Glutamine Synthetase from *Pseudomonas syringae* pv. *tabaci*: Properties and Inhibition by Tabtoxinine-β-lactam

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Glutamine synthetase from *Pseudomonas syringae* pv. *tabaci* was purified 500-fold. Maximum activity was observed with 10 mM-glutamate, 20 mM-ATP and 4 mM-NH₄Cl. The enzyme exhibited substrate inhibition; higher levels of glutamate, MgATP or NH₄Cl decreased its activity. The γ-glutamyltransferase activity was inhibited by Mg²⁺ (75% at 10 mM-Mg²⁺). The enzyme was heat stable and there appeared to be only one form present. Tabtoxinine-β-lactam, a hydrolytic product of tabtoxin produced by pv. *tabaci*, inactivated the enzyme. This inhibition was linear with respect to the concentration of the inhibitor, and enzyme activity could not be recovered by dialysis, acetone precipitation or incubation with crude cell lysate. Mg²⁺ and ammonium ions were required for binding of the inhibitor: incubation of tabtoxinine-β-lactam with the enzyme in the presence of both Mg²⁺ and ammonium ions resulted in a greater decrease in synthetase activity than incubation with either one or neither component. Tabtoxinine-β-lactam did not inhibit the γ-glutamyltransferase activity of the enzyme if ADP was used in the assay, but did when ATP was used.

INTRODUCTION

A number of plant pathogens produce toxins whose targets are shared by the host and pathogen. In these cases, to paraphrase Demain (1974), what keeps the potential suicide from succeeding? Although this question has been extensively considered with regard to antibiotic-producing micro-organisms, there is very little information available about the situation in plant pathogens. To a large extent this is because the mechanism of action of most phytopathogen-produced toxins is unknown.

Essentially all the information we currently have comes from studies on phaseolotoxin, produced by *Pseudomonas syringae* pv. *phaseolicola*, which inhibits ornithine carbamoyltransferase (Patil et al., 1972). The evidence strongly suggests that pv. *phaseolicola* possesses at least two mechanisms for self protection. First, it produces a form of ornithine carbamoyltransferase which is relatively unaffected by phaseolotoxin (Ferguson et al., 1980). Interestingly, this form occurs only at temperatures allowing phaseolotoxin production; at high temperatures when no phaseolotoxin is produced, a sensitive form of the enzyme occurs (Staskawicz et al., 1980). Second, induced mutants of *Salmonella typhimurium* and *Escherichia coli* resistant to the toxin lose their transport or permease system for phaseolotoxin uptake (Staskawicz & Panopoulos, 1980). Possibly then, phaseolotoxin may be selectively excluded from the oligopeptide transport systems in pv. *phaseolicola*. Other mechanisms of self protection may be involved too (e.g. internal compartmentalization separating phaseolotoxin from its target), but as yet there is no experimental evidence for them.

A closely related pathovar, pv. *tabaci*, produces tabtoxin (Fig. 1; Stewart, 1971; Taylor et al., 1972) which, when hydrolysed by the host and/or bacteria, releases tabtoxinine-β-lactam.

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Abbreviations: IM buffer, imidazole/MgCl₂ buffer; MSO, L-methionine sulfoximine.
We have shown that tabtoxinine-β-lactam inhibits glutamine synthetase from pea seeds (Thomas et al., 1983). There are similarities between this host/pathogen interaction and that of pv. phaseolicola. For instance, the chlorotic symptoms can be reversed by addition of the product of the target enzyme [i.e. arginine (Patil et al., 1972) or glutamine (Sinden & Durbin, 1968)]. If the self-protection mechanisms in the two bacteria are similar, one would expect that either glutamine synthetase from pv. tabaci would be unaffected by tabtoxinine-β-lactam, or there are two forms of the enzyme differing in sensitivity. Indeed, multiple forms of glutamine synthetase have been found in other bacteria such as Rhizobium japonicum (Darrow & Knotts, 1977), Agrobacterium tumefaciens (Fuchs & Keister, 1980) and Bacillus caldolyticus (Wedler et al., 1980). However, only one form of glutamine synthetase was found in three species of Pseudomonas (Meyer & Stadtman, 1981).

Here we report the purification and characteristics of glutamine synthetase from pv tabaci, and its inhibition by tabtoxinine-β-lactam. The characteristics of the enzyme are distinctly different from other reported bacterial glutamine synthetases, and it is markedly inhibited by tabtoxinine-β-lactam. Possible mechanisms of self protection including cellular compartmentalization and detoxification are discussed.

**METHODS**

*Cell growth.* Pseudomonas syringae pv. tabaci TD1, a virulent, tabtoxin-producing clone of strain ATCC 11528, was grown in shake culture overnight in a medium containing 30 mM-K₂HPO₄, 7 mM-KH₂HPO₄, 0.4 mM-MgSO₄, 55 mM-glucose and 10 mM-histidine. The culture was centrifuged, and the bacteria were resuspended at one-tenth of the original culture volume in saline (0.85% NaCl) and used to inoculate fresh medium, at an initial density of 10⁸ cells ml⁻¹, which was then incubated for an additional 24 h.

*Enzyme purification.* The following procedures were done at 4 °C, except for DEAE-cellulose and Sepharose 6B chromatography (20 °C). Cells (up to 100 g wet wt) were collected by centrifugation (14500 g, 5 min), washed once in 20 mM-imidazole (pH 7-5) and 10 mM-MgCl₂ (IM buffer), and resuspended in IM buffer containing 1 mM-β-mercaptoethanol at the ratio of 1 g wet wt of cells to 2 ml buffer. The cells were disrupted by three passages through a French press (110 MPa) and the lysate was clarified by centrifugation (40000 g, 30 min). A protein fraction containing glutamine synthetase activity was precipitated by adding (NH₄)₂SO₄ to give 50% saturation. The precipitate was collected by centrifugation (40000 g, 10 min), resuspended at half the original lysate volume in IM buffer and dialysed against column buffer (IM buffer plus 50 mM-KCl adjusted to pH 8-3). This crude preparation was centrifuged (40000 g, 10 min) and the supernate applied to a column of DEAE-cellulose (1 x 20 cm) equilibrated with column buffer. Non-adsorbed protein was washed from the column with column buffer until the effluent had no absorbance at 280 nm, and the enzyme eluted by applying a linear gradient (400 ml of 50 to 700 mM-KCl in IM buffer (pH 8-3). Fractions having glutamine synthetase activity were pooled (usually 30 ml total), precipitated by the addition of (NH₄)₂SO₄ to 50% saturation, centrifuged (40000 g, 10 min) and resuspended in 1 ml IM buffer. After dialysis against 20 mM-imidazole (pH 7-5) and 1 mM-MgCl₂, the preparation was heat-treated for 30 min at 60 °C, centrifuged for 2 min (Eppendorf microfuge, 12000 g) and subjected to gel filtration on a Sepharose 6B column (1.8 x 118 cm) eluted with buffer containing 20 mM-imidazole (pH 7-5) and 1 mM-MgCl₂. Fractions (1 ml) were monitored with respect to A₂₈₀ and transferase activity; the active fractions were pooled (5 to 10 ml total volume).
Table 1. Purification procedure and yields of glutamine synthetase from *P. syringae pv. tabaci*

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity</th>
<th>Percentage yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified crude lysate</td>
<td>0.077</td>
<td>100</td>
</tr>
<tr>
<td>DEAE effluent</td>
<td>5.0</td>
<td>41</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>6.6</td>
<td>31</td>
</tr>
<tr>
<td>Sepharose 6B effluent</td>
<td>41.6</td>
<td>25</td>
</tr>
</tbody>
</table>

* Units are µmol γ-glutamylhydroxamate formed (mg protein)⁻¹ min⁻¹ at 24 °C.

**Analytical procedures.** The γ-glutamyltransferase assay of Stumpf et al. (1951) was modified to allow direct comparisons with the assay of Shapiro & Stadtman (1970). The assay mixture contained 0.5 ml of 40 mM-imidazole (pH 7.2), 60 mM-NH₂OH. HCl, 3 mM-MnCl₂, 20 mM-K₂HPO₄, 20 mM-glutamine and either 0.4 mM-ATP or 0.4 mM-ADP. The transferase assay using ADP was used to monitor enzyme purification. Mg²⁺ sensitivity and enzyme identification on non-denaturing polyacrylamide gels. The transferase assay using either ADP or ATP was used to study toxin inhibition. In this assay, the assay components and the enzyme were incubated for 30 min before the addition of the inhibitor and glutamine. Synthetase activity was measured by phosphate liberation (Shapiro & Stadtman, 1970). The assay was modified by stopping a 0.2 ml reaction mixture with 1 ml 52 mM-FeSO₄ in 0.015 M-H₂SO₄, and colour was developed with 1 ml 8 mM-(NH₄)₆MoO₃O₁₄ in 0.55 M-H₂SO₄. All assays were run for 15 min at 23 ± 2 °C.

**RESULTS**

Typical purification and yield of glutamine synthetase from *pv. tabaci*, based on transferase activity, are presented in Table 1. Routinely, a 500-fold increase in specific activity was obtained with yields ranging between 25 and 40%. The enzyme was heat stable for 1 h at 60 °C; this aided in the purification procedure. Glutamine synthetase eluted as a single peak from DEAE-cellulose at approximately 300 mM-KCl. The enzyme preparation exhibited a single band on native PAGE. Based on SDS-PAGE the subunits have a molecular weight of approximately 60,000.

Hexadecyltrimethylammonium bromide, often reported to aid in glutamine synthetase purification, destroyed enzyme activity in crude extracts. Very low yields, due to weak binding, were obtained with crude and semi-purified glutamine synthetase when either Affi-gel blue (Bio-Rad) or ADP-agarose (Sigma) affinity chromatography was attempted. Purified glutamine synthetase from *pv. tabaci* did not focus and less than 5% of the original activity was recovered on a 20 ml PBE chromatofocusing column (Pharmacia) previously equilibrated with 25 mM-imidazole (pH 7.4), and eluted with Polybuffer 74 (pH 4.0), presumably due to sequestration of the Mg²⁺ cofactor by Polybuffer 74. Glutamine synthetase from *pv. tabaci* was also isolated by the methods of Shapiro & Stadtman (1970) and Streicher & Tyler (1980); however, yield and/or purity was not as good as with the method described here.

Glutamate concentrations between 50 and 100 mM, a range commonly used in glutamine synthetase assays, inhibited glutamine synthetase from *pv. tabaci*. A Lineweaver–Burk plot over a 200-fold glutamate concentration range illustrates this (Fig. 2). The inhibition was
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Fig. 2. Response of glutamine synthetase to various glutamate concentrations. Assay components: MgCl₂, ATP, NH₄Cl (10 mM each) and 50 mM-imidazole (pH 7.5).

Fig. 3. Activity of glutamine synthetase at various concentrations of Mg, ATP. Assay components: NH₄Cl and glutamate (10 mM each) and 50 mM-mm-imidazole (pH 7.5). Separate blanks lacking enzyme were used at each Mg, ATP concentration to correct for extraneous phosphate.

Fig. 4. Activity of glutamine synthetase at various concentrations of NH₄Cl. Assay components: MgCl₂, ATP, glutamate (10 mM each) and 50 mM-imidazole (pH 7.5).

Fig. 5. Response of glutamine synthetase to Mg²⁺ in the transferase assay. Assay components: 40 mM-imidazole (pH 7.2), 60 mM-NH₂OH, 3 mM-MnCl₂, 20 mM-K₂AsO₄, 0.4 mM-ADP and 20 mM-glutamine.

-independent of ammonium concentration in the range 5–50 mM-NH₄Cl. The enzyme showed a linear response from 0.5 mM- to 2.5 mM-glutamate (correlation coefficient 0.99). High concentrations of ATP inhibited the enzyme to a lesser extent (Fig. 3). The response of the enzyme to ammonium was linear over the range 0.4 mM to 1 mM (correlation coefficient 0.99; Fig. 4). Ammonium concentrations greater than 10 mM slightly decreased the velocity. Maximum velocity was observed at 10 mM-glutamate, 20 mM-Mg, ATP and 4 mM-NH₄Cl.

After dialysis to replace Mg²⁺ with Mn²⁺, the γ-glutamyltransferase activity of glutamine synthetase from pv. tabaci cells was assayed with various concentrations of MgCl₂ (Fig. 5). Velocity was maximum in the absence of Mg²⁺, and at 10 mM it had decreased by 75%. The adenylylated form of the enzyme in other bacterial systems is the only form sensitive to high levels of Mg²⁺ (Shapiro & Stadtman, 1970; Stadtman et al., 1970; Streicher & Tyler, 1980). Adenylylated glutamine synthetase from E. coli had lowered activity without Mg²⁺, optimal activity at 3 mM-Mg²⁺, and significantly lowered activity at Mg²⁺ concentrations greater than 30 mM. In contrast, the presence of any Mg²⁺ decreased the transferase activity of glutamine synthetase from pv. tabaci.
Table 2. Effect of incubation of enzyme, tabtoxinine-β-lactam and components of the assay mixture on inhibition of pv. tabaci glutamine synthetase activity

Preincubation was for 30 min; all treatments contained 50 mM-imidazole (pH 7.5), 0.1 mM-tabtoxinine-β-lactam and glutamine synthetase. The concentrations of glutamate, Mg. ATP, Mg. ADP and NH₄Cl were each 10 mM. The incubation treatment containing ADP was corrected for phosphate contamination of the ADP and inhibition of synthetase activity by ADP.

<table>
<thead>
<tr>
<th>Preincubation mixture</th>
<th>Assay incubation</th>
<th>Percentage of control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg. ATP + NH₄Cl</td>
<td>Glutamate</td>
<td>11</td>
</tr>
<tr>
<td>Mg. ATP</td>
<td>NH₄Cl + glutamate</td>
<td>42</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Mg. ATP + glutamate</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Mg. ATP + NH₄Cl</td>
<td>96</td>
</tr>
<tr>
<td>Mg. ADP + NH₄Cl</td>
<td>Mg. ATP + glutamate</td>
<td>91</td>
</tr>
</tbody>
</table>

* Mean of four experiments. The control consisted of 0.1 mM-tabtoxinine-β-lactam added at assay initiation: 100% = 0.14 μmol P_i released. A control without tabtoxinine-β-lactam was 220% of the control with it.

Tabtoxinine-β-lactam at 0.25 mM inhibited the synthetase activity of pv. tabaci enzyme by 90%; tabtoxin and tabtoxinine-δ-lactam did not inhibit this activity. The inhibition of glutamine synthetase by tabtoxinine-β-lactam was linear over the range 0.01 mM to 0.5 mM (correlation coefficient 0.99; Fig. 6). Various components of the synthetase assay were first incubated with 0.1 mM-tabtoxinine-β-lactam and the enzyme for 30 min, at which time the remaining assay components were added along with the glutamate to initiate the assay. The preincubation mixture containing both Mg. ATP and NH₄Cl showed a marked decrease in activity over the control, the mixture containing Mg. ATP was intermediate and the other treatments showed negligible decrease (Table 2).

Recovery of enzyme activity was attempted from glutamine synthetase inhibited by incubation with 2 mM-tabtoxinine-β-lactam by: (i) 24 h dialysis against IM buffer, (ii) 24 h dialysis against synthetase assay mixture containing glutamate, (iii) incubation for 30 min with crude cell lysate at 24 °C, and (iv) precipitation by the addition of 0.5 vol. acetone (−20 °C) followed, after centrifugation, by resuspension of the pellet in IM buffer. No glutamine synthetase activity was recovered by any of the above treatments.

Tabtoxinine-β-lactam at 0.5 mM inhibited the γ-glutamyltransferase activity of glutamine synthetase from pv. tabaci when ATP, but not ADP, was present. The transferase assay using ATP incorporates a 30 min incubation of assay components and enzyme before the addition of glutamine to start the reaction. Tabtoxinine-β-lactam was added at 30 min along with the glutamine; the assay using ADP was done in the same manner. Separate experiments using the transferase assay with ADP but without this 30 min incubation period showed that tabtoxinine-β-lactam did not inhibit the enzyme when ADP was used in the assay. Using ATP, the
transferase activity in the presence of tabtoxinine-β-lactam was 10% of the control activity, whereas the analogous reaction using ADP was 102% of its control. In parallel experiments, tabtoxinine-β-lactam inhibited the transferase activity of glutamine synthetase from both pea seed and \textit{E. coli} when either ADP or ATP was used in the assay.

\textbf{DISCUSSION}

Glutamine synthetase from \textit{pv. tabaci} has two unusual characteristics when compared with other bacterial glutamine synthetases. First, moderate to high levels of each substrate used decreased its activity. The inhibition observed with each substrate was not affected by either saturating (inhibiting) or optimal concentrations of the other two substrates. Second, the enzyme was sensitive to low concentrations of Mg\textsuperscript{2+} in the transferase assay. It was much more sensitive than adenylated glutamine synthetase from \textit{E. coli}. This sensitivity to Mg\textsuperscript{2+} may indicate that the enzyme from \textit{pv. tabaci} was isolated in an adenylated state. However, if it responds like other adenylated enzymes, one would have expected to see negligible synthetase activity in the presence of Mg\textsuperscript{2+}: this was not observed.

Incubation of the synthetase assay components indicated that both NH\textsubscript{4}Cl and Mg\textsubscript{2+}ATP were required for inhibition by tabtoxinine-β-lactam. Incubation of the enzyme with the toxin in the presence of these substrates decreased the remaining activity over the non-incubated treatment. The assay chemicals were not totally free of ammonium; separate experiments with shorter preincubation periods showed that the decrease in activity of the NH\textsubscript{4}Cl-free incubation treatment was the result of this residual ammonium. Inhibition by tabtoxinine-β-lactam of transferase activity of the \textit{pv. tabaci} enzyme was different from that of the enzyme from pea (Thomas et al., 1983) or \textit{E. coli}. Also, this inhibition was different from that caused by MSO. Tabtoxinine-β-lactam inhibits transferase activity of glutamine synthetase from pea and \textit{E. coli} when either ADP or ATP is present in the assay mixture: it inhibited the \textit{pv. tabaci} enzyme only if ATP was present. All three enzymes are inhibited by MSO when either ADP or ATP is present in the mixture. This may indicate that ATP is an absolute requirement for the binding of tabtoxinine-β-lactam to \textit{pv. tabaci} glutamine synthetase and that ADP will suffice for its binding to the pea and \textit{E. coli} enzymes along with the binding of MSO to all three enzymes.

It seems unlikely that the self protection of \textit{pv. tabaci} from tabtoxinine-β-lactam is the result of multiple forms of the enzyme. Purification by three different methods yielded an enzyme which was not separable by electrophoresis, heat stability or Mg\textsuperscript{2+} sensitivity, characteristics often used for distinguishing different forms. Further, heat treatment of crude lysate resulted in no loss of total glutamine synthetase activity. It is possible that another form of the enzyme may be produced under other growth conditions, but we found no evidence to support this.

We are currently investigating the possibility that this self protection is the result of either strict intracellular compartmentalization or excretion of tabtoxinine-β-lactam upon formation. Tabtoxin and tabtoxinine-β-lactam have not been found within cells of \textit{pv. tabaci} (R. D. Durbin, unpublished results). The unique substrate interactions observed with \textit{pv. tabaci} glutamine synthetase may also play a role in the tolerance of the organism. Altered substrate affinity has been reported to confer resistance to MSO in mutants of \textit{Salmonella typhimurium} (Miller & Brenchley, 1981). The glutamine synthetases from these mutants are still irreversibly inactivated by MSO, but they also have significantly higher \(K_m\) values for both glutamate and ammonium. These bacteria are capable of growing at a slightly lower rate in a concentration of MSO which is completely inhibitory to wild-type \textit{S. typhimurium}.

Tabtoxin does not inhibit glutamine synthetase from pea (Thomas et al., 1983) or \textit{pv. tabaci}, and tabtoxin at concentrations of at least 500 μM does not inhibit the growth of \textit{pv. tabaci}, whereas 250 μM-tabtoxinine-β-lactam will inhibit the growth of \textit{pv. tabaci} for a period of 6 to 30 h, depending on the strain used. It is conceivable that the formation of tabtoxin is a detoxification mechanism; however, the proportions of tabtoxin and tabtoxinine-β-lactam formed in culture can be altered by manipulating the medium (Durbin & Uchytil, 1984).

Elucidation of the mechanism of glutamine synthetase inhibition by tabtoxinine-β-lactam and a complete understanding of the self protection exhibited by \textit{pv. tabaci} may be important for
understanding the host–parasite interactions of tabtoxin-producing *P. syringae* pathovars. In addition, the unusual characteristics of *pv. tabaci* glutamine synthetase have the potential of adding information to our understanding of this complex enzyme in relationship to its regulation and occurrence in other organisms. It is apparent that glutamine synthetases from pseudomonads are more diverse than was previously thought.

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**REFERENCES**


