The inactivation of ornithine transcarbamoylase by N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine

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Phaseolotoxin, a tripeptide inhibitor of ornithine transcarbamoylase, is a phytotoxin produced by *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo-blight in beans. *In vivo* the toxin is cleaved to release N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine, the major toxic chemical species present in diseased leaf tissue. This paper reports on the interaction between N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine and ornithine transcarbamoylase. N^{δ} -(N' -Sulpho-diaminophosphinyl)-L-ornithine was found to be a potent inactivator of the enzyme, in contrast with phaseolotoxin, which previously has been reported to inhibit the enzyme reversibly. Inactivation by N^{δ} -(N' -[^{35}S]sulpho-diaminophosphinyl)-L-ornithine resulted in the incorporation of ^{35}S into ethanol-precipitated protein. The stoichiometry of ^{35}S incorporation was approximately 1 mol/mol of active sites. Inactivation was second-order and a rate constant of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 0°C in 50 mM-Tris/HCl, pH 9.0, was obtained. Carbamoyl phosphate, a substrate of ornithine transcarbamoylase, protected the enzyme from inactivation. A dissociation constant of $3 \mu\text{M}$ for the enzyme–carbamoyl phosphate complex was calculated. L-Ornithine, the second substrate for ornithine transcarbamoylase, protected the enzyme only at high concentrations. The results are consistent with N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine being a potent affinity label that binds via the carbamoyl phosphate-binding site of ornithine transcarbamoylase. Cleavage of phaseolotoxin to N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine *in vivo* appears to be an important function in the physiology of the disease.

Phaseolotoxin is a chlorosis-inducing tripeptide phytotoxin produced by *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo-blight of beans (*Phaseolus vulgaris* L.) (Mitchell, 1976; Moore *et al.*, 1984). An extensive kinetic analysis of the inhibition of ornithine transcarbamoylase (OTCase) from *Escherichia coli* W by purified phaseolotoxin has been performed (Templeton *et al.*, 1984). Phaseolotoxin was found to be a

reversible inhibitor of OTCase and displayed mixed inhibition with respect to carbamoyl phosphate with an apparent K_i and apparent K_i' of $0.2 \mu\text{M}$ and $10 \mu\text{M}$ respectively. Inhibition was non-competitive with respect to L-ornithine with an apparent K_i of $0.9 \mu\text{M}$. These results suggested that phaseolotoxin was binding primarily to the carbamoyl phosphate-binding site of OTCase but that it could also bind to the OTCase–carbamoyl phosphate complex, although this event was kinetically less significant (Templeton *et al.*, 1984).

Although phaseolotoxin is produced in liquid cultures of *Ps. syringae* pv. *phaseolicola* (Mitchell, 1976), there is strong evidence that the toxin is cleaved *in vivo* by plant peptidases (Mitchell & Bielecki, 1977). On the basis of the recent structural revision of phaseolotoxin (Moore *et al.*, 1984),

Abbreviations used: OTCase, ornithine transcarbamoylase; PSorn, N^{δ} -(N' -sulpho-diaminophosphinyl)-L-ornithine.

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the peptidase product would be N^{δ} -(N' -sulphodiaminophosphinyl)-L-ornithine (PSorn), with the structure, as proposed by Moore *et al.* (1984), shown in Scheme 1. Bean plants were grown in the presence of $^{35}\text{SO}_4^{2-}$ and infected with *Ps. syringae* pv. *phaseolicola*. Extracts of the chlorotic tissue were found to contain up to $1.5\text{ }\mu\text{g}$ of PSorn/g fresh wt., but the concentrations of phaseolotoxin were approximately 100-fold less (Mitchell & Bielecki, 1977). Phaseolotoxin has been shown to be easily cleaved by peptidases (Mitchell, 1976) *in vitro*. It was therefore important to investigate whether the cleavage of phaseolotoxin to PSorn affected the inhibition of OTCase.

Previously published data suggest that PSorn may inhibit OTCase in a manner different from phaseolotoxin. Kwok *et al.* (1979) reported that a chemically unidentified toxin preparation isolated from chlorotic leaves, which possibly contained significant quantities of PSorn, was an affinity label for OTCase. In addition, Kwok & Patil (1982) showed that phaseolotoxin treated with leucine aminopeptidase was a more potent inhibitor of OTCase under standard assay conditions. The present paper reports the results of experiments on the mode of action of purified PSorn on OTCase from *E. coli* W.

Experimental

Materials

Carbamoyl phosphate (disodium salt), L-citrulline, L-ornithine, Tris, leucine aminopeptidase and bovine serum albumin were from the Sigma Chemical Co., St. Louis, MO, U.S.A. OTCase was purified from *E. coli* W as described by Templeton *et al.* (1984). [^{35}S]Phaseolotoxin was prepared from a liquid culture of *Ps. syringae* pv. *phaseolicola* (strain Plant Diseases Division Culture Collection 4419) supplied with $^{35}\text{SO}_4^{2-}$ (Amersham International, Amersham, Bucks., U.K.), as described by Mitchell & Bielecki (1977). [^{35}S]PSorn ($92.46\text{ }\mu\text{Ci}/\mu\text{mol}$) was prepared by treatment of [^{35}S]phaseolotoxin with leucine aminopeptidase as described by Mitchell & Bielecki (1977) for the preparation of non-radioactively-labelled PSorn. The purified PSorn was radiochemically pure on a two-dimensional t.l.c./t.l.c. system (Mitchell, 1976). Sephadex G-50 was obtained from Pharmacia, Uppsala, Sweden.

Enzyme and protein assay

OTCase was assayed as described by Templeton *et al.* (1984) and had a specific activity of 3000 units/mg of protein. Protein was estimated by assuming $A_{280}^{1\%} = 9.56$ (Templeton *et al.*, 1984) or by the method of Peterson (1977) with crystalline bovine serum albumin as a standard. In all experi-

ments enzyme concentration was calculated with respect to the subunit concentration, i.e. the active-site concentration.

Incorporation of radioactivity into ethanol-precipitable protein

Enzyme ($5.2\text{ }\mu\text{M}$) and [^{35}S]PSorn ($0\text{--}6.25\text{ }\mu\text{M}$) were incubated in $50\text{ }\mu\text{l}$ of 50 mM -Tris/HCl, pH 9.0, at 0°C for 30 min. Portions ($5\text{ }\mu\text{l}$) were removed and assayed for enzyme activity. Bovine serum albumin ($50\text{ }\mu\text{l}$ of a 10 mg/ml solution) was added to each remaining $45\text{ }\mu\text{l}$ sample as a carrier. Protein was precipitated with $900\text{ }\mu\text{l}$ of 95% (v/v) ethanol, and the mixture was kept on ice for 30 min. Precipitated material was collected on Whatman 2.1 cm GF/F glass-fibre filters that had been pre-soaked in ethanol. The filters were washed ($1 \times 15\text{ ml}$) with aq. 50% (v/v) ethanol solution containing 10 mM - K_2HPO_4 and 10 mM -ornithine to remove non-specifically bound [^{35}S]PSorn. Omission of this step resulted in non-specific binding of approx. 10% of the original radioactivity. Filters were washed with 95% (v/v) ethanol ($2 \times 15\text{ ml}$) and dried in an oven at 80°C for 3 h. The radioactivity precipitated on filters was measured in 10 ml of Triton/toluene scintillant.

Gel-permeation chromatography of inactivated OTCase

OTCase, inactivated with [^{35}S]PSorn as described above, was chromatographed on a Sephadex G-50 column ($1\text{ cm} \times 14\text{ cm}$; $V_0 = 6\text{ ml}$) equilibrated in 0.1% sodium dodecyl sulphate. Fractions (1 ml) were collected directly into scintillation vials, and measured for radioactivity in Bray's (1960) scintillation fluid.

In separate experiments, inactivated enzyme ($50\text{ }\mu\text{l}$) was treated separately with either 0.1% sodium dodecyl sulphate, 5 M -urea or 5 M -guanidinium chloride (final concentrations) at 37°C for 30 min. Each enzyme preparation ($100\text{ }\mu\text{l}$) was then chromatographed on Sephadex G-50 as described above.

Inactivation of OTCase by PSorn

OTCase (10 nM) and PSorn (10 nM) were incubated at 0°C in 1 ml of 50 mM -Tris/HCl, pH 9.0. The temperature was maintained at 0°C with an ice/water bath. Portions ($10\text{ }\mu\text{l}$) were removed at the appropriate times and assayed for OTCase activity. The concentration of active enzyme (e) at time t was calculated from the following formula:

$$e = e_0(v/v_0)$$

where e_0 is the initial concentration of enzyme and v_0 and v are the enzyme velocities initially and at time t respectively. Experiments were performed in the presence of substrate in a similar fashion.

Carbamoyl phosphate or L-ornithine was mixed with enzyme in a volume of 990 μ l and the reaction was started by the addition of 10 μ l of PSorn.

Results and discussion

An experiment was designed to determine whether 35 S remained bound to OTCase inactivated with [35 S]PSorn after precipitation with ethanol. The proportion of enzyme activity remaining (e/e_0) was plotted against the initial molar ratio of [35 S]PSorn to enzyme ($[^{35}\text{S}]\text{PSorn}/e_0$) (Fig. 1). The slope of this line indicated that 0.95 mol of PSorn was required to inactivate 1 mol of active sites. A plot of nmol of 35 S incorporated against the initial molar ratio of [35 S]PSorn to enzyme was linear and the stoichiometry of 35 S incorporation was 0.85 mol per mol of active sites. These data show that approx. 1 mol of [35 S]PSorn was bound per mol of inactivated enzyme.

Co-migration of 35 S and protein during gel-filtration chromatography provided further evidence that there was a covalent link between [35 S]PSorn and OTCase. OTCase and [35 S]PSorn (run separately) partitioned distinctly, with elution volumes of 6.5 ml and 13 ml respectively (Fig. 2a). In contrast, when enzyme (5.2 μ M) was inactivated

with [35 S]PSorn and chromatographed on a column of Sephadex G-50 equilibrated in 50 mM-Tris/HCl, pH 8.0, containing 0.1% sodium dodecyl sulphate, the protein peak was 35 S-labelled and most of the radioactivity was eluted in the void volume (Fig. 2b). Similar results were obtained when inactivated enzyme was treated with 5 M-urea or 5 M-guanidinium chloride before gel-permeation chromatography.

Kinetics of inactivation of OTCase by PSorn

Under pseudo-first-order conditions ($i > e$), enzyme inactivation occurred too rapidly to be measured accurately. The inactivation of OTCase was therefore measured under second-order conditions (Fig. 3). The rate constant of $10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ is high, but lower than that expected for a simple diffusion process (10^9 – $10^{11} \text{ M}^{-1} \cdot \text{s}^{-1}$) (Fersht, 1977). Second-order kinetics indicated that the concentration of a Michaelis-type binding complex was negligible under these conditions. Similar conclusions have been made by Knott-Hunziker *et al.* (1980) and Arisawa & Then (1983) for the

Fig. 1. Stoichiometry of inactivation of OTCase by [35 S]PSorn

Enzyme (5.2 μ M) and various amounts (0–6.25 μ M) of [35 S]PSorn were incubated at 0°C in 50 mM-Tris/HCl, pH 9.0. The reaction volume was 50 μ l. After 30 min 5 μ l samples were removed and assayed for OTCase activity. The remainder was used for the measurement of ethanol-precipitable radioactivity as described in the Experimental section. The results were plotted as (O) e/e_0 against [PSorn]/ e_0 , where e_0 and e are the enzyme activity initially and after 30 min respectively, and (●) nmol of 35 S incorporated against [35 S]PSorn/ e_0 .

Fig. 2. Gel-permeation chromatography of OTCase after inactivation with [35 S]PSorn

OTCase was inactivated with [35 S]PSorn as described in the Experimental section. Chromatography was performed on Sephadex G-50 equilibrated in 50 mM-Tris/HCl, pH 8.0, containing 0.1% sodium dodecyl sulphate. Fractions (1 ml) were collected directly into scintillation vials and the radioactivity was measured. (a) ■, OTCase (A_{280}); ○, [35 S]PSorn, run separately. (b) ○, Inactivated enzyme samples.

Fig. 3. *Inactivation of OTCase by PSorn*
Inactivation of OTCase by PSorn was performed as described in the Experimental section. The results were plotted as $1/e - 1/e_0$ against time, where e_0 and e are the concentrations of active enzyme initially and at time t respectively.

inactivation of β -lactamase by 6- β -bromopenicilanic acid and 6-acetylmethylenepenicillanic acid respectively.

Protection of inactivation by substrate

The structural homology between PSorn and the substrates of OTCase suggested that it was acting at the active site. More conclusive evidence can be obtained if the rate of inactivation is decreased in the presence of substrate. OTCase has a compulsory-order mechanism with carbamoyl phosphate the first substrate to bind (Legrain & Stalon, 1976). Rates of inactivation were measured in the presence of various concentrations of carbamoyl phosphate (Fig. 4). Low concentrations of carbamoyl phosphate significantly decreased the rate of inactivation.

The dissociation constant for the enzyme-carbamoyl phosphate complex can be calculated from these data by using the equations derived from the following expressions:

where A represents carbamoyl phosphate, I represents PSorn, E-I is the inactivated complex and E is free enzyme.

The derivation is essentially that described by Dixon & Webb (1979), modified slightly because of the order of the reaction.

From the previous expressions:

$$e = e_t + x + z \quad x = e_f \cdot a / K_{ia}$$

where e and e_t are the concentrations of total and

Fig. 4. *Inactivation of OTCase by PSorn in the presence of carbamoyl phosphate*

Inactivation of OTCase by PSorn in the presence of carbamoyl phosphate was performed as described in the Experimental section. The concentrations of carbamoyl phosphate were: ●, 0 μ M; Δ , 2.5 μ M; ■, 5.0 μ M; ○, 10 μ M; □, 15 μ M.

Fig. 5. *Calculation of the dissociation constant for the OTCase-carbamoyl phosphate complex*

The reciprocals of the apparent rate constants obtained from the data of Fig. 4 were plotted against the concentration of carbamoyl phosphate. A value of 3 μ M was obtained for the dissociation constant for the OTCase-carbamoyl phosphate complex.

free enzyme and x and z are the concentrations of EA and EI respectively. Solving for e_f :

$$e_f = (e - z)K_{ia}/(K_{ia} + a)$$

Assuming PSorn binds only to free enzyme:

$$-de_f/dt = ke_f(i - z)$$

where i is the concentration of PSorn. In the presence of carbamoyl phosphate, substitution gives the expression:

$$-de_f/dt = [kK_{ia}/(K_{ia} + a)](e - z)(i - z)$$

and thus:

$$-de_f/dt = k_{app}(e - z)(i - z)$$

This is the form of a second-order reaction, and thus at equimolar concentrations of PSorn and enzyme it will integrate to:

$$1/e - 1/e_0 = k_{app}t$$

In reciprocal form $1/k_{app}$ is defined as:

$$1/k_{app} = 1/k + a/K_{ia}k$$

Therefore a plot of $1/k_{app}$ against [carbamoyl phosphate] will have an intercept of $1/k$ on the $1/k_{app}$ axis, a slope of $1/K_{ia}k$ and an intercept of $-K_{ia}$ on the substrate concentration axis.

The results from Fig. 4 were replotted in this manner (Fig. 5). The plot was linear and a dissociation constant for carbamoyl phosphate of $3\mu\text{M}$ was obtained. This value is lower than that of $15\mu\text{M}$ obtained from a steady-state kinetic analysis at pH 8.0 by Legrain & Stalon (1976), but this may be due to the different pH.

The rate of OTCase inactivation was measured in the presence of 3mM- and 10mM-L-ornithine. The rate constant k was not affected by 3mM-L-ornithine; however, 10mM-L-ornithine decreased the rate of inactivation by 37%, suggesting that L-ornithine was binding to free enzyme at high concentrations. Legrain & Stalon (1976) reported that the species of ornithine with zero net charge ($pK_a = 8.88$) was able to bind to free enzyme in such a way as to preclude the binding of carbamoyl phosphate. At pH 9.0 there are significant concentrations of this species, and this may explain the limited protection of OTCase inactivation by ornithine.

A possible mechanism for the inactivation of OTCase by PSorn is outlined in Scheme 1. From the proposed mechanism we predict that free ornithine will be released into the medium and an amino acid residue on the enzyme will be phosphorylated with the phosphinyl substituent of PSorn. The observation that phaseolotoxin is unable to inactivate OTCase (Templeton *et al.*, 1984) suggests that a free carboxy group on the L-ornithine residue is required for complete binding

Scheme 1. Proposed mechanism of inactivation of OTCase by PSorn

Nucleophilic attack of PSorn by a basic residue (B) of the enzyme (OTCase) occurs as shown, with the release of ornithine and irreversible inactivation of OTCase.

to and hence inactivation of OTCase. Alternatively, the additional bulk of the two extra amino acids may be affecting the binding of the toxin in some way.

Mitchell & Bielecki (1977) found that PSorn was the major toxic product found in infected bean leaves, implying that phaseolotoxin was cleaved to PSorn by the plant *in vivo*. The significance of this observation is now apparent. The conversion of phaseolotoxin, a reversible inhibitor, into PSorn, a potent inactivator of OTCase, is an important part of the disease process. The conversion of a bacterial phytotoxin into a more potent form has been reported in the case of tabtoxin, which is cleaved to tabtoxinine- β -lactam, a potent inactivator of glutamine synthetase (Uchytel & Durbin, 1980; Thomas *et al.*, 1983).

Ferguson & Johnson (1980) and Staskawicz *et al.* (1980) reported that pre-incubation of purified phaseolotoxin with crude extracts of OTCase significantly increased enzyme inhibition. It is possible that during the pre-incubation period peptidases present in the OTCase preparation degraded phaseolotoxin to PSorn. In addition, it would appear that the toxin preparation used by Kwok *et al.* (1979) contained significant quantities of PSorn, and this would explain the inactivation kinetics they observed for OTCase. Their conclusion that the toxin formed 'a loose Michaelis-type' complex before inactivation was not borne out by the present study.

From these data it is clear that PSorn is a potent inactivator of OTCase from *E. coli* W, and indeed to our knowledge is one of the most potent naturally occurring enzyme inactivators reported. It appears likely that OTCase is an important, if

not the primary, site of action of PSorn in the bean leaf, but further physiological studies will be required to confirm this.

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