The inhibition of ornithine transcarbamoylase from *Escherichia coli* W by phaseolotoxin

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The mechanism of inhibition of ornithine transcarbamoylase by the bacterial toxin phaseolotoxin [*N*- δ -(phosphosulphamyl)ornithylalanylhomarginine] was investigated. Ornithine transcarbamoylase was purified by affinity chromatography from *Escherichia coli* W argR⁻ by using *N*- δ -(phosphonoacetyl)ornithine as the ligand. Under steady-state conditions phaseolotoxin inhibition was reversible and exhibited mixed kinetics with respect to carbamoyl phosphate. The apparent K_i and apparent K'_i were 0.2 μ M and 10 μ M respectively. Inhibition with respect to ornithine was non-competitive, with an apparent K_i of 0.9 μ M. These data are consistent with competitive binding of phaseolotoxin to the carbamoyl phosphate-binding site of the enzyme. The toxin also appears to be able to bind to the enzyme–carbamoyl phosphate complex, although, since K'_i is 50 times greater than K_i , this event is kinetically much less significant. In the presence of phaseolotoxin ornithine transcarbamoylase exhibited a transient phase of activity before a steady state. This is consistent with low rates of association and dissociation for the toxin with enzyme and the enzyme–toxin complex. Rate constants of $2.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $5 \times 10^{-3} \text{ s}^{-1}$ were estimated for the association and dissociation constants respectively.

Pseudomonas syringae pv. *phaseolicola* (Burkholder; Young, Dye & Wilkie) is the causal agent of halo-blight in beans (*Phaseolus vulgaris* L.). Once infection has occurred, a toxin is produced that diffuses into the surrounding leaf tissue, giving rise to the characteristic chlorotic halo. Prolonged infection can result in apical chlorosis, and nodulation is totally inhibited in infected plants (Hale & Shanks, 1983). The toxin has been shown to be transported to these areas through the phloem (Mitchell & Bieleski, 1977). It was isolated and purified from culture filtrates of *Ps. syringae* pv. *phaseolicola* and its structure determined as *N*- δ -(phosphosulphamyl)ornithylalanylhomarginine by Mitchell (1976*a,b*). The toxin was given the trivial name of phaseolotoxin, and its structure is given in Fig. 1(*a*). When applied to bean leaves, the purified toxin quantitatively

reproduced the characteristics of the disease (Mitchell, 1978).

Chlorotic tissue was found to contain elevated concentrations of the amino acid ornithine (Patel & Walker, 1963; Ferguson & Johnson, 1980), and it has been shown by several workers that phaseolotoxin is a potent inhibitor of OTCase (EC 2.1.3.3) from bean (Tam & Patil, 1972; Ferguson *et al.*, 1980; Staskawicz *et al.*, 1980). As shown in Fig. 1, the *N*- δ -(phosphosulphamyl)ornithyl group on the toxin is structurally similar to carbamoyl phosphate and ornithine, the substrates of OTCase. The exact mode of inhibition of phaseolotoxin is unknown, however. Tam & Patil (1972) claimed that phaseolotoxin is a reversible inhibitor that decreases OTCase activity by binding to a putative allosteric binding site. Kwok *et al.* (1979) showed that toxin isolated from infected bean leaves was an affinity label for OTCase. In addition, there have been other reports showing that pre-incubation of toxin and enzyme decreases enzyme activity. No definitive experiments have been performed to distinguish enzyme inactivation from enzyme inhibition. Furthermore, a fixed-time

Abbreviation used: OTCase, ornithine transcarbamoylase.

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Fig. 1. Structures of (a) phaseolotoxin, (b) ornithine and (c) carbamoyl phosphate

assay has been used to assay OTCase activity and phaseolotoxin inhibition, and it has been unclear whether initial velocities have been measured or if steady-state conditions apply.

Phaseolotoxin has been shown to be a potent inhibitor of OTCase from a variety of sources (Ferguson *et al.*, 1980; Ferguson & Johnson, 1980). We have chosen to use OTCase from *Escherichia coli* W because (a) this enzyme has been well characterized kinetically (Legrain & Stalon, 1976), the enzyme having a compulsory-order mechanism with carbamoyl phosphate binding to free enzyme, and (b), unlike *E. coli* K12, the W strain only contains one gene for OTCase. The kinetic data best fitted a Theorell–Chance mechanism, where the concentration of the ternary complex is negligible (Legrain & Stalon, 1976). The aim of the present study has been to characterize the mode of action of phaseolotoxin on OTCase from *E. coli* W.

Experimental

Materials

L-Ornithine, L-citrulline, L-canavanine sulphate, carbamoyl phosphate (dilithium salt), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, epoxy-activated Sepharose 6B, acrylamide and molecular-mass markers were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Whale myoglobin was purchased from Calbiochem (San Diego, CA, U.S.A.). QAE (quaternary aminoethyl)-Sephadex A-25, Sephadex G-100 and Sephadex G-50 were obtained from Pharmacia (Uppsala, Sweden). Sodium dodecyl sulphate and *NN'*-methylenebis-

acrylamide were obtained from BDH Chemicals (Poole, Dorset, U.K.). L-Lactate mono-oxygenase, prepared by the method of Sullivan *et al.* (1977), was available in our laboratory. All other chemicals were of reagent grade.

Enzyme and protein assays

OTCase was assayed at 37°C in a final volume of 100 µl that contained 5 mM-carbamoyl phosphate, 3 mM-ornithine and 50 mM-Tris/HCl buffer, pH 8.0. Carbamoyl phosphate was dissolved immediately before use because of its instability in solution. After incubation for 10 min, the reaction was quenched by the addition of 3 ml of chromogenic reagent (Boyde & Rahmatullah, 1980) for the determination of citrulline. Blanks containing substrate only and enzyme only were included as routine controls. Removal of protein from the sample before citrulline determination was not necessary. When increased sensitivity was required, 1 ml only of chromogenic reagent was added, and this allowed the detection of less than 1 nmol of citrulline.

The assay conditions for measurement of OTCase were such that the enzyme was saturated in carbamoyl phosphate and at the K_m for ornithine (Legrain & Stalon, 1976). Therefore velocities under these conditions were doubled to give a true *V*. One unit of activity is the amount of enzyme that catalyses the formation of 1 µmol of citrulline/min. Specific activity is units/mg of protein under these conditions.

Protein was measured by the method of Lowry *et al.* (1951) as modified by Peterson (1977), with crystalline bovine serum albumin as a standard.

Construction of an *argR*⁻ mutant

E. coli W was obtained from the Department of Microbiology, University of Otago, New Zealand, and maintained on nutrient agar at 4°C. An *argR*⁻ mutant was isolated essentially as described by Maas (1961) and Novick & Maas (1961). Cells mutagenized with *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine were selected on arginine-free plates (Novick & Maas, 1961) containing 0.1 mg of canavanine sulphate/ml. The mutant selected had OTCase activity over 20 times higher than the wild-type.

N-δ-(Phosphonoacetyl) ornithine synthesis

N-δ-(Phosphonoacetyl)ornithine was synthesized as described by Hoogenraad (1978) except that the ion-exchange step was performed with QAE-Sephadex A-25 (HCO₃⁻ form), as for phaseolotoxin purification (Mitchell, 1976b). Verification of the structure of the synthesized compound was achieved with ¹H n.m.r. and ¹³C n.m.r. ¹H-n.m.r. spectrum: δ (p.p.m.) (2H₂O; reference tetramethylsilane) 3.86 [1H, t, CH(CO₂H)-NH₂], 3.27 (2H, t, NH-CH₂-CH₂), 2.75 (2H, d, *J* = 21 Hz, PO₄-CH₂-C=O), 1.87 and 1.72 (4H, multi, CH₂-CH₂-CH₂-CH). ¹³C-n.m.r. spectrum: δ (p.p.m.) (2H₂O; reference dioxan) 25.42 and 28.49 (s, NH-CH₂-CH₂-CH₂), 39.51 (d, *J* = 240 Hz, PO₄-CH₂-C=O), 40.12 (s, NH-CH₂-CH₂-CH₂-), 55.63 [s, CH₂-CH₂-CH(CO₂H)-NH₂], 170.72 [s, CH₂(C=O)-NH-], 175.83 [s, CH(CO₂H)-NH₂].

Purification of OTCase from *E. coli* W *argR*⁻

E. coli W *argR*⁻ was grown in shake cultures at 37°C in a medium containing 1% (w/v) glucose, 1% (w/v) yeast extract, 0.7% K₂HPO₄ and 0.3% KH₂PO₄, pH 7.0. Cells were harvested by centrifugation at late-exponential phase and washed once in distilled water. The pellet was resuspended in breakage buffer (50 mM-Tris/HCl buffer, pH 8.0, containing 2 mM-2-mercaptoethanol and 1 mM-EDTA) and sonicated in a Branson sonifier for 15 min at 20% duty cycle and power 8. Cell debris was removed by centrifugation (9000g for 10 min), and the supernatant was filtered through a Whatman GF/F glass-fibre filter.

The construction of the *N*-δ-(phosphonoacetyl)-ornithine affinity column, application and elution of OTCase were as described by Hoogenraad *et al.* (1980). The crude cell-free extract was applied to the *N*-δ-(phosphonoacetyl)ornithine affinity column, which had been equilibrated in breakage buffer. The column was washed with 200 ml of breakage buffer and then 500 ml of breakage buffer containing 0.2 M-KCl. Finally, the enzyme was eluted in half-strength breakage buffer containing 10 mM-carbamoyl phosphate. The enzyme was

concentrated by ultrafiltration and chromatographed on a gel-permeation column (Sephadex G-100; 42 cm × 2.5 cm) equilibrated in 50 mM-Tris/HCl buffer, pH 8.0.

Electrophoresis

Analytical discontinuous gel electrophoresis was performed in slabs as described by Gabriel (1971) with the use of system 1 for a 7.5%-acrylamide gel. The only modification was that the concentration of the stacking gel was doubled to 2.5% acrylamide. An activity stain was developed for OTCase based on the general method introduced by Nimmo & Nimmo (1982) for the location of phosphate-producing enzymes. Immediately after electrophoresis, gels were incubated at 37°C in assay buffer containing 10 mM-CaCl₂. After 10 min the gels were washed thoroughly in distilled water and stored in 0.2% NaN₃ solution.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) with an acrylamide concentration of 12.5%. Gels were run and stained as described above. Samples were solubilized in 62.5 mM-Tris/HCl buffer, pH 6.7, containing sodium dodecyl sulphate (3%, w/v), urea (6 M), 2-mercaptoethanol (50 mM) and Bromophenol Blue (0.005%) by heating at 70°C for 5 min. The molecular-mass markers were bovine serum albumin (68 000 Da), ovalbumin (45 000 Da), glyceraldehyde-3-phosphate dehydrogenase (36 000 Da), carbonic anhydrase (29 000 Da), lactoglobulin (18 300 Da) and lysozyme (14 200 Da).

Amino acid analysis

Samples of the purified protein (7–9 μg) were hydrolysed in redistilled 6 M-HCl containing 1% phenol for 24, 48 and 72 h. For quantification norleucine was mixed with the protein solution before hydrolysis so that 2 nmol was added per analysis. Analyses were carried out in duplicate on a Waters Millipore high-pressure liquid-chromatography system with a fluorescence detection system. The column and buffer system were essentially as described by Benson (1977). Tryptophan was estimated by the method of Edelhoch (1967) with a Shimadzu variable-wavelength spectrophotometer.

High-pressure liquid gel-permeation chromatography

High-pressure liquid gel-permeation chromatography was carried out on a Waters high-pressure liquid-chromatography system with a model 440 absorbance detector with an extended wavelength facility. A TSK G3000SW permeation column (Toyo Soda Manufacturing Co., Yamaguchi-Ken, Japan) was used for all chromatograms. The solvent was 50 mM-phosphate buffer, pH 8.0, the flow

rate 1 ml/min and the back-pressure 2750–3100 kPa (400–450 lbf/in²).

Phaseolotoxin purification

Phaseolotoxin was purified from liquid cultures of *Ps. syringae* pv. *phaseolicola* (Plant Diseases Division Culture Collection 5006) as described by Mitchell (1976b). The final preparation was the only ninhydrin-positive spot on a two-dimensional t.l.c./electrophoresis system (Mitchell, 1976b) and was quantified by phosphorus determination (Ames & Dubin, 1960). The toxin was stored as a 7.75 mM solution in aq. 50% (v/v) methanol. Under these conditions phaseolotoxin can be stored without loss of activity for several years (Mitchell, 1984). Before use, samples of toxin were evaporated to dryness at room temperature and dissolved in 50 mM-Tris/HCl buffer, pH 8.0.

Results and discussion

Enzyme purification

The purification of OTCase has been facilitated by the use of *argR*⁻ mutants, which contain increased contents of the enzymes in the pathway of arginine biosynthesis (Legrain & Stalon, 1976). The mutant used in this study was isolated by selecting for resistance to the arginine analogue L-canavanine (Maas, 1961; Novick & Maas, 1961). Normally L-canavanine binds to the ArgR protein, preventing transcription of the arginine-biosynthetic enzymes and leading to arginine starvation. An *argR*⁻ mutant that is resistant to L-canavanine can grow in the absence of exogenously supplied arginine. The mutant isolated in the present study had OTCase activity approximately 20-fold higher than the wild-type.

OTCase was purified by the affinity method of Hoogenraad *et al.* (1980) and Sephadex G-100 chromatography as described in the Experimental section. A typical purification scheme is shown in Table 1. The final specific activity, 2914 units/mg, is similar to the value of 2888 units/mg obtained by Legrain & Stalon (1976) with conventional techniques. The purified enzyme has an $A_{280}^{1\%}$ value of 9.56 and an A_{280}/A_{260} ratio of 2.1:1.

The final enzyme preparation was homogeneous by several criteria. The enzyme was included in the Sephadex G-100 chromatogram ($K_D = 0.1$), the A_{280} profile was symmetrical and the enzyme was eluted with a specific activity of between 2800 and 3100 units/mg of protein. Polyacrylamide-gel electrophoresis under non-denaturing conditions gave a single band (Fig. 2). An activity stain showed

Fig. 2. Analytical electrophoresis of purified OTCase. Purified enzyme solutions were subjected to electrophoresis as described in the Experimental section. OTCase (20 μ g) was electrophoresed under non-denaturing conditions, and stained for (1) protein and (2) OTCase activity. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed on (3) a mixture of protein standards (10 μ g of each protein) containing: bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, β -lactoglobulin and lysozyme; (4) purified OTCase (10 μ g); (5) stored OTCase (20 μ g).

Table 1. Purification of OTCase from *E. coli* W

For experimental details see the text. Abbreviation: PALO, *N*- δ -(phosphonoacetyl)ornithine.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Cell-free extract	460	25 400	1656	15.3	100	0
PALO affinity chromatography	49	23 700	9.3	2547	93	166
Gel-permeation chromatography (Sephadex G-100)	26	22 400	7.7	2914	88	190

OTCase activity associated with this band (Fig. 2). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that the preparation was homogeneous (Fig. 2) with an apparent minimum molecular mass of 37500 Da. The subunit molecular mass of 37500 Da differs slightly from the 35000 Da reported by Legrain & Stalon (1976). The molecular mass that we obtained is consistent with the molecular mass obtained from other sources (Table 2). High-pressure liquid gel-permeation chromatography of the native enzyme showed a single peak with an elution volume of 14 ml (Fig. 3). When the enzyme was run in the presence of standards it partitioned between bovine serum albumin and bovine serum albumin dimer (Fig. 3b). From these data a molecular mass for the native OTCase of approx. 95000 Da was calculated. These results confirm that OTCase from *E. coli* W and anabolic OTCase from other sources is a trimer with subunits of molecular mass approx. 37500 Da (Legrain *et al.*, 1977).

Pure OTCase, when stored at a high concentration (17 mg/ml), was stable at pH 9.0 and 4°C for 3–4 weeks. Prolonged storage resulted in a decrease in specific activity to 2000 units/mg. We found that the most convenient procedure was to store portions of the enzyme at –80°C and thaw them as required. A small amount of activity was lost during freeze-thawing, but prolonged storage at –80°C did not affect activity. Long-term storage of OTCase at 4°C resulted in the appearance of an electrophoretically distinct diffuse band with an apparent molecular mass of approx. 35000 Da (Fig. 2). We did not investigate whether there was a direct correlation between the appearance of this band and the loss of activity.

Amino acid analysis

The results from the amino acid analysis of the purified *E. coli* W enzyme are shown in Table 2. The enzyme from *E. coli* is very similar to the enzyme from other sources. In particular, it is characterized by the relatively high number of acidic residues (aspartate and glutamate) and a small number of basic amino acids (which is consistent with the low pI of the microbial enzyme). The enzyme is also rich in the hydrophobic amino acids, such as leucine and alanine. The predominant difference between the *E. coli* enzyme and the enzyme from other sources is the apparent substitution of alanine for isoleucine residues.

Effect of phaseolotoxin on the standard OTCase assay

Table 3 shows the effect of phaseolotoxin on the standard assay system without pre-incubation of enzyme and toxin. The concentration of phaseolotoxin was varied 10000-fold; 100 µM-phaseolotoxin

Fig. 3. High-pressure liquid chromatography/gel-permeation chromatography of purified OTCase

Chromatographic conditions were as described in the Experimental section. (a) Purified OTCase (90 µg). (b) A mixture of protein standards containing (100 µg of each) lactate mono-oxygenase, bovine serum albumin, ovalbumin and whale myoglobin, plus 90 µg of OTCase.

almost completely inhibited OTCase activity, 5 µM gave just over 50% inhibition, and concentrations under 0.1 µM barely affected the assay system. These results are similar to those obtained by Ferguson *et al.* (1980) with crude enzyme extracts from various sources.

It was essential to determine whether phaseolotoxin was binding the OTCase in a reversible or an irreversible fashion. Enzyme samples were chromatographed on a Sephadex G-50 column (14 cm × 1 cm; $V_0 = 5.6$ ml) equilibrated in 50 mM-

Table 2. *Amino acid composition of OTCase*

Samples of purified *E. coli* W OTCase were analysed as described in the Experimental section. The μmol of residues/mg of protein is either the mean $\pm$ s.d. or the extrapolated value from 12 determinations. The mol of residues/mol of subunit has been rounded off to the nearest integer. Abbreviation: N.D., not determined.

Amino acid	Amino acid composition					
	(mol of residue/mol of subunit)					
Source ...	<i>E. coli</i> W (this study)	<i>E. coli</i> W (this study)	<i>S. faecalis</i> (Marshall & Cohen, 1972)	Bovine (Marshall & Cohen, 1972)	Rat (Lusty <i>et al.</i> , 1979)	Human (Kalousek <i>et al.</i> , 1978)
$(\mu\text{mol/mg of protein})$						
Asp	0.795 $\pm$ 0.025	30	36	27	30	30
Thr	0.555	21	20	24	26	21
Ser	0.349	13	15	21	25	21
Glu	1.138 $\pm$ 0.027	43	41	34	39	37
Gly	0.805 $\pm$ 0.046	30	26	21	24	28
Ala	1.033 $\pm$ 0.044	39	26	21	24	26
Val	0.521 $\pm$ 0.018	20	26	15	17	16
Met	0.290	11	13	10	11	8
Ile	0.267 $\pm$ 0.011	10	18	15	17	16
Leu	0.966 $\pm$ 0.026	36	31	43	50	44
Tyr	0.273	10	10	11	12	9
Phe	0.339 $\pm$ 0.012	13	18	11	13	12
His	0.230	9	8	7	8	9
Lys	0.635 $\pm$ 0.018	24	23	26	25	27
Arg	0.340 $\pm$ 0.021	13	13	11	14	12
Pro	0.232	9	9	15	15	17
Trp	0.122	5	3	5	4	5
Cys	N.D.	—	2	3	2	3
Subunit M_r ...		37500	38900	36000	39900	38000

Table 3. *Effect of phaseolotoxin on the standard OTCase assay*

Enzyme assays were performed in a total volume of 100 μ l at 37°C containing 5 mM-carbamoyl phosphate, 3 mM-ornithine, 50 mM-Tris/HCl buffer, pH 8.0, and increasing concentrations of phaseolotoxin. The reaction was started by the addition of enzyme (6.0 munits), and stopped after 10 min with colour reagent. Each result was the mean of duplicate determinations (S.E.M. < 5%) and typical of several experiments performed under these conditions.

Concn. of phaseolotoxin (μ M)	Citrulline produced (nmol)	Inhibition of control assay (%)
0	29.5	0
0.01	28.5	3
0.10	27.7	6
0.50	24.5	17
1.0	22.0	25
5.0	12.6	57
10.0	7.8	74
100	2.1	93

Tris/HCl buffer, pH 8.0. Samples containing enzyme (0.13 μ M) and phaseolotoxin (100 μ M) were incubated at 37°C for 10 min in the presence of carbamoyl phosphate (0.25 mM). Enzyme activity was inhibited by 97% under these conditions (Table 3). Chromatography of these samples (100 μ l) resulted in the recovery of 75% of the initial enzyme activity. When 0.13 μ M enzyme (100 μ l) alone was chromatographed, 80% of the initial enzyme activity was recovered. These data provide evidence that the inhibition is reversible and are consistent with the results obtained by Tam & Patil (1972), who showed that inhibition of OTCase by phaseolotoxin was reversible by dialysis.

Initial velocities

Enzyme reaction in the presence of the toxin only became linear after 1 min of incubation (Fig. 4a). This pre-steady-state period was consistently found with incubations of toxin and enzyme, but was absent from control assays. In a separate ex-

Fig. 4. *Time course of OTCase inhibition by phaseolotoxin*

(a) OTCase activity was measured under the following conditions: 0.5 mM-carbamoyl phosphate, 3 mM-ornithine, 50 mM-Tris/HCl buffer, pH 8.0, and phaseolotoxin. The total volume was 480 μ l. Each reaction was started by the addition of enzyme (77 munits), and 100 μ l samples were quenched in 1 ml of chromogenic reagent. The concentrations of phaseolotoxin were: $\bullet$, 0 μ M; $\square$, 0.42 μ M; $\blacksquare$, 0.83 μ M; $\blacktriangle$, 1.67 μ M; $\circ$, 3.33 μ M. (b) Phaseolotoxin and enzyme were pre-incubated (total volume of 960 μ l) at 37°C for 5 min. The reaction was started by the addition of 1 mM-carbamoyl phosphate and 1 mM-ornithine. Samples (100 μ l) were removed at the appropriate times and quenched with 1 ml of chromogenic reagent. The concentrations of phaseolotoxin were: $\bullet$, 0 μ M; $\blacktriangle$, 0.83 μ M; $\circ$, 1.66 μ M; $\blacksquare$, 3.33 μ M; $\triangle$, 4.16 μ M.

periment in which samples were taken every 15 s for the first minute, a non-linear period of enzyme activity was confirmed. Pre-incubation of enzyme with toxin before the addition of substrates did not alter this phenomenon (Fig. 4b). These results can be explained by phaseolotoxin having low rate constants for the formation and breakdown of the enzyme-toxin complex, with respect to the substrates. Previous workers have measured steady-state velocities by simply determining the amount of citrulline produced during a fixed time (Tam & Patil, 1972; Ferguson *et al.*, 1980; Staskawicz *et al.*, 1980). This has led to an overestimate of the true velocity if enzyme is added to toxin

and substrates simultaneously, but an underestimation of the velocity when enzyme and toxin were pre-incubated.

Determination of inhibition constants

Steady-state velocities were measured with various concentrations of phaseolotoxin at four different concentrations of carbamoyl phosphate but at a constant concentration of ornithine (Fig. 4a). The data were plotted by the method of Dixon (1953) (Fig. 5a), and the apparent K_i determined by this method was $0.2 \mu\text{M}$. The mode of inhibition was mixed or competitive with respect to carbamoyl phosphate. When these results were re-plotted

Fig. 5. Kinetics of OTCase inhibition by phaseolotoxin

(a) Dixon plot of the inhibition of OTCase by phaseolotoxin with respect to carbamoyl phosphate. Steady-state velocities were measured as described for Fig. 4(a) at various concentrations of phaseolotoxin. The concentrations of carbamoyl phosphate were: ●, 5 mM; ○, 1 mM; ■, 0.5 mM; ▲, 0.3 mM. The concentration of ornithine was constant at 3.0 mM. The apparent K_i obtained was $0.2 \mu\text{M}$. (b) The results from (a) were re-plotted by the method of Cornish-Bowden (1974). The concentrations of carbamoyl phosphate were: ●, 5 mM; ■, 1 mM; ▲, 0.5 mM. The apparent K_i' obtained was $10 \mu\text{M}$. (c) Dixon plot of the inhibition of OTCase by phaseolotoxin with respect to ornithine. Steady-state velocities were measured at various phaseolotoxin concentrations, 1 mM-carbamoyl phosphate and ornithine concentrations of: ●, 0.5 mM; ■, 3.0 mM; ▲, 6.0 mM. The apparent K_i obtained was $0.9 \mu\text{M}$.

Scheme 1. Mechanism of OTCase inhibition by phaseolotoxin

Key: E, OTCase; A, carbamoyl phosphate; B, ornithine; P, citrulline; Q, phosphate; I, phaseolotoxin; k_{+1} – k_{-4} , rate constants.

Fig. 6. Calculation of the rate constant for the dissociation of the enzyme–phaseolotoxin complex (k_{-3})

Values of k' were calculated as described in the text from experiments performed as described in Fig. 4(a). Carbamoyl phosphate concentrations were: ●, 5 mM; □, 1 mM; ■, 0.5 mM; ▲, 0.3 mM. The value of 0.3 min^{-1} was obtained for k_{-3} .

in accordance with the method of Cornish-Bowden (1974), the mode of inhibition was mixed with a K'_i of $10 \mu\text{M}$, 50 times higher than the K_i (Fig. 5b).

The mode of inhibition with respect to ornithine was determined by varying the concentration of phaseolotoxin at different concentrations of ornithine. The Dixon plot (Fig. 5c) revealed non-competitive kinetics with an apparent K_i of $0.9 \mu\text{M}$.

Phaseolotoxin appears to be competing for the carbamoyl phosphate-binding site of OTCase; the apparent K_i of $0.2 \mu\text{M}$ is 1000 times less than the K_m for carbamoyl phosphate and 75 times less than its dissociation constant (Legrain & Stalon, 1976). The toxin also appears to be able to bind to the enzyme–carbamoyl phosphate complex, which is not surprising, since many amino acids and peptides are inhibitors of OTCase (Legrain & Stalon, 1976). In this case it is possible that phaseolotoxin is binding to the ornithine-binding site of the enzyme. A

mechanism that would accommodate the kinetic data is shown in Scheme 1.

The presence of a transient phase of enzyme activity before a steady state is reached (Fig. 4a) shows that phaseolotoxin shares a characteristic of tight-binding inhibitors in that it has low rates of association to and dissociation from the enzyme. When there is a significant transient phase before a steady-state velocity is attained, it is possible to estimate the individual rate constants of the K_i (k_{+3} and k_{-3} of Scheme 1) (Williams & Morrison, 1979; Cha, 1975, 1976).

Cha (1975) outlined four possible mechanisms describing slow-binding inhibition. The one that best fits that of phaseolotoxin is a special case of mechanism D, where there is no equilibrium between EI and EIS. The velocity at any time is:

$$v = v_s + (v_0 - v_s) e^{-k't} \quad (1)$$

where v_s and v_0 are the steady-state and initial velocities respectively, t is time and

$$k' = \frac{\left(k_{+3} + \frac{k_{+4}[A]}{K_a}\right)[I]}{1 + [A]/K_a} + k_{-3} \quad (2)$$

where K_a is the Michaelis constant for carbamoyl phosphate. Integration of eqn. (1) gives:

$$P = v_s - \frac{1}{k'} (v_s - v_0) (1 - e^{-k't}) \quad (3)$$

at $t = 0$:

$$P_0 = \frac{1}{k'} (v_0 - v_s) \text{ or } k' = \frac{1}{P_0} (v_0 - v_s) \quad (4)$$

Since P_0 can be determined by extrapolation of v_s to the y -axis (Fig. 4a), a value of k' can be estimated. A plot of k' against $[I]$ should be linear and the intercept on the y -axis will give an estimate of k_{-3} (eqn. 2).

k' values were calculated from the results in Fig. 4(a) and from the data of other experiments performed at different concentrations of carbamoyl phosphate. Plots of k' against $[I]$ were linear (Fig. 6) and gave a value of 0.3 min^{-1} ($5 \times 10^{-3} \text{ s}^{-1}$) for k_{-3} . By using a value of K_i of $0.2 \mu\text{M}$, k_{+3} was calculated to be $2.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The on/off constants for the toxin are significantly lower than the equivalent rate constants calculated for carbamoyl phosphate by Legrain & Stalon (1976). The 'on' constant for carbamoyl phosphate (k_{+1}) of $6.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ is 260 times faster than k_{+3} , and the value for k_{-1} of 97 s^{-1} is 20000 times faster than the 'off' constant for phaseolotoxin (k_{-3}). These data explain the transient phase of activity before the steady-state kinetics for phaseolotoxin inhibition of OTCase.

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