Inhibition of Glucosamine Synthase by Bacilysin and Anticapsin

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L-Glutamine : D-fructose-6-phosphate amidotransferase ('glucosamine synthase', EC 5.3.1.19) from Escherichia coli MRE 600 was purified at least 75-fold. It catalysed the formation of 21.1 μmol glucosamine 6-phosphate (mg protein)^{-1} in 30 min at 37 °C. Its molecular weight, estimated by gel filtration, was about 90000 and it was inhibited by thiol group reagents. Anticapsin, the C-terminal amino acid of the dipeptide antibiotic bacilysin, and to a lesser extent bacilysin itself, inhibited glucosamine synthase activity. Kinetic studies indicated that the inhibition was non-competitive with respect to fructose 6-phosphate as substrate but partly competitive with respect to L-glutamine. Incubation of the enzyme with anticapsin brought about a time-dependent and irreversible inhibition. It is suggested that anticapsin behaves as a glutamine analogue and that a reaction of its epoxide group with a thiol group of glucosamine synthase results in its linkage to the enzyme by a covalent bond.

INTRODUCTION

Bacilysin is a dipeptide antibiotic produced by Bacillus subtilis A 14. Its structure (Fig. 1) was established by Walker & Abraham (1970). The dipeptide antibiotics tetaine, produced by Bacillus pumilus (Borowski, 1953; Kaminski & Sokolowska, 1973), and bacilllin, produced by Bacillus subtilis sp. KM 208 (Atsumi et al., 1975), are identical with bacilysin. Bacilysin inhibits the growth of many Gram-positive and Gram-negative bacteria and of Candida albicans (Kenig & Abraham, 1976; Chmara et al., 1980). The C-terminal epoxy amino acid of bacilysin, named anticapsin, is produced by B. subtilis A 14 (Walker & Abraham, 1970) and by a strain of Streptomyces griseoplanus (Whitney et al., 1970; Neuss et al., 1970). Anticapsin shows weak antibacterial activity but has considerable activity against C. albicans (Kenig & Abraham, 1976).

Bacilysin is carried into bacterial and C. albicans cells by a peptide transport system (Perry & Abraham, 1979; Chmara et al., 1981; Chmara et al., 1982) and is then hydrolysed by

![Fig. 1. Structure of bacilysin. The N-terminal amino acid is L-alanine and the C-terminal amino acid is anticapsin.](image-url)

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intracellular peptidases to L-alanine and anticapsin (Kenig et al., 1976; Chmara et al., 1982). Whitney & Funderburk (1970) and Kenig et al. (1976) have demonstrated that anticapsin strongly inhibits the synthesis of glucosamine in Escherichia coli and Staphylococcus aureus by inhibition of glucosamine synthase.

Kenig et al. (1976) showed that anticapsin competes with the L-glutamine substrate for glucosamine synthase, and raised the question whether the epoxide group of anticapsin reacts at the active site of the enzyme to form a covalent bond. The experiments described here have been carried out to investigate the nature of this reaction.

**METHODS**

**Organisms.** E. coli MRE 600, used for the production of glucosamine synthase, was a laboratory strain. E. coli K12 strain 3000 Hfr was used for studies of the inhibition of intracellular glucosamine synthase.

**Reagents.** Fructose 6-phosphate dipotassium salt, L-glutamine, B-glutamine, DTT, dithioerythritol, glucose 6-phosphate, sucrose, bovine serum albumin, hydroxyapatite spheroidal, EDTA, N-ethylmaleimide, and 5,5'-dithiobis(2-nitrobenzoic acid) were from BDH. EGTA was from Sigma and HEPES, bis-Tris, ACES, MOPS and PIPES buffers were from Calbiochem. 2-Nitro-5-thiocyanobenzoic acid and 6,6-dithiodinicotinic acid were from Serva (Heidelberg, FRG). Cellex D (DEAE) and Bio-Gel P-2 were from Bio-Rad and Sephacryl S-300 and Blue dextran 2000 were from Pharmacia. BaciIysin, prepared by the method of Perry & Abraham (1979), was a gift from Dr D. Perry. Anticapsin was a gift from Dr N. Neuss, Eli Lilly, Indianapolis, USA. L-γ-Glutamylhydroxamate, Gly-Phe, Gly-Leu, Ala-Ala, Met-Leu, aldolase, haemoglobin and chymotrypsin were from Sigma.

**Buffers.** Buffer A contained 25 mM-potassium phosphate buffer pH 7.6, 1 mM-EGTA, 1 mM-KCl, 5 mM-DTT. Buffer B contained 25 mM-potassium phosphate buffer pH 7.4, 1 mM-EGTA, 1 mM-KCl, 1 mM-DTT, 10 mM-L-glutamine, 0.05 mM-glucose 6-phosphate, 500 mM-sucrose. Buffer C contained 25 mM-potassium phosphate buffer pH 7.5, 1 mM-EGTA, 1 mM-DTT, 0.5 mM-L-glutamine, 0.05 mM-glucosamine 6-phosphate, 600 mM-sucrose. Buffers D and E were similar to buffer C, but 25 mM-potassium phosphate, pH 7.5 in buffer C, was replaced with 2.5 mM and 400 mM-potassium phosphate buffer, pH 6.8, respectively.

**Protein and peptidase assays.** The protein content of crude preparations of glucosamine synthase (Table 1, stages 1 and 2) was determined by the method of Lowry as described by Layne (1957), with bovine serum albumin as a standard. For partially purified preparations (stages 2 to 4) the Coomassie dye technique of Bradford (1976) was used. The peptidase activity of crude and purified preparations of the enzyme was determined by the method of Payne (1972).

**Assay of glucosamine synthase.** Enzyme activities were measured by the method of Ghosh et al. (1960) as modified by Kenig et al. (1976) except that the reaction mixture contained 15 mM-D-fructose 6-phosphate dipotassium salt, 10 mM-L-glutamine, 1 mM-DTT or EGTA, 25 mM-potassium phosphate buffer, pH 7.4, and enzyme preparations (0.5 or 1 ml) at various stages of purification. The amount of glucosamine 6-phosphate formed was determined after 30 min. One unit of activity is defined as the amount of enzyme which catalyses the synthesis of 1 μmol of glucosamine 6-phosphate in 30 min under the conditions of the assay.

**Molecular weight.** This was determined by filtration of the enzyme in buffer C through a column (70 cm × 27 cm diam.) of Sephacryl S-300. Aldolase (mol. wt 158000), haemoglobin (mol. wt 67000) and chymotrypsinogen A (mol. wt 25000) were used as markers.

**pH profile.** Purified glucosamine synthase was assayed in 37.5 mM-potassium phosphate (pH 6.0) and at pH values from 6.0 to 8.0 in the following buffers: bis-Tris (pKₐ 6.5-70), PIPES (pKₐ 6.80), ACES (pKₐ 9.0), MOPS (pKₐ 7.20) and HEPES (pKₐ 7.55).

**Production and purification of glucosamine synthase.** E. coli MRE 600 was grown in 1.5 l of media in 6-l whirlflasks (Mitchell, 1949) for about 18 h at 37°C. The medium had the following composition (g l⁻¹): Bacto-peptone (Difco), 1.5; Lab-Lemco (Oxoid), 1.5; yeast extract (Difco), 5; NaCl, 3.5; K₂HPO₄, 3.68; KH₂PO₄, 1.32 and glucose, 2.0. The glucose was sterilized by autoclaving separately. The inoculum for each flask was 50 ml of an overnight culture grown in the same medium. The bacteria were harvested by centrifugation at 4°C, washed twice with buffer A, resuspended in 250 ml of buffer B and stored at −27°C until used.

Preparations of frozen bacteria (45–60 g) were thawed and subjected to sonic disruption with a MSE 100 W ultrasonic disintegrator at less than 10°C. The crude extract was centrifuged at 25000 × g for 30 min, and the supernate then centrifuged at 105000 × g for 60 min. The volume of the resulting solution was adjusted to give a concentration of protein of 10 mg ml⁻¹.

The enzyme was purified at 4°C. Crude cell-free extracts were diluted fourfold with buffer B and applied to a column (25 cm × 5 cm diam.) of Cellex D (OH⁻ form) equilibrated with buffer B. Elution was carried out initially with 100 ml of this buffer and then with six successive 200 ml volumes of the buffer in which the concentration of KCl was raised in steps to 300 mM. The flow rate was 240–300 ml h⁻¹. The active fractions from the column were
Table 1. Purification of glucosamine synthase from E. coli MRE 600

<table>
<thead>
<tr>
<th>Stage</th>
<th>Material from:</th>
<th>Vol. (ml)</th>
<th>Protein (mg)</th>
<th>Total Protein (mg ml⁻¹)</th>
<th>Total Units* (units ml⁻¹</th>
<th>Sp. act. (units mg⁻¹)</th>
<th>Fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Extract supernatant</td>
<td>250</td>
<td>2500</td>
<td>700</td>
<td>10</td>
<td>2.8</td>
<td>0.28</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Cellex D (DEAE)</td>
<td>240</td>
<td>288</td>
<td>950†</td>
<td>1.2</td>
<td>3.96</td>
<td>3.3</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Cellex D†</td>
<td>220</td>
<td>136</td>
<td>814</td>
<td>0.62</td>
<td>3.7</td>
<td>6.0</td>
<td>21.7</td>
</tr>
<tr>
<td>4</td>
<td>Hydroxyapatite</td>
<td>110</td>
<td>47</td>
<td>425</td>
<td>0.43</td>
<td>3.87</td>
<td>9.0</td>
<td>32.3</td>
</tr>
<tr>
<td>5</td>
<td>Sephacryl S-300</td>
<td>120</td>
<td>13</td>
<td>274</td>
<td>0.11</td>
<td>2.28</td>
<td>21.1</td>
<td>75.6</td>
</tr>
</tbody>
</table>

* 1 unit is defined as the amount of enzyme required to synthesize 1 μmol glucosamine 6-phosphate in 30 min at 37 °C.
† The increase in activity could have been due to the removal of an inhibitor in the crude extract.
‡ The enzyme was eluted from Cellex D at KCl concentrations of 150–200 mM.

combined, diluted fourfold with buffer C and applied to a column (25 cm × 5 cm diam.) of Cellex D (OH⁻ form) previously equilibrated with buffer C. Elution was with five successive 200 ml volumes of the same buffer in which the concentration of KCl was raised in steps from 100 to 300 mM. Fractions (each 40 ml) were collected at flow rate 240 ml h⁻¹ and those showing enzymic activity were combined.

The active eluate from the preceding column was diluted fourfold with buffer D and applied to a hydroxyapatite column (25 cm × 1.5 cm diam.) previously equilibrated with the same buffer. A linear gradient of increasing phosphate concentration, formed from 300 ml buffer D and 300 ml buffer E, was used for elution. Fractions (each 20 ml) were collected at flow rate of 60 ml h⁻¹. The active fractions of the eluate were combined, diluted to 120 ml with buffer C and the resulting solution divided into three equal portions. Each portion was applied separately to a Sephacryl S-300 column (70 cm × 2.6 cm diam.) equilibrated with buffer C. Fractions (each 12 ml) were collected during elution with buffer C at flow rate of 24 ml h⁻¹ and assayed for glucosamine synthase activity.

Inactivation of glucosamine synthase by anticapsin. Glucosamine synthase [198 μg, sp. act. about 21 units (mg protein)⁻¹] was incubated at 37 °C in a solution (2 ml) containing: 25 mM-potassium phosphate buffer pH 7.4, 1 mM-DTT, 1 mM-EGTA, 500 mM-Sucrose, and anticapsin at various concentrations. Samples (0-2 ml) were removed from the mixture at intervals and diluted 20-40-fold into the standard assay solution for determination of residual enzyme activity. An inactivation constant, K_inact, was calculated or determined graphically from linear plots of the half life of the glucosamine synthase (τ) versus (anticapsin concentration)⁻¹ (Fig. 3) by use of the relationship given by Meloche (1967):

\[
\tau = \frac{1}{K_{\text{inact}}} (\frac{1}{I}) + \tau
\]

where I is the concentration of anticapsin and T is the minimum half-inactivation time of the enzyme at infinite concentration of anticapsin.

RESULTS AND DISCUSSION

Purification and properties of glucosamine synthase

The results of a typical purification of glucosamine synthase from E. coli MRE 600 are given in Table 1. The final specific activity [21.1 units (mg protein)⁻¹] was higher than that reported by Ghosh et al. (1960) for purified glucosamine synthase from E. coli B (7.3 units mg⁻¹). The success of the procedure used here depended on the use of DTT or dithioerythritol, EGTA and sucrose as stabilizing agents. In buffers B and C both a crude extract and a purified preparation retained 80% of their activity for two months at 4 °C. Normman et al. (1973) reported similar findings with glucosamine synthase from Blastocladidella emersonii.

The crude extract containing glucosamine synthase catalysed the hydrolysis of bacilysin and a number of other dipeptides at comparable rates. The dipeptidases, stimulated by Co²⁺, were eluted from Cellex D (Table 1, stage 2) before the synthase and when the concentration of KCl was 50 and 100 mM, respectively. Three peaks of dipeptidase activity were observed when Gly-Phe and Gly-Leu were used as substrates but only two peaks with Ala-Ala, Met-Leu and bacilysin. The purified synthase (Table 1, stage 4) showed virtually no peptidase activity.

The molecular weight of the glucosamine synthase from E. coli MRE 600 was estimated by gel filtration to be 90000 ± 9000. This was similar to the reported values of 90000 and 105000 for...
Table 2. Inhibition of glucosamine synthase activity by bacilysin, anticapsin and other reagents

A mixture of the enzyme preparation (21 units mg\(^{-1}\)) and one of the inhibitors listed was incubated in the standard assay solution at 37 °C for 30 min. The inhibition is the decrease in the amount of glucosamine 6-phosphate formed as a percentage of that formed in a control solution containing no inhibitor.

<table>
<thead>
<tr>
<th>Inhibitor*</th>
<th>Conc (µM)</th>
<th>Cell-free extract</th>
<th>Purified enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM</td>
<td>2000</td>
<td>68</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>–</td>
<td>79</td>
</tr>
<tr>
<td>DTNB</td>
<td>500</td>
<td>62</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
<td>74</td>
</tr>
<tr>
<td>NTCB</td>
<td>500</td>
<td>48</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
<td>66</td>
</tr>
<tr>
<td>DTDN</td>
<td>1000</td>
<td>44</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
<td>62</td>
</tr>
<tr>
<td>pCMB</td>
<td>500</td>
<td>72</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
<td>70</td>
</tr>
<tr>
<td>DON</td>
<td>100</td>
<td>36</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>–</td>
<td>44</td>
</tr>
<tr>
<td>Azaserine</td>
<td>1000</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>–</td>
<td>18</td>
</tr>
<tr>
<td>Bacilysin</td>
<td>200</td>
<td>83</td>
<td>37</td>
</tr>
<tr>
<td>Anticapsin</td>
<td>1.25</td>
<td>76</td>
<td>96</td>
</tr>
</tbody>
</table>

* NEM, N-ethylmaleimide; DTNB, 5,5'-dithionitrobenzoic acid; NTCB, nitrothiocyanobenzoic acid; DTDN, 6,6'-dithiodicinicotinic acid; pCMB, p-chloromercuribenzenesulphonic acid; DON, 6-diazo-5-oxo-1-norleucine.

enzymes from *E. coli* ATCC 12743 and *B. subtilis* W 23, respectively (Kornfeld, 1967). In contrast, values of 360000 (Winterburn & Phelps, 1971) and 340000 (Kornfeld, 1967) have been reported for the enzyme isolated from rat liver. The mammalian enzyme may thus contain four subunits. The pH profile of the enzyme studied here showed a maximum activity at pH 7.2–7.4 in phosphate, HEPES, PIPES and bis-Tris buffers. Activity maxima reported for the enzymes from *E. coli*, Neurospora crassa (Ghosh et al., 1960) and *S. aureus* (Kenig et al., 1976) are 7.9, 6.7 and 6.9, respectively.

**Inhibition and inactivation of glucosamine synthase activity**

*E. coli* glucosamine synthase was inhibited by reagents that react with thiol groups (Table 2) and by 6-diazo-5-oxonorleucine, which competes with glutamine for the enzyme from rat liver (Bates & Handschumacher, 1969).

Bacilysin showed only moderate inhibitory activity against the purified enzyme but a considerably higher activity against the crude enzyme. This difference could be accounted for by the presence of dipeptidases in the crude preparation which hydrolysed bacilysin to L-alanine and anticapsin (Perry & Abraham, 1979). Among all the inhibitors tested, anticapsin was by far the most active, causing almost total inactivation of the purified enzyme at a concentration of 1.25 µM. All the inhibitors except bacilysin showed higher activity against the purified enzyme than against the crude enzyme. This was probably due to non-specific binding to other proteins in the crude preparations.

**Inhibition and inactivation by anticapsin.** Plots of the reciprocals of the initial rates of formation of glucosamine 6-phosphate against the reciprocals of the concentration of the glutamine substrate in the presence of various concentrations of anticapsin indicated (on the assumption that Michaelis–Menten kinetics were followed) that the inhibition of the enzyme by anticapsin was partly competitive and partly non-competitive (Cornish-Bowden, 1976). The apparent \( K_m \) for glutamine was \( 4 \times 10^{-4} \) M which does not differ significantly from the values reported by
Mode of action of bacilysin and antcapsin

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Ghosh et al. (1960) for the enzyme from *E. coli* B (6.5 × 10⁻⁴ M). A plot of the reciprocal of the rate against antcapsin concentration gave an apparent value for *K*_m of 1.25 × 10⁻⁷. The resulting value of 3.1 × 10⁻⁴ for *K*_i/*K*_m does not differ greatly from that obtained by Kenig et al. (1976) for the enzyme from *S. aureus*.

Similar plots in which the concentration of the fructose 6-phosphate substrate was varied gave *K*_m for the latter of 4 × 10⁻³ M, similar to that reported by Ghosh et al. (1960) for the partly purified enzyme from *E. coli* B (2 × 10⁻³ M), and indicated that the inhibition by antcapsin was non-competitive, as reported by Kenig et al. (1976b) for the staphylococcal enzyme.

These data indicated that antcapsin had a very high affinity for a site on the glucosamine synthase molecule which was close to that occupied by glutamine. However, further experiments indicated that the inhibition by antcapsin could not be described entirely in terms of reversible inhibitor–enzyme complexes.

**Inactivation of glucosamine synthase by antcapsin.** Progressive inactivation of the glucosamine synthase occurred when the enzyme was incubated with antcapsin (Fig. 2). The inactivation was not reversed by dilution, dialysis, or gel filtration through Bio-Gel P-2. The inactivation followed pseudo-first order kinetics at high concentrations of antcapsin (50–500μM) but at lower concentrations the rate progressively decreased after 20 min, possibly due to inactivation of antcapsin. Plots of the time for half inactivation (τ) against the reciprocal of the antcapsin concentration, which intersected the ordinate (Fig. 3), were consistent with the reversible formation of a noncovalent complex (EI) preceding irreversible inactivation as shown below, where E represents the enzyme, I antcapsin and E_{inact} irreversibly inactivated enzyme.

E + I ⇌ EI \( \xrightarrow{k_2} \) E_{inact}

The formation of such an enzyme–inhibitor complex may exhibit saturation kinetics prior to covalent modification of the enzyme (Meloche, 1967). Further evidence for formation of a reversible enzyme–antcapsin complex prior to inactivation was provided by the hyperbolic relationship between the experimental values of *k*_{obs} for inactivation (Fig. 4). Extrapolation to infinite inhibitor concentration gave a minimum time for half inactivation (T) as 1.85 min (Fig. 3), and indicated that the inactivation exhibited saturation kinetics. From these data a value for *K*_{inact}([k⁻¹ + k₂],) of 3.2 × 10⁻⁵ M and a maximum value for the inactivation rate constant *k*₂(ln 2/T) of 0.374 min⁻¹ were calculated.
Fig. 3. Plot of the time for half inactivation of glucosamine synthase ($\tau$) against the reciprocal of the concentration of anticapsin.

Fig. 4. Dependence of the pseudo-first order rate constant ($k_{obs}$) for the inactivation of glucosamine synthase on the concentration of anticapsin. The reaction was carried out under the conditions given in Fig. 2.

The addition of L-glutamine or L-$\gamma$-glutamylhydroxamate to a mixture of glucosamine synthase and anticapsin protected the enzyme from inactivation, but the addition of D-glutamine did not. Inactivation of the purified glucosamine synthase preparation (0.198 mg) by 50 $\mu$g anticapsin was determined as described in Fig. 2. Pseudo-first order constants found for inactivation ($k_{obs}$) in the presence of 5 mM-L-glutamine and 5 mM-L-$\gamma$-glutamylhydroxamate were 0.092 and 0.116 min$^{-1}$, respectively. The corresponding values in the presence of 5 mM-D-glutamine and in the absence of a protecting agent were 0.246 and 0.252 min$^{-1}$, respectively. These results are consistent with the view that anticapsin reacts with the enzyme at or near its L-glutamine binding site.

Glucosamine synthase from E. coli MRE 600 resembled other amidotransferases in its utilization of glutamine and its inactivation by thiol group reagents (Buchanan, 1973).

The evidence for the presence of a thiol group in the glucosamine synthase together with the known reaction of the thiol group of cysteine with the epoxide group in the anticapsin moiety of bacilysin (Rogers et al., 1965) suggested that the irreversible inhibition of the enzyme by anticapsin was a consequence of the formation of a covalent bond when the epoxide group for the latter was attacked by the thiol group of a cysteine residue in the enzyme.

In vivo inactivation. Glucosamine synthase in E. coli K12 was inactivated by bacilysin as it was by the enzyme in a crude extract. In both cases the inactivation was presumably caused by anticapsin liberated from bacilysin by peptidases. Incubation of the bacteria for 30 min at 37 °C in the presence of 9.25 $\mu$M-bacilysin resulted in 52.5% inhibition comparable with 46.7% inhibition of the extracted enzyme. Gel-filtration of the extract from bacilysin-treated cells (92.5 $\mu$M) after centrifugation at 105 000 g caused no restoration of glucosamine synthetase activity.

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REFERENCES


