine linkage (C. Dumora & A.-M. Lacoste, unpublished work), probably because of a different conformation of the protein.

Phosphonoacetaldehyde hydrolase from the two species appears to be very specific for its substrate, since a series of phosphonates and of phosphate esters tested behaved as inert

molecules. Only acetonylphosphonate was described as a competitive inhibitor of the enzyme of *B. cereus* (Olsen *et al.*, 1988). In contrast, in preliminary experiments on the enzyme of *Ps. aeruginosa*, we have shown that a series of alkylphosphonic acids from C_1 to C_4 exhibited inhibitory properties increasing with the increase in the carbon chain length. In the present paper we show that the effects of these phosphonic acids are more complex than a mere inhibition of enzyme activity, since an activation occurs at low concentrations of these compounds. Therefore we investigated the kinetic behaviour of the enzyme over a wide range of concentrations of n-butylphosphonic acid, which we had previously found to be the most inhibitory ligand. From these results we propose a model for the mechanism of this regulation.

MATERIALS AND METHODS

Materials

Methylphosphonic acid, ethylphosphonic acid, n-propylphosphonic acid and n-butylphosphonic acid were purchased from Richmond Organics, Richmond, VA, U.S.A. 2-Aminoethylphosphonic acid and phosphonoacetaldehyde were synthesized as described by Kosolapoff (1947) and Isbell *et al.* (1969) respectively. Yeast alcohol dehydrogenase and NADH were from Boehringer–Mannheim. 2-Amino-2-methylpropane-1,3-diol was obtained from Fluka.

Phosphonoacetaldehyde hydrolase

The enzyme was purified to electrophoretic homogeneity from cells of *Ps. aeruginosa* A237 (Institut Pasteur, Paris) grown in a culture medium containing 2-aminoethylphosphonic acid as both phosphorus and carbon sources (Dumora *et al.*, 1989).

Enzyme assays

The reaction mixture contained 100 μmol of 2-amino-2-methylpropane-1,3-diol/HCl buffer, pH 8.5, 10 μmol of MgCl_2 , various amounts (0.1–2 μmol) of 2-phosphonoacetaldehyde, various amounts (0–20 μmol) of n-butylphosphonic acid and the enzyme, in a final volume of 1 ml. The activity of the enzyme was determined by monitoring spectrophotometrically the release of acetaldehyde, by using the coupled assay with alcohol dehydrogenase and NADH. The decrease in absorbance, as NADH was

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consumed to reduce acetaldehyde, was measured at 340 nm and 25 °C on a Perkin-Elmer spectrophotometer.

Optimization procedure

The experimental points were fitted by a non-linear least-squares method programmed on an Apple computer and also by the program TRIDAM developed on a PC-compatible computer by J. Langla & J. P. Mazat (ENSAM, Bordeaux, France).

RESULTS AND DISCUSSION

The regulation pattern of phosphonoacetaldehyde hydrolase by n-butylphosphonic acid was studied with concentrations ranging from 0.05 to 4 mM. At the lowest concentrations (0.05–0.3 mM) this molecule appears to be an activator, whereas at higher concentrations it is an inhibitor (Fig. 1). This effect is independent of the concentration of substrate, since it is seen at both saturating and half-saturating concentrations. This behaviour is rather unusual, although it has already been described in some instances (Brown & Reichard, 1969; Brown *et al.*, 1969; Dungan & Datta, 1973).

To explain the opposite effects of n-butylphosphonate at low and high concentrations, it is necessary to assume the existence of two different classes of sites for the binding of this molecule, called modulator M: one class of sites where the modulator acts as an activator, and the other class of sites being inhibitory. We have previously developed a model based on an allosteric MWC K-system (Monod *et al.*, 1965) with the existence of two different sites per subunit for the same modulatory ligand (Mazat *et al.*, 1977). This model is depicted in Scheme 1, where the sites 1 and 2 are the binding sites for the modulator M and where the third site binds the substrate S. This model has been applied to the results of Fig. 1.

As a total inhibition of enzyme activity is observed, we have supposed that the T form of the enzyme is catalytically inactive ($V_{MT} = 0$). Thus the equation of enzyme activity is:

$$v = V_{MR} \frac{[S]}{1 + \frac{[S]}{K_{RS}}} \bar{R} \quad (1)$$

with

$$\bar{R} = \frac{1}{1 + L_0 \cdot A_s \cdot A_{M1} \cdot A_{M2}}$$

Fig. 1. Variation of phosphonoacetaldehyde hydrolase activity as a function of n-butylphosphonic acid concentration

The experimental points were fitted according to eqn. (1) with the parameter values listed in Table 1. Phosphonoacetaldehyde concentrations: ●, 0.2 mM; ○, 2 mM.

Scheme 1. Allosteric model for phosphonoacetaldehyde hydrolase involving two classes of sites (1 and 2) for the modulator M (n-butylphosphonic acid)

S is the substrate phosphonoacetaldehyde, and L_0 is the allosteric equilibrium constant between the R and T forms in the absence of ligand.

where L_0 is the allosteric equilibrium constant in the absence of any ligand ($L_0 = [T_0]/[R_0]$) and A_s , A_{M1} and A_{M2} are the so-called allosterians (Mazat & Patte, 1976):

$$A_s = \left(\frac{1 + \frac{[S]}{K_{TS}}}{1 + \frac{[S]}{K_{RS}}} \right), A_{M1} = \left(\frac{1 + \frac{[M]}{K_{M1T}}}{1 + \frac{[M]}{K_{M1R}}} \right) \text{ and } A_{M2} = \left(\frac{1 + \frac{[M]}{K_{M2T}}}{1 + \frac{[M]}{K_{M2R}}} \right)$$

S is phosphonoacetaldehyde and M is n-butylphosphonic acid.

In these equations K_{RS} and K_{TS} are the dissociation constants of S for the R and the T forms of the enzyme respectively, K_{M1R} and K_{M1T} are the dissociation constants of M for the site 1 in the R and T forms respectively, and K_{M2R} and K_{M2T} have the same significance for the site 2; V_{MR} is the maximal rate of the R form of the enzyme ($V_{MT} = 0$).

In eqn. (1) we have assumed that the Michaelis constant of the R form is equal to the dissociation constant K_{RS} .

Fig. 2 describes the activity of the enzyme as a function of

Fig. 2. Variation of phosphonoacetaldehyde hydrolase activity as a function of phosphonoacetaldehyde concentration at different concentrations of n-butylphosphonic acid

The experimental points were fitted according to eqn. (1) with the parameter values listed in Table 1. n-Butylphosphonic acid concentrations: ■, 0 mM; □, 0.05 mM; ○, 1 mM; ●, 2 mM; △, 4 mM.

Table 1. Values of the parameters of eqn. (1) giving the best fit of the experimental results

The curves of Figs. 1 and 2 are drawn with these values.

Parameter of the model	Value
L_0	63.7
V_{MR}	946 $\mu\text{M} \cdot \text{min}^{-1}$
K_{RS}	70 μM
K_{TS}	90 μM
K_{M1R}	5.1 μM
K_{M1T}	5.8 μM
K_{M2R}	10 mM
K_{M2T}	1.93 mM

substrate concentration at different concentrations of the modulator M. The experimental points have been fitted as indicated in the Materials and methods section. The values giving the best fit, summarized in Table 1, allow us to draw the curves in Figs. 1 and 2.

The values reported in Table 1 deserve some comments. First of all, the L_0 value indicates that the enzyme is predominantly in the inactive form in the absence of any ligand. The observed maximum value of activity, about 25 $\mu\text{M} \cdot \text{min}^{-1}$, compared with the V_{MR} value of 946 $\mu\text{M} \cdot \text{min}^{-1}$, also indicates that the enzyme is predominantly in the T (inactive) form in the presence of substrate. For this reason an activation is seen as soon as there is a small amount of enzyme shifting from the T form to the R form.

The dissociation constants for the substrate in the two forms are nearly the same (70 and 90 μM), but slightly lower than 200 μM previously found (Dumora *et al.*, 1989), and correspond to the substrate concentration that gives half-maximal activity whatever the concentration of the modulator. This means that the substrate has only a weak role in the shift of the allosteric equilibrium and explains why the activities as a function of the substrate concentration, at different modulator concentrations, are nearly hyperbolic (Fig. 2).

Site 1 binds the modulator with the highest affinity. Once more, the dissociation constants are nearly equal: 5.1 μM and 5.8 μM . However, the modulator M has a slight higher affinity for the R form and this is the other reason why a small activation peak is observed.

The affinity of M for site 2 is much lower, but now the higher affinity is for the T form, the ratio of the dissociation constants (10/1.93) being much greater than the opposite ratio (5.8/5.1) for site 1, so that inhibition results at high M concentrations. Note that even if the dissociation constant ratio is not very high, owing to the dimeric structure of the enzyme, the value to be taken into account is the ratio squared, which is around 25.

In conclusion, the model developed in Scheme 1 accounts quantitatively for the experimental results of Fig. 1. Of course, other models might explain the observed modulation of the activity of the phosphonatase, especially 'Induced fit' models proposed by Koshland *et al.* (1966). The advantage of the MWC model is that the activatory or inhibitory properties of a ligand are clearly structurally interpreted in terms of a shift of the

allosteric equilibrium due to the difference in the affinities for the R and the T forms. Any other model would also have to involve two sites for the modulator M.

As noticed above, other examples of a regulatory ligand with activatory and inhibitory properties exist. This is the case with ribonucleoside diphosphate reductase (Brown & Reichard, 1969; Brown *et al.*, 1969), for which two classes of sites were demonstrated by binding experiments. The regulation by dATP shows the same pattern as the regulation of phosphonatase by M (see also Mazat *et al.*, 1977). In the case of ribonucleoside diphosphate reductase, the existence of two regulatory sites affords a fine regulatory pattern that allows the demand of the cell for deoxyribonucleotides to be met better. In our case, however, the physiological reason for such complex regulation is unclear. It may be looked for in the mode of evolution of enzymes. Gene duplication is now well recognized as a driving force in molecular evolution (Ohno, 1980), and many proteins have emerged in this way, leading to the concept of superfamilies of proteins. This process has also been used by evolution to construct large proteins from duplication of small polypeptides. When the duplicated sequence contains a binding site, this site is also duplicated. One of the best examples of this situation is found in the structure of Ca^{2+} -binding proteins (Goodman *et al.*, 1979), which contain several homologous sequences each carrying a binding site for Ca^{2+} with different affinities according to the variation in the sequence. The two sites of phosphonatase could well be the relics of an ancestral duplicated site, perhaps even of the active site itself. The answer to this question will be sought through sequence determination of the gene.

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