BBA 66256

MECHANISM OF INHIBITION OF SPINACH β-CYSTATHIONASE BY RHIZOBITOXINE

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(Received August 24th, 1970)

SUMMARY

Inactivation of spinach β-cystathionase by rhizobitoxine is an example of an active-site-directed irreversible inhibition, and may be represented by the following mechanism:

$$EP + R \rightleftharpoons EPR \rightarrow EPR_I$$

EP, the holoenzyme form of β-cystathionase (P, pyridoxal phosphate); R, rhizobitoxine; EPR, a dissociable enzyme–rhizobitoxine complex; EPR_I, inactivated enzyme.

This proposed mechanism is based on the following observations:

1. The inactivation is progressive and ultimately complete as would be expected for an irreversible process. Irreversibility of the inactivation was demonstrated directly by gel filtration.

2. The rate of inactivation is first order with respect to active enzyme.

3. A plot of the pseudo first-order rate constant as a function of rhizobitoxine concentration shows saturation kinetics, consistent with the formation of a dissociable complex EPR. The equilibrium constant (K_i) for the dissociation of EPR is $8.0 \cdot 10^{-5}$ M; the rate of the inactivation at infinite rhizobitoxine concentration is equivalent to a minimum half-time of inactivation ($T_{\frac{1}{2}}$) of 0.2 min.

4. The rate of dissociation of EPR is rapid relative to the rate of inactivation.

5. The substrates cystathionine and djenkolate, as well as the competitive inhibitor β-cyanoalanine, protect the enzyme from inactivation by rhizobitoxine. The kinetics of this protection are consistent with a competition between these amino acids and rhizobitoxine for the active enzyme.

6. The rhizobitoxine-inactivated enzyme can be reactivated specifically by incubation with pyridoxal phosphate, suggesting that the irreversible step in inactivation involves formation of a bond between rhizobitoxine and pyridoxal phosphate.

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

INTRODUCTION

Certain strains of the root nodule bacterium *Rhizobium japonicum* synthesize a toxin that inhibits greening of new leaf growth of the host soybean plant[1]. The toxin, which has been called "rhizobitoxine", causes the same effect when applied to many other plant species[2]. It also inhibits the growth of *Salmonella typhimurium*[3]. This toxin has been isolated from the root nodules and leaves of diseased plants[1] and from the culture medium of *R. japonicum*[4]. Although the precise structure of the toxin remains to be elucidated, it is known to be a basic sulfur-containing amino acid which yields an ether derivative of homoserine upon desulfurization[5]. This partial knowledge of its structure led to experiments with *S. typhimurium* which suggested that rhizobitoxine acts by interfering with the metabolism of cystathionine[3]. This suggestion was based chiefly on the observations that supplementation with homocysteine or methionine (but not serine, homoserine or cystathionine) prevented the inhibitory effect of rhizobitoxine on the growth of *S. typhimurium*, and that rhizobitoxine caused a marked inhibition of β-cystathionase* purified from *S. typhimurium*.

The question remains as to whether the same mode of action of rhizobitoxine is operative in higher plants. As a preliminary step in resolving this question, the effect of rhizobitoxine on the spinach β-cystathionase described in the accompanying report[6] was examined.

MATERIALS AND METHODS

General methods

All incubations were at 30°. Except where stated otherwise, all gel filtrations were performed at 4° with Sephadex G-25 (Pharmacia) equilibrated with 5 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA and 0.14 mM 2-mercaptoethanol. Proteins were determined by the method of LOWRY *et al.*[7].

Chemicals

Rhizobitoxine was isolated and purified from whole-culture extracts of *R. japonicum* Strain 94 (U.S.D.A. Rhizobium Collection, Beltsville, Md.) by a modification of a previously described procedure[4,8]. The medium contained the following components in a final volume of 1 l: 10 g of mannitol, 0.11 g of $CaCl_2$, 0.25 g of $MgSO_4 \cdot 7\ H_2O$, 1 g of casamino acid hydrolyzate (Difco technical grade containing 8% N and 37% NaCl), the hot-water extract from 5 g of active dry yeast, 0.19 g of K_2HPO_4 and 0.124 g KH_2PO_4. The phosphate buffer was sterilized separately and added to the medium after autoclaving. The final pH of the medium was 6.8.

378 l of medium were inoculated with 10 l of an exponentially growing culture of *R. japonicum*, and the culture stirred and aerated (0.1 l of air per min per l of culture, antifoamant added) at 28° until the bacteria reached the end of their exponential phase of growth (4–5 days). Cell yield was about $1 \cdot 10^9$/ml or 2.7 g (wet weight)/l. To extract rhizobitoxine from both the cells and the medium, the whole culture was steamed for 30 min, centrifuged, and the supernatant solution concentrat-

* International Union of Biochemistry recommendations have not yet appeared for this enzyme, which catalyzes an elimination at the β-carbon atom of the C_3 moiety of cystathionine to yield homocysteine, pyruvate and ammonia.

ed 20-fold by vacuum distillation at 60°. Storage of the concentrate at 5° resulted in considerable precipitation of medium ingredients which were centrifuged and discarded.

The clarified concentrate from 189 l of culture was adjusted to pH 7.0 and passed through a column (7.6 cm × 54 cm) of Dowex 50W-X4 resin (200–400 mesh) in the NH_4^+ form[9] at 7.8 ml/min. The column, maintained at 5° throughout the purification, was washed with 7 bed volumes of water (in 9 h) and eluted with 0.02 NH_4OH (7.8 ml/min, 23-ml fractions). Rhizobitoxine was recovered in eluate Fractions 85–179 and unknown Y, the naturally occurring component of rhizobitoxine[1], in Fractions 95–230.

Aliquots (0.1 ml) of eluate fractions were evaporated to dryness to rid samples of NH_3 and assayed for rhizobitoxine by descending chromatography on paper buffered with 0.04 M sodium phosphate (pH 2.5)[9]. The developing solvent was 80% phenol, and the detection reagent was ninhydrin in 95% ethanol buffered with 5% collidine. Rhizobitoxine (R_F = 0.15) forms a yellow reaction-product with ninhydrin on paper[8], and separates from the contaminating amino acids unknown Y (R_F = 0.34), lysine (R_F = 0.28) and arginine (R_F = 0.43).

Fractions containing rhizobitoxine were combined, evaporated to dryness at 40°, and re-chromatographed on a column (0.75 cm × 50 cm) of resin, generally as described above. The column was successively eluted with 360 ml of 0.002 M, 280 ml of 0.0033 M and 1000 ml of 0.01 M NH_4OH. Fractions each of 10 ml were collected at a column flow rate of 17 ml/h. Rhizobitoxine was recovered in Fractions 46–127 and unknown Y in Fractions 124–129; however, elution volumes vary considerably with amounts of materials being chromatographed. Evaporation of selected fractions yielded rhizobitoxine that was pure by criteria of paper chromatography in three solvents[1] and paper electrophoresis for 90 min at 2000 V in 0.025 M sodium phosphate (pH 6.9). The yield of rhizobitoxine was about 1 mg/l of culture.

Reagent solutions of rhizobitoxine were prepared using an assumed molecular weight of 414 (L. D. OWENS, J. F. THOMPSON AND K. BIEMANN, unpublished). Solutions (0.25 M) of pyridoxal phosphate were prepared by dissolving 165 mg of pyridoxal phosphate (Calbiochem, A grade) in a mixture of 0.6 ml of 1 M KOH and 0.25 ml of 2 M Tris–HCl (pH 8.65), followed by dilution with water to a final volume of 2.5 ml. These solutions remained stable for many months when stored in the dark at −65°.

Preparation and assay of spinach β-cystathionase

The enzyme used in these studies is Fraction 3 which had been concentrated up to 35-fold by "dialysis" against Aquacide II (Calbiochem) powder, then subjected to gel filtration[6]. The enzyme was assayed spectrophotometrically[6].

Determination of the rate of inactivation of β-cystathionase by rhizobitoxine

The inactivation reaction was started by addition of enzyme to an incubation mixture containing 0.2 M Tris–HCl (pH 8.65) and the additions described in the experimental details of the figures and tables. Inactivation was allowed to proceed for the designated time, then stopped by addition of L-cystathionine. The resultant concentration of cystathionine was at least 20 mM, and was sufficient to prevent further inactivation with all concentrations of rhizobitoxine used. The protective effect of cystathionine was instantaneous, since no difference in activity was observed

between starting an enzyme assay with cystathionine (5 mM), or with a mixture of cystathionine (5 mM) and rhizobitoxine (0.01 mM). After addition of cystathionine, the solution was stored at 4° for a maximum of 30 min before assay of β-cystathionase. Control experiments showed that no significant loss in enzyme activity occurred during this storage period. In the study of the effect of pyridoxal phosphate on inactivation, it was necessary to remove pyridoxal phosphate by gel filtration before the enzyme could be assayed spectrophotometrically. In this particular study, gel filtration served also to stop the inactivation.

Preparation of inactivated β-cystathionase

Preparations of inactivated enzyme were obtained by incubation for 12 min of β-cystathionase in 0.2 M Tris–HCl (pH 8.65) containing 0.1 mM rhizobitoxine. Inactivation was stopped by addition of cystathionine to a final concentration of 8.35 mM followed by gel filtration.

Determination of the rate of reactivation

Reactivation was started by addition of inactivated enzyme to a reaction mixture containing 0.2 M Tris–HCl (pH 8.65) and the additions described in the experimental details of the figures and tables. The reaction was allowed to proceed for the desired time, then stopped either by gel filtration, or preferably by dilution followed immediately by gel filtration. The precise method of stopping the reactivation is described in the experimental details of the figures and tables. Recovery of protein after gel filtration was quantitative. Enzyme assays were performed immediately after gel filtration.

RESULTS

Time-course of inactivation

The time-course of inactivation of β-cystathionase by rhizobitoxine is shown in Fig. 1, in which the ordinate is plotted linearly in Curve 1 and logarithmically in Curves 2 and 3. Inactivation proceeds in two phases. In the primary phase, the major proportion of the enzyme activity was rapidly lost. In the secondary phase, the small proportion of residual activity disappeared at a very low rate. These results could be explained according to two alternative hypotheses: (a) β-Cystathionase exists in two forms (isoenzymes), one relatively susceptible to rhizobitoxine, the other relatively resistant. (b) β-Cystathionase exists in one form only, which is completely converted by rhizobitoxine to a form with a small residual activity. None of the experiments described in this paper distinguish between these hypotheses.

The relatively small amount of enzyme activity that remained during the secondary phase was determined at the limit of sensitivity of the assay, and its accurate determination was therefore impractical. In this experiment, an approximate value of 7% was obtained by extrapolation (represented by the broken line in Curve 1). Repeated determinations at more frequent time intervals than those reported in Fig. 1 yielded a mean value of 13 ± 6%. It may be calculated from the equations below that the uncertainty in this value is reflected by only a small error in the determination of kinetic constants. For the same reasons as described above, it was also impractical to determine whether the very low rate of inactivation reported

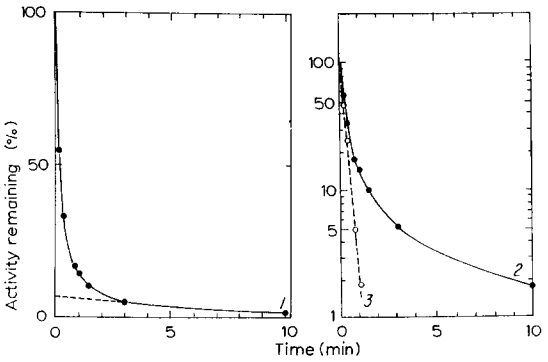

Fig. 1. Time-course of inactivation. The ordinate of Curve 1 represents a linear plot of the enzyme activity remaining in the presence of rhizobitoxine (B), expressed as a percentage of the enzyme activity remaining in the absence of rhizobitoxine (A). The ordinate of Curve 2 is the same $[(B/A) \times 100]$ as that of Curve 1, except for its presentation on a logarithmic scale. Curve 3 shows the time-course of inactivation during the rapid phase only, and is expressed on a logarithmic scale by the expression $[(B - C)/(A - C)] \times 100$. C is the residual enzyme activity and, as specified in the text, equals 13% of A.

above represents a small finite rate, or whether the residual activity was completely resistant to inactivation.

The time-course shown in Curve 1 is presented as a semi-logarithmic plot in Curve 2. The biphasic nature of the inactivation is again evident. Curve 3 shows the time-course of inactivation during the rapid phase only and was obtained from Curve 2 by subtraction of the residual enzyme activity (see experimental details of Fig. 1). Within experimental error, the inactivation shown in Curve 3 proceeds to completion in a logarithmic fashion, indicating that the rate of inactivation is first order with respect to active enzyme concentration.

The kinetic studies to be reported below were determined for the rapid phase of inactivation only. Inactivation is characterized[10] by either a pseudo first-order rate constant:

$$k = \frac{1}{t} \ln \frac{A-C}{B-C}$$

or (in the reciprocal form) as the time required for 50% inactivation:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

The time of inactivation (t) is expressed in minutes. The other terms comprising the above equations are described in the legend of Fig. 1.

Effect of rhizobitoxine concentration on rate of inactivation

The results of Fig. 2 demonstrate that the rate of inactivation is a function of rhizobitoxine concentration. A model to explain this relationship will be presented below.

Irreversibility of inactivation

The type of inactivation described above, which is progressive and ultimately

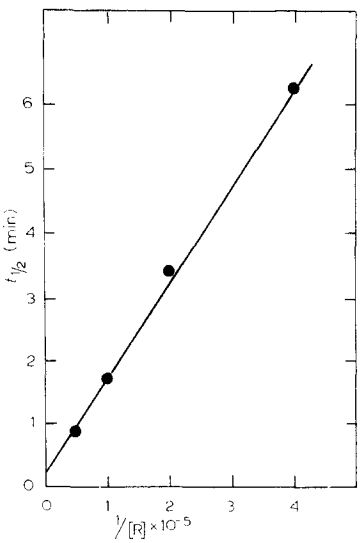

Fig. 2. Inactivation half-time as a function of the reciprocal of rhizobitoxine concentration. Each value of $t_{1/2}$ was determined from the time required for the value $[(B - C)/(A - C)] \times 100$ (see legend of Fig. 1) to decrease to 50%. The straight line was fitted by the method of least squares. [R] = molar concentration of rhizobitoxine.

complete, is characteristic of an irreversible inhibition[11,12]. The irreversible nature of the inhibition was confirmed by the demonstration that gel filtration did not significantly affect the specific activity of rhizobiotoxine-inactivated enzyme (Table I).

Protection of enzyme by β-cyanoalanine, cystathionine or djenkolate

The protective effect of various amino acids is shown in Table II. In the absence of added amino acid, 83% of the enzyme was inactivated by rhizobitoxine. Addition of β-cyano-L-alanine, L-cystathionine or L-djenkolate reduced the inactivation to 19, 28.5 or 49%, respectively, indicating a marked protection by these amino acids. A minor protective effect was observed with L-cystine and L-homocystine. No significant protection was observed with any of the following amino acids: D-cystathionine, L-homoserine, L-serine, L + *meso*-lanthionine, L-methionine, L-lysine, or L-diaminopimelate.

TABLE I

IRREVERSIBILITY OF INACTIVATION

Enzyme was incubated for 7.5 min either in the presence or absence of 0.0125 mM rhizobitoxine. At the completion of the incubation one aliquot was assayed directly, the other after gel filtration.

Additions	*Relative specific activity (%)*	
	Before gel filtration	*After gel filtration*
None	100	105
Rhizobitoxine	19	23.5

TABLE II

EFFECT OF VARIOUS AMINO ACIDS ON INACTIVATION

Enzyme was incubated for 6.5 min with the amino acids (0.715 mM) as shown, either in the absence or presence of rhizobitoxine (0.0143 M). Inactivation is expressed as the percentage difference between the rates of enzyme activity determined in the presence and absence of rhizobitoxine.

Added amino acid	*Inactivation (%)*
None	83
β-Cyano-L-alanine	19
L-Cystathionine	28.5
L-Djenkolate	49
L-Cystine	66
L-Homocystine	73

The effect of increasing concentrations of cystathionine on the rate of inactivation is shown in Fig. 3. Curve 1 shows the rate of inactivation obtained with 0.02 mM rhizobitoxine. In the presence of the same concentration of rhizobitoxine, ad-

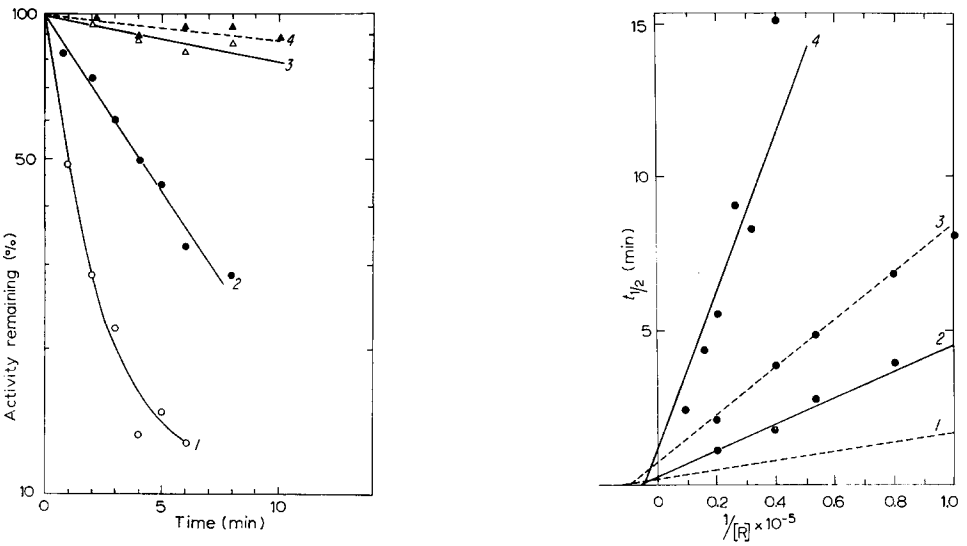

Fig. 3. Effect of cystathionine on inactivation. Enzyme was incubated with the following additions: Curve 1, 0.02 mM rhizobitoxine; Curve 2, 0.02 mM rhizobitoxine + 0.3 mM L-cystathionine (cystathionine/rhizobitoxine = 15/1); Curve 3, 0.02 mM rhizobitoxine + 10 mM cystathionine (cystathionine/rhizobitoxine = 500/1); Curve 4, none. The ordinate represents the enzyme activity remaining, expressed as a percentage of the initial activity.

Fig. 4. Competition between rhizobitoxine and β-cyanoalanine. Details of the experimental points used in the derivation of Curve 1 (β-cyanoalanine absent) are represented in Fig. 2. Curves 2–4 represent the following respective concentrations of β-cyanoalanine: $3.75 \cdot 10^{-5}$, $7.5 \cdot 10^{-5}$ and $4 \cdot 10^{-4}$ M. Enzyme was incubated with β-cyanoalanine and rhizobitoxine at the concentrations shown in a final volume of 0.4 ml. At the completion of the incubation 0.1 ml of 0.1 M cystathionine was added followed by enzyme assay in a final volume of 1.0 ml. From the determined values of K_m and K_A for cystathionine and β-cyanoalanine, respectively, it may be calculated that even the highest concentration of β-cyanoalanine used permitted an enzyme assay of over 94% of that expected in the absence of β-cyanoalanine. No corrections were made for this small effect of β-cyanoalanine on the assay of β-cystathionase. All lines were fitted by the method of least squares.

dition of 0.3 mM cystathionine (Curve 2) markedly decreases the rate, while addition of 10 mM cystathionine (Curve 3) reduces the rate of inactivation to a low value not very different from that observed in the absence of rhizobitoxine (Curve 4). The increased protection against inactivation as the ratio of cystathionine/rhizobitoxine was raised is consistent with a competition between rhizobitoxine and cystathionine for enzyme*.

It was impractical to study directly the kinetics of competition between rhizobitoxine and cystathionine or djenkolate, since cystathionine and djenkolate are both active substrates for spinach β-cystathionase, and therefore change in concentration during kinetic measurements. However, β-cyanoalanine is a competitive inhibitor, but not a substrate of spinach β-cystathionase[6], and is ideally suited for kinetic competition studies. Fig. 4 shows the results of a kinetic study in which $t_{\frac{1}{2}}$ is plotted as a function of the reciprocal of rhizobitoxine concentration for various concentrations of β-cyanoalanine. A model to explain these results will be presented below.

TABLE III

PERCENTAGE RECOVERY OF ENZYME ACTIVITY UNDER VARIOUS CONDITIONS

Separate aliquots of inactivated enzyme were incubated for 15 min in the presence of the additions shown, then subjected to gel filtration and determination of enzyme activity. The recovery is expressed as percentage of the activity before inactivation with rhizobitoxine.

Expt.	*Addition*				*Recovery (%)*
	Pyridoxal phosphate (1 mM)	*Pyridoxal phosphate (25 mM)*	*Cystathionine (10 mM)*	*Rhizobitoxine (0.1 mM)*	
1	—	—	—	+	26.5
2	+	—	—	+	79
3	+	—	+	+	90
4	—	+	—	+	102
5	+	—	—	+	10
6	+	—	+	+	85.5

Reactivation by pyridoxal phosphate

Experiments described above have provided evidence that the conversion of active to inactive enzyme is an irreversible reaction. These experiments were performed in the absence of added pyridoxal phosphate during inactivation and assay of the enzyme. The series of experiments reported below demonstrate that added pyridoxal phosphate can bring about reactivation of the enzyme.

The effect of various treatments on the restoration of activity of a preparation of inactivated enzyme is shown in Table III. Incubation with 1 mM pyridoxal phosphate (Expt. 2) resulted in recovery of 79% of the original activity. The presence of 10 mM cystathionine added either alone (separate experiments not shown) or in combination with 1 mM pyridoxal phosphate (Expt. 3) had no significant effect. Complete

* An alternative explanation that protection by cystathionine results from its chemical reaction with rhizobitoxine is most unlikely on the basis of the observed kinetics. Furthermore, no reaction between cystathionine and rhizobitoxine could be detected by paper chromatography and paper electrophoresis.

reactivation is obtained in the presence of 25 mM pyridoxal phosphate (Expt. 4). Reactivation is specific for pyridoxal phosphate, and was not observed with pyridoxal, pyridoxine 5'-phosphate, pyridoxamine, pyridoxamine 5'-phosphate, 4-pyridoxic acid and 4-deoxypyridoxine. In the presence of 0.1 mM rhizobitoxine, no net reactivation occurred (Expt. 5). However, rhizobitoxine was without effect in the presence of 10 mM cystathionine (Expt. 6).

These combined results show that complete reactivation could be achieved in the presence of pyridoxal phosphate, and that cystathionine was neither required nor did it affect the reactivation. Rhizobitoxine alone (but not in the presence of cystathionine) inhibits reactivation. The reason for this effect is probably that inactivation proceeds at a faster rate than reactivation (see below), rather than a direct effect of rhizobitoxine on the reactivation by pyridoxal phosphate.

Time-course of reactivation

The percentage of inactive enzyme remaining is logarithmic with time (Fig. 5), showing that the rate of reactivation is first order with respect to inactive enzyme.

Effect of pyridoxal phosphate concentration on rate of reactivation

The results of Fig. 6 show the effect of pyridoxal phosphate concentration on the rate of reactivation. While these results do not distinguish between a linear and

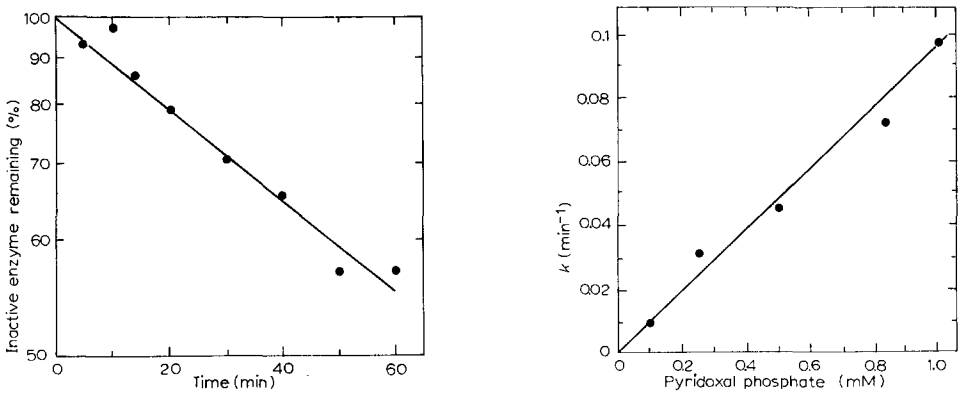

Fig. 5. Time-course of reactivation. Preparations of inactivated enzyme used in this and other studies consisted of a mixture of active and inactive enzyme, whose relative proportions were determined as follows: Complete reactivation obtained by incubation for 15 min in the presence of 25 mM pyridoxal phosphate yields a value (D) which represents the total amount of enzyme (active *plus* inactive). A corresponding incubation in the absence of pyridoxal phosphate yields a value (E) which represents the amount of active enzyme only. The difference ($D - E$) between these two assays therefore represents the amount of inactive enzyme originally present in the inactivated enzyme preparation. The amount of inactive enzyme remaining after partial reactivation can be obtained similarly by subtracting the value (F) of the enzyme assay obtained after partial reactivation from the value D. The ordinate is therefore given by the expression $[(D - F)/(D - E)] \times 100$. The time-course of reactivation was determined in the presence of 0.05 mM pyridoxal phosphate, and was stopped at the times shown by gel filtration.

Fig. 6. Effect of pyridoxal phosphate concentration on the rate of reactivation. Inactivated enzyme preparation was incubated for 15 min with pyridoxal phosphate at the concentrations shown above, and stopped by gel filtration. The pseudo first-order rate constants (k) are given by the expression $1/15 \ln [(D - E)/(D - F)]$ (ref. 10). The method of determination of the values D, E and F is given in the legend of Fig. 5.

hyperbolic relationship, they do demonstrate that the rate of reactivation is not higher than first order with respect to pyridoxal phosphate.

Effect of pyridoxal phosphate on rhizobitoxine inactivation

At comparable concentrations of pyridoxal phosphate and rhizobitoxine, reactivation is much slower than inactivation. Thus the rate of reactivation with 0.05 mM pyridoxal phosphate was characterized by a $t_{\frac{1}{2}}$ of approx. 74 min (Fig. 5), whereas a $t_{\frac{1}{2}}$ of inactivation of approx. 0.5 min with 0.05 mM rhizobitoxine may be calculated from Eqn. 4 (below). Since the rates of inactivation and reactivation are dependent upon the concentrations of rhizobitoxine and pyridoxal phosphate, respectively, it should be possible to equalize the rates of the two processes by using low concentrations of rhizobitoxine and high concentrations of pyridoxal phosphate. Under these conditions, pyridoxal phosphate should prevent the inactivation by rhizobitoxine. This prediction was confirmed by incubation of enzyme with a low concentration ($7.5 \cdot 10^{-6}$ M) of rhizobitoxine and a high concentration (25 mM) of pyridoxal phosphate. Incubation for 10 min with pyridoxal phosphate alone had no effect on enzyme activity. Incubation with rhizobitoxine resulted in inhibition to 27% of the original activity. Incubation with rhizobitoxine in the presence of pyridoxal phosphate*, by contrast, resulted in a relatively small net loss of enzyme activity to 74% of its original activity. This figure approximates that expected from the values of separate assays of the rate of inactivation and reactivation determined with either $7.5 \cdot 10^{6}$ M rhizobitoxine or 25 mM pyridoxal phosphate, respectively.

DISCUSSION

The results obtained are consistent with inactivation of β-cystathionase by rhizobitoxine proceeding *via* the following equations:

$$EP + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EPR \qquad (1)$$

$$EPR \xrightarrow{k_2} EPR_I \qquad (2)$$

EP, the holoenzyme form of β-cystathionase (P, pyridoxal phosphate); R, rhizobitoxine; EPR, a dissociable β-cystathionase–rhizobitoxine complex; EPR_I, inactivated enzyme.

The following rate equation for enzyme inactivation may be derived from Eqns. 1 and 2:

$$v_i = \frac{V_i [R]}{[R] + K_i} \qquad (3)$$

where v_i is the velocity of inactivation at non-saturating concentration of rhizobitoxine; V_i, the velocity of inactivation at saturating concentration of rhizobitoxine; and $K_i = ([EP] [R])/[EPR]$.

* A non-enzymic reaction of pyridoxal phosphate with rhizobitoxine, in which a ninhydrin-positive compound is formed, was demonstrated by paper chromatography and electrophoresis. However, this reaction is not responsible for the protective effect of pyridoxal phosphate, since the inhibitory effect of rhizobitoxine was not significantly decreased by prior incubation with a 130-fold molar excess of pyridoxal phosphate.

Eqn. 3 may be expressed in terms of $t_{\frac{1}{2}}$ and $T_{\frac{1}{2}}$ (minimum half-time of inactivation obtained at a saturating concentration of rhizobitoxine) by substitution of the term $t_{\frac{1}{2}}/T_{\frac{1}{2}} = V_i/v_i$:

$$t_{\frac{1}{2}} = \frac{T_{\frac{1}{2}} K_i}{[\mathrm{R}]} + T_{\frac{1}{2}} \tag{4}$$

Protection by β-cyanoalanine, cystathionine or djenkolate is represented as follows:

$$EP + \mathrm{A} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\mathrm{PA} \tag{5}$$

where A is the amino acid β-cyanoalanine, cystathionine or djenkolate, and EPA is an enzyme–amino acid complex. The following rate equation for enzyme inactivation in the presence of A may be derived from Eqns. 1, 2 and 5:

$$v_i = \frac{V_i}{1 + \dfrac{K_i}{[\mathrm{R}]} + \dfrac{K_i\,[\mathrm{A}]}{K_\mathrm{A}\,[\mathrm{R}]}} \tag{6}$$

where K_A is the equilibrium constant for the dissociation of EPA in Eqn. 5. Eqn. 6 can be expressed in the form:

$$t_{\frac{1}{2}} = \frac{T_{\frac{1}{2}}}{[\mathrm{R}]}\left(K_i + \frac{K_i\,[\mathrm{A}]}{K_\mathrm{A}}\right) + T_{\frac{1}{2}} \tag{7}$$

The main properties and supporting evidence for the scheme described above are summarized below:

(1) The rate of inactivation is first order with respect to active enzyme concentration. This relationship is given by the kinetic equation

$$v_i = k_2\,[E\mathrm{PR}] = \frac{k_2\,[\mathrm{R}]\,[EP]}{K_i} \tag{8}$$

The pseudo first-order rate constant (and corresponding value of $T_{\frac{1}{2}}$) reported in the figures, is equal to the constant $k_2[\mathrm{R}]/K_i$ in Eqn. 8.

(2) The overall conversion (represented by combined Eqns. 1 and 2) of active to inactive enzyme is irreversible. This is supported by the time-course of the inactivation (Fig. 1), and by failure to reverse the inactivation by gel filtration (Table I).

(3) The inactivation involves the initial formation of a dissociable complex EPR. Formation of this complex is consistent with the saturation kinetics exhibited by a plot of the pseudo first-order rate constant as a function of rhizobitoxine concentration. These kinetics are illustrated in Fig. 2, which shows that the relationship between inactivation half-time and the reciprocal of rhizobitoxine concentration is a straight line (as predicted from Eqn. 4) with a slope equal to $T_{\frac{1}{2}} K_i$, and intercept equal to $T_{\frac{1}{2}}$. Values of 0.2 min and $8.0 \cdot 10^{-5}$ M for $T_{\frac{1}{2}}$ and K_i, respectively, were determined.

The following experiment was designed to test the hypothesis that EPR was in rapid equilibrium with EP and R. Such a condition would require that the rate con-

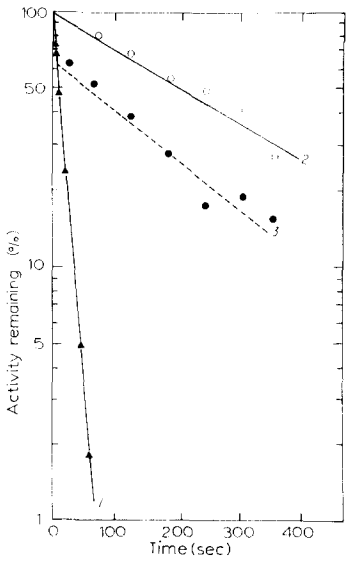

Fig. 7. Dissociation of enzyme–rhizobitoxine complex. Curves 1 and 2 represent inactivation obtained during the incubation of enzyme in the presence of $2 \cdot 10^{-4}$ and $6.25 \cdot 10^{-6}$ M rhizobitoxine, respectively. The incubation mixture for Curve 3 was initially the same as that for Curve 1, but at 6 sec the incubation mixture was diluted 32-fold with 0.2 M Tris–HCl (pH 8.65) to yield a concentration of rhizobitoxine equal to that of Curve 2. The values of the ordinate are given by the expression $[(B - C)/(A - C)] \times 100$ (see legend of Fig. 1). All lines were fitted by the method of least squares.

stant (k_{-1}) for the dissociation of EPR was much greater than the rate constant (k_2) for the conversion of EPR to EPR$_I$.

The rate of inactivation was first determined in the presence of a high concentration ($2 \cdot 10^{-4}$ M) of rhizobitoxine, at which the enzyme is predominantly complexed as EPR. In Fig. 7, Curve 1 shows that the presence of $2 \cdot 10^{-4}$ M rhizobitoxine results in a rate of inactivation experimentally indistinguishable from the maximum rate with a $T_{\frac{1}{2}}$ of 12 sec. It follows that at $2 \cdot 10^{-4}$ M rhizobitoxine, the enzyme is predominantly in the form EPR. The rate of inactivation was then determined in the presence of a low concentration ($6.25 \cdot 10^{-6}$ M) of rhizobitoxine (Curve 2). The $t_{\frac{1}{2}}$ value of approx. 193 sec corresponds to approx. 6%* of the active enzyme in the form EPR. In curve 3 the enzyme was initially incubated with $2 \cdot 10^{-4}$ M rhizobitoxine, then at 6 sec the incubation mixture was diluted to yield a concentration of rhizobitoxine of $6.25 \cdot 10^{-6}$ M. Before dilution, it is known (Curve 1) that the active enzyme is predominantly in the form EPR. If k_{-1} is much greater than k_2, dilution will result in the rapid establishment of a new equilibrium concentration of EPR represented by approx. 6% of the total active enzyme, and the rate of inactivation will be equal to that represented in Curve 2. If, on the other hand, the condition of k_{-1} being much greater than k_2 is not satisfied, the relative proportion of EPR will decrease relatively slowly upon dilution. Under these conditions, the rate of inactivation upon dilution will decrease slowly from that represented in Curve 1.

Curve 3 shows that dilution at 6 sec resulted in a rate of inactivation which was

* The percentage of enzyme complexed as EPR at a particular rhizobitoxine concentration is given by the expression $(T_{1/2}/t_{1/2}) \times 100$.

essentially identical to that represented in Curve 2. Statistical analysis* showed that the two rates did not differ significantly. Furthermore, the new equilibrium value of *E*PR appears to have been attained very rapidly, since the slower rate of inactivation was attained at the first determination after dilution. These results therefore confirm the hypothesis that Eqn. 1 is rapidly reversible.

(4) Protection by certain amino acids results from a competition between these amino acids and rhizobitoxine for the enzyme. The relationship (Fig. 4) between $t_{\frac{1}{2}}$ and the reciprocal of rhizobitoxine concentration at various concentrations of β-cyanoalanine is consistent with such a competition. Eqn. 7 predicts that this plot results in a series of straight lines whose slope increases with increasing concentration of β-cyanoalanine, and which intersect the ordinate at a value of $T_{\frac{1}{2}}$. Fig. 4 shows that the slopes of the lines do indeed increase with increasing concentration of β-cyanoalanine. Statistical analysis* showed that the lines intercepted the ordinate at values which did not differ significantly from the predicted value for $T_{\frac{1}{2}}$ of 0.2 min.

The competitive kinetics with rhizobitoxine were determined above only with β-cyanoalanine. However, since β-cyanoalanine is a competitive inhibitor of spinach β-cystathionase, the same mechanism of protection presumably applies also to cystathionine and djenkolate.

The competitive kinetics described above are generally considered to be strong evidence that the irreversible inhibitor combines with the active site of the enzyme. It does indeed seem reasonable to assume that the active site of β-cystathionase is involved in the initial binding of rhizobitoxine. However, as discussed by OGSTON[14] and BAKER[15], such an assumption is not necessarily valid since kinetic results identical to those described above would apply if a combination of R (or A) at one site decreased the affinity of A (or R) at a different site. A number of mechanisms have been suggested[16] by which the binding of R or A to the enzyme can be mutually exclusive, even when R and A do not bind to the same site.

The type of inhibition described here has been designated by a variety of terms, including "active-site-directed irreversible inhibition"[15], "bifunctional reagent inhibition"[17], and "affinity labeling inhibition"[18]. It is characterized by a high degree of specificity, resulting from the combined requirement for formation of an enzyme–inhibitor complex (Eqn. 1), and subsequent irreversible formation of inactivated enzyme (Eqn. 2). The complete reactivation obtained with pyridoxal phosphate indicates that rhizobitoxine is irreversibly bound to the pyridoxal phosphate prosthetic group of the enzyme. Formation of this bond could inactivate the enzyme by two mechanisms: (a) The pyridoxal phosphate–rhizobitoxine compound (PR) remains bound to the enzyme, which is inactive because the attachment of rhizobitoxine to pyridoxal phosphate prevents its normal catalytic function. Reactivation would then proceed by conversion of PR to pyridoxal phosphate. This conversion could occur by displacement by pyridoxal phosphate either of intact PR, or of the R moiety only of PR. (b) Attachment of rhizobitozine to pyridoxal phosphate greatly decreases its affinity for the enzyme, resulting in the formation of apoenzyme. Reactivation would then proceed by conversion of the apoenzyme to the holoenzyme.

For spinach β-cystathionase, the value of K_i is $8.0 \cdot 10^{-5}$ M, and that for K_m with cystathionine is $1.3 \cdot 10^{-4}$ M (ref. 6). With Salmonella β-cystathionase, K_m for

* Data was analyzed statistically by the two-sample *t* test[13] at the 0.25 level.

cystathionine ($3.6 \cdot 10^{-4}$ M) is similar to that for the spinach enzyme, but K_i ($2.2 \cdot 10^{-8}$ M) is much lower[3]. The derivation of K_i with Salmonella β-cystathionase was based on the assumption that the inhibition was reversible. On the basis of the observations reported here with spinach β-cystathionase, this assumption may have been unwarranted, thereby invalidating the value reported above. If the data of OWENS *et al.*[3] is reinterpreted on the basis that inactivation followed the same kinetics as that of spinach β-cystathionase, values for $T_{\frac{1}{2}}$ and K_i were found to lie within the approximate range of 0.6–1.3 min, and $6.2 \cdot 10^{-8}$–$2.7 \cdot 10^{-7}$ M, respectively. Regardless of the kinetics of inactivation of Salmonella β-cystathionase, it therefore appears that the K_i for this enzyme is at least two orders of magnitude less than that for the spinach enzyme.

The present demonstration that rhizobitoxine inhibits the β-cystathionase of spinach does not, of course, prove that this is the mechanism whereby the toxin causes pathology in the intact plant. Further studies of the specificity of the inhibition and its *in vivo* occurrence will be needed to elucidate the molecular mechanism whereby rhizobitoxine acts as a disease determinant. If rhizobitoxine does prove to react specifically with β-cystathionase, it may become an important tool in determining the physiological roles of the two pathways known in plants to be potential routes for homocysteine biosynthesis: the transsulfuration pathway, which requires β-cystathionase and the direct sulfhydration pathway, which does not require β-cystathionase[19].

ACKNOWLEDGEMENT

We wish to thank Mr. Thomas F. Moon of the Epidemiology and Biometry Branch, National Institute of Child Health and Human development, for assistance in the statistical analyses.

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