

ANTICAPSIN, AN ACTIVE-SITE DIRECTED IRREVERSIBLE
INHIBITOR OF GLUCOSAMINE-6-PHOSPHATE SYNTHETASE
FROM *ESCHERICHIA COLI*

H. CHMARA*, H. ZÄHNER† and E. BOROWSKI

Department of Pharmaceutical Technology and Biochemistry, Technical University,
80-952 Gdansk, Poland

†Institut für Biologie II, Universität Tübingen,
Auf der Morgenstelle 28, D-7400 Tübingen, BRD

(Received for publication December 26, 1983)

Glucosamine-6-phosphate synthetase from *Escherichia coli* K-12 is progressively inactivated by L-β-(2,3-epoxycyclohexyl-4-on)alanine (anticapsin). With increasing concentrations of anticapsin the reaction exhibits rate saturation: the minimum inactivation half-time is 1.15 minutes, with a K_{inact} of 2.5 μM.

Glutamine and competitive inhibitors protect against inactivation. Fructose-6-phosphate promotes the inactivation rate. It is concluded that anticapsin is an active-site directed glutamine analog in the reaction catalyzed by glucosamine-6-phosphate synthetase.

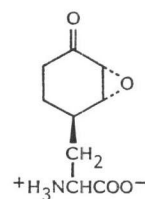
Glutamine is the donor of the nitrogen atom in the conversion of fructose-6-phosphate to glucosamine-6-phosphate catalyzed by enzymes from both microbial¹⁾ and mammalian cells²⁾. This reaction is catalyzed by the enzyme glucosamine-6-phosphate synthetase (2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.19, formerly L-glutamine: D-fructose-6-phosphate amidotransferase EC 2.6.1.16). The product glucosamine-6-phosphate undergoes sequential transformations leading to the formation of UDP-N-acetylglucosamine³⁾.

Several glutamine analogs are potent inhibitors of the glucosamine-6-phosphate synthetase activity. Preliminary studies in this laboratory have shown that the glucosamine-6-phosphate synthetase is inhibited by anticapsin in cell-free extracts from bacteria, yeast⁴⁾ and tumor cells (CHMARA & BOROWSKI, unpublished data). Anticapsin, L-β-(2,3-epoxycyclohexyl-4-on)alanine, Fig. 1, produced by *Streptomyces griseoplanus* NRRL 3507⁵⁾ is also the C-terminal amino acid of the dipeptide antibiotic tetaïne (bacilysin) produced by *Bacillus pumilus* strain theta⁶⁾.

Recently it has been postulated that anticapsin acts as a glutamine analog and irreversibly inhibits the glucosamine-6-phosphate synthetase activity by covalently binding to the active site of the enzyme^{4,7)}.

The present paper describes the results of studies on the interaction of anticapsin with partially purified glucosamine-6-phosphate synthetase from *Escherichia coli* K-12 and shows that the enzyme is irreversibly inactivated by the inhibitor.

Fig. 1. The structure of the epoxyamino acid anticapsin.



Materials and Methods

Cells of *E. coli* K-12 strain 3000 Hfr were grown with vigorous aeration in CGPY⁸⁾ medium

for about 18 hours at 35°C. After two washings with buffer A at 4°C, cells were resuspended in small volume of buffer B and stored at -22°C until used. Glucosamine-6-phosphate synthetase was prepared using the procedure described by GHOSH *et al.*¹¹ up to the elution from DEAE-cellulose, followed by chromatography on DEAE-cellulose column as described by NORRMANN *et al.*⁹. The fractions with high glucosamine-6-phosphate synthetase activity were purified at least on the Sephacryl S-300 column. All purification steps were conducted in buffer B. The partially purified glucosamine synthetase had a specific activity of 7.2 $\mu\text{mol GlcN-6-P}$ per mg of protein under standard assay conditions. The enzyme stored in buffer B at 4°C was relatively stable for several weeks.

The enzyme activity was assayed at 37°C for 30 minutes in 1-ml reaction mixture containing 15 mM fructose-6-phosphate dipotassium salt, 10 mM L-glutamine, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis (β -aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 25 mM potassium phosphate buffer pH 7.5 and enzyme protein. The formation of glucosamine-6-phosphate was determined by the previously described method⁷. Protein was determined by the LOWRY method as described by LAYNE¹⁰. For a change of buffer a sample of enzyme in buffer B is filtered through a Bio-Gel P-2 column previously equilibrated with buffer C.

For inactivation experiments by anticapsin or glutamine analogs 0.25~0.30 mg of enzyme protein in buffer C was incubated at 30°C with various concentrations of inhibitors and in the presence of fructose-6-phosphate at 15 mM. To follow the inactivation process the enzyme inhibitor complex was separated by the centrifuge-column procedure¹¹. Aliquots 200 μl were withdrawn at several time intervals during the course of the reaction and transferred to the top of a small (1 ml Sephadex G-25 in buffer B) column and centrifuged 1 minute at 200 $\times g$. Appropriate aliquots of effluent were used for determination of residual enzyme activity by standard assay procedures.

Buffers: Buffer A; 25 mM potassium phosphate buffer pH 7.5, 1 mM EGTA, 5 mM dithiothreitol; Buffer B; 25 mM potassium phosphate buffer pH 7.5, 1 mM EGTA, 2 mM dithiothreitol, 10 mM L-glutamine and 500 mM sucrose; Buffer C; 25 mM potassium phosphate buffer pH 7.5, 1 mM EGTA, 1 mM dithiothreitol and 500 mM sucrose.

Chemicals

Anticapsin was a gift from Dr. N. NEUSS, Eli Lilly, Indianapolis. 6-Diazo-5-oxo-L-norleucine, azaserine, albizziin, glutamic acid- γ -monohydroxamate, glutamic acid- γ -methyl ester and glutamic acid- γ -hydrazide were from Sigma (St. Louis, Mo.). All other chemicals were purchased from Serva (Heidelberg).

Results

Inactivation by Anticapsin and Some Glutamine Analogs

Incubation in 25 mM potassium phosphate buffer pH 7.5 containing: 1 mM EGTA, 1 mM dithiothreitol and one of the substrates: the fructose-6-phosphate at 15 mM and partially purified glucosamine-6-phosphate synthetase with anticapsin resulted in a time dependent loss of enzyme activity. The ratio of the remaining activity in the presence of anticapsin (E) to that of the control (E_0) measured at the same times of incubation was plotted vs. time in a semilogarithmic graph as shown in Fig. 2. The inactivation followed pseudo-first-order reaction kinetics, the rate of which was directly related to the inhibitor concentration. Using the slopes of the straight lines, the values of k_{app} were calculated.

The dependence of the observed rate constant for inactivation on the anticapsin concentration (Fig. 3) indicated that the inhibitor reversibly binds to the enzyme prior to irreversible inactivation:



where: E is the enzyme, I is the inhibitor, $E \cdot I$ is the reversible enzyme-inhibitor complex, $E-I$ is the enzyme irreversibly modified, K_{inact} is the dissociation constant of $E \cdot I$ and k_{max} is the maximum inactivation rate constant at infinite inhibitor concentration.

Fig. 2. Inactivation of glucosamine-6-phosphate synthetase by anticapsin.

Partially purified enzyme (0.25 to 0.3 mg, specific activity 7.2 μmol glucosamine-6-phosphate (GlcN-6-P) mg of protein) was incubated at 30°C with various concentrations of anticapsin: 1, 250; 2, 100; 3, 4.0; 4, 2.5; 5, 2.0; 6, 1.0; 7, 0.75 and 8, 0.5 μM . At the indicated times, 200 μl aliquots were removed and centrifuged through a small Sephadex G-25 column. The effluent was assayed for any remaining enzymatic activity.

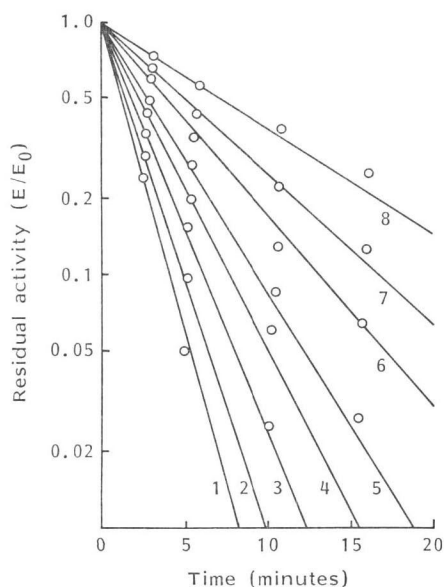


Fig. 3. Dependence of the pseudo-first-order rate constants of inactivation on the anticapsin concentration.

Enzyme was reacted with several concentrations of anticapsin under the conditions given in the legend to Fig. 2. The inset is a double reciprocal plot of the data according to the KITZ-WILSON equation¹²⁾.

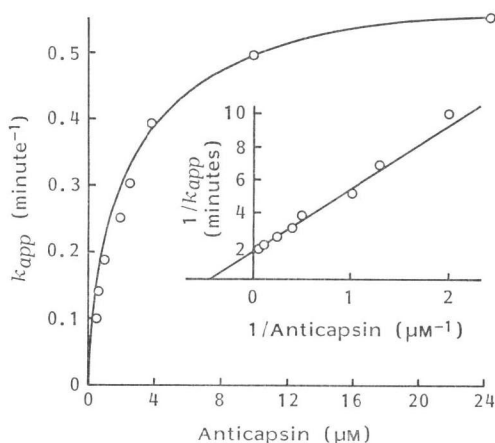


Fig. 3 (inset) shows the double reciprocal plot according to the KITZ-WILSON¹²⁾ equation:

$$\frac{1}{k_{app}} = \frac{K_{inact}}{k_{max}} \cdot \frac{1}{(I)} + \frac{1}{k_{max}}$$

which allows the calculation of K_{inact} of 2.5 μM and k_{max} of 1×10^{-2} /second. The maximum in-

activation rate constant corresponds to a minimal inactivation half-time of 1.15 minutes.

The presence of the substrate fructose-6-phosphate accelerates the inactivation. In its absence, the value of K_{inact} increased to 13.3 μM ; in contrast the maximum inactivation rate constant showed no significant change (9×10^{-3} /second). The acceleration of inactivation by fructose-6-phosphate is ascribed to ordered binding of first fructose-6-phosphate and then anticapsin.

The glutamine analog 6-diazo-5-oxo-L-norleucine also strongly inactivates the glucosamine-6-phosphate synthetase from *E. coli* K-12 (data not shown). The determined K_{inact} value of 96 μM is only one order of magnitude higher than the value for inactivation caused by anticapsin. Other glutamine analogs such as azaserine and albizziin at concentrations of 5 mM do not cause significant inactivation of the enzyme.

At a concentration of 1 mM complete inactivation of the enzyme could be achieved within 30 minutes by thiol-group reagents: iodoacetamide and *N*-ethylmaleimide. At a concentration of 100 μM these reagents caused inactivation expressed by inactivation rate constants of 0.4×10^{-3} /second and 3.3×10^{-3} /second respectively.

Inhibitory Properties of Anticapsin on Glucosamine-6-phosphate Synthetase Activity

The glucosamine-6-phosphate synthetase activity was assayed in the presence of various concen-

Fig. 4. Competitive inhibition of glucosamine-6-phosphate synthetase by anticapsin.

Enzyme was assayed as described in "Materials and Methods". Assays were performed at the indicated concentrations of anticapsin (μM).

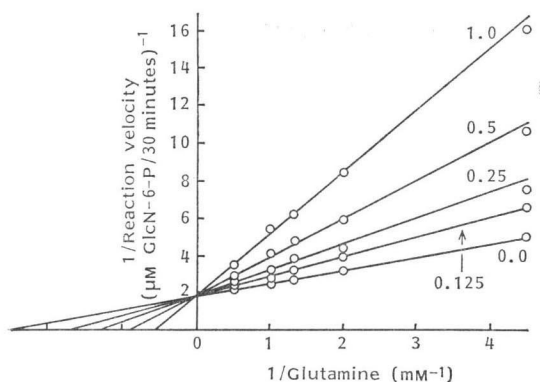
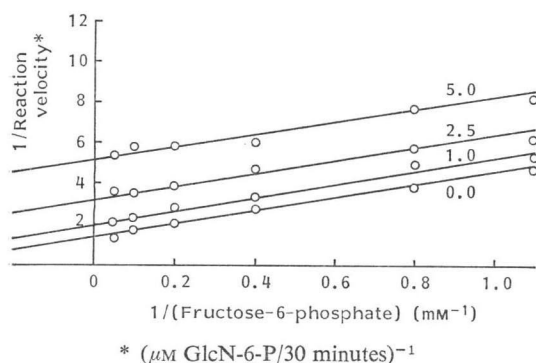


Fig. 5. Uncompetitive inhibition of glucosamine-6-phosphate synthetase by anticapsin.

Enzyme was assayed as described in "Materials and Methods". Assays were performed at the indicated concentrations of anticapsin (μM) and a fixed concentration of L-glutamine of 5 mM.



at a concentration range of $0.125 \sim 1 \mu\text{M}$ and in the presence of glutamine at concentrations from $0.25 \sim 2 \text{ mM}$ acted as a competitive inhibitor. Apparent K_m values for glutamine at different anticapsin concentrations could be calculated from these data. A replot¹⁴⁾ of these apparent K_m values vs. the corresponding anticapsin concentration yielded a linear relationship (data not shown) and apparent K_m and K_i may be calculated. The experiment gave a K_m value of $400 \mu\text{M}$ and a K_i value of $0.275 \mu\text{M}$.

The low ratio of K_i/K_m of 6.8×10^{-4} demonstrates that anticapsin has a higher affinity to the active site than the natural substrate glutamine. Anticapsin appears to be a linear uncompetitive inhibitor with respect to fructose-6-phosphate (Fig. 5). This result indicates that anticapsin binds predominantly to enzyme·fructose-6-phosphate complexes. This behavior is indicative of an ordered sequential mechanism of binding¹⁵⁾.

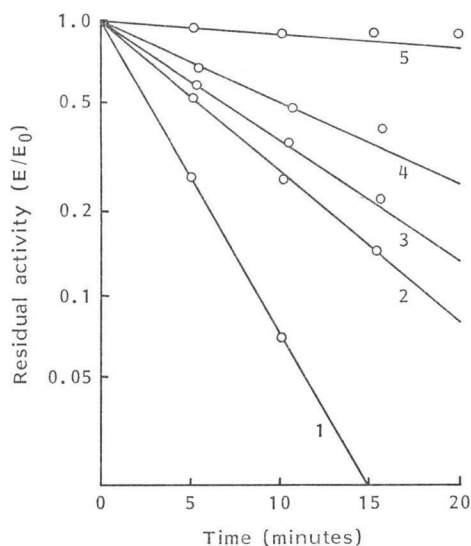
Protection against Inactivation by Anticapsin

If anticapsin is reacting to become covalently bound to the glutamine binding site of glucosamine-

Fig. 6. Effect of L-glutamine and competitive inhibitors on glucosamine-6-phosphate synthetase inactivation by anticapsin.

Enzyme was incubated as in Fig. 2, with: 1, $10 \mu\text{M}$ anticapsin; 2, $10 \mu\text{M}$ anticapsin and 10 mM *N*-acetylglutamine; 3, $10 \mu\text{M}$ anticapsin and 10 mM albizziin; 4, $10 \mu\text{M}$ anticapsin and 1 mM glutamic acid- γ -monohydroxamate; 5, $10 \mu\text{M}$ anticapsin and 1 mM L-glutamine.

Experiments was conducted in the absence of fructose-6-phosphate in the reaction mixture. $200 \mu\text{l}$ aliquots were removed, and the activity remaining was determined as before.



trations of anticapsin and at different concentrations of glutamine, one of the substrates. The data was plotted according to the LINEWEAVER-BURK equation¹³⁾. As seen in Fig. 4 anticapsin

6-phosphate synthetase in the process of inactivation, one should expect that the presence of the substrate glutamine, as well as competitive inhibitors of the enzyme activity, must impair the inactivation reaction. Fig. 6 shows that L-glutamine at a concentration of 1 mM completely protected the enzyme against inactivation caused by anticapsin. The competitive inhibitors albizziin and γ -glutamylhydroxamate at the same concentration showed only limited protective action. Other compounds incapable of binding to the glutamine binding site, such as D-glutamine, glutamic acid- γ -hydrazide and glutamic acid- γ -methyl ester do not protect the enzyme (data not shown). It is noteworthy that N-acetylglutamine at a relatively high concentration of 10 mM also prevents inactivation of the enzyme.

Discussion

Glucosamine-6-phosphate synthetase was prepared from *E. coli* K-12 strain 3000 Hfr to about 60% of purity as checked by polyacrylamide gel electrophoresis. The molecular weight of glucosamine-6-phosphate synthetase was estimated by gel filtration, on Sephacryl S-300 and to be $90,000 \pm 5,000$.

Incubation of partially purified glucosamine-6-phosphate synthetase from *E. coli* K-12 with the competitive inhibitor anticapsin (Fig. 4) causes time-dependent, pseudo-first order loss of enzyme activity. Gel filtration of the inactivated enzyme does not cause its reactivation, consistent with mechanism of inactivation involving covalent modification of the enzyme⁽⁶⁾. Saturation kinetics are observed with a K_{inact} of $2.5 \mu\text{M}$ and k_{max} of 1×10^{-2} /second, excluding the possibility that inactivation occurs by a nonspecific bimolecular mechanism. The binding of anticapsin to the glutamine binding site of the enzyme occurs by the sequential ordered mechanism. The observation that the rate of inactivation is diminished in the presence of glutamine and of competitive inhibitors, indicated that covalent modification occurs at the enzyme active site.

All thus far examined glutamine analogs: 6-diazo-5-oxo-L-norleucine, azaserine, albizziin, 2-amino-4-oxo-5-chloropentanoic acid⁽¹⁷⁾ and α -amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid⁽¹⁸⁾ covalently bind to the active site cysteine residue that is essential for glutamine utilization.

The identification of the covalently modified amino acid residue (probably cysteine) in the active site of the enzyme upon the action of anticapsin requires further studies. The exceptional effectiveness of anticapsin as an enzyme inactivator can not be explained just by the high affinity to the enzyme binding site and chemical reactivity of the moiety participating in the formation of a covalent bond. We postulate that due to the specific structure of anticapsin, proximity and orientation effects occur upon the binding of the inhibitor by the enzyme glutamine binding site.

Based on the obtained results it can be concluded that anticapsin is an active-site directed glutamine analog in the reaction catalyzed by glucosamine-6-phosphate synthetase.

Acknowledgments

We are indebted to the Deutsche Forschungsgemeinschaft SFB 76, Federal Republic of Germany and Institute of Experimental Biology, Polish Academy of Sciences, Warsaw for support during the course of this work.

References

- 1) GHOSH, S.; H. I. BLUMENTHAL, E. DAVIDSON & S. ROSEMAN: Enzymatic synthesis of glucosamine-6-phosphate. *J. Biol. Chem.* 235: 1265~1273, 1960
- 2) KORNFELD, R.: Studies on L-glutamine-D-fructose-6-phosphate amidotransferase. *J. Biol. Chem.* 242: 3135~3141, 1967
- 3) WARREN, L.: The biosynthesis and metabolism of amino sugars and amino sugar-containing heterosaccharides. *In Glycoproteins. Ed. GOTTSCHALLK*, p. 1123, Elsevier, Amsterdam, 1972
- 4) CHMARA, H.; S. MILEWSKI, M. DZIEDUSZYCKA, M. SMULKOWSKI, P. SAWLEWICZ & E. BOROWSKI: Epoxy-

peptides — a novel group of metabolic inhibitors in prokaryotic and eukaryotic microorganisms. *Drugs Expl. Clin. Res.* 8: 11~22, 1982

- 5) NEUSS, N.; B. B. MOLLOY, R. SHAH & N. DE LA HIGUERA: The structure of anticapsin, a new biologically active metabolite of *Streptomyces griseoplanus*. *Biochem. J.* 118: 571~575, 1970
- 6) KAMIŃSKI, K. & T. SOKOŁOWSKA: The probable identity of bacilylsin and tetaine. *J. Antibiotics* 26: 184~185, 1973
- 7) KENIG, M.; E. VANDAMME & E. P. ABRAHAM: The mode of action of bacilylsin and anticapsin and biochemical properties of bacilylsin-resistant mutants. *J. Gen. Microbiol.* 94: 46~54, 1976
- 8) LUGTENBERG, E. J. J. & P. G. DE HAAN: A simple method following the fate of alanine containing components in murein synthesis in *Escherichia coli*. *Antonie van Leeuwenhoek. J. Microbiol. Serol.* 37: 537~552, 1971
- 9) NORRMANN, J.; R. B. MYERS, T. W. GIDDLINGS & E. C. CANTINO: Partial purification of L-glutamine: D-Fructose-6-phosphate amidotransferase from zoospores of *Blastocladiella emersonii*. *Biochim. Biophys. Acta* 302: 173~177, 1973
- 10) LAYNE, B.: Spectrophotometric and turbidimetric methods for measuring protein. *Methods Enzymol.* 3: 447~454, 1957
- 11) PENEFSKY, H. S.: Reversible binding of P₁ by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem.* 252: 2891~2899, 1977
- 12) KITZ, R. & I. B. WILSON: Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* 237: 3245~3249, 1962
- 13) SEGEL, I. H.: *Biochemical Calculations.* p. 246, John Wiley & Sons Inc., New York, 1976
- 14) SEGEL, I. H.: *Enzyme Kinetics.* p. 108, John Wiley & Sons Inc., New York, 1975
- 15) SPECTOR, T. & W. W. CLELAND: Meanings of K_i for conventional and alternate substrate inhibitors. *Biochem. Pharmacol.* 30: 1~7, 1981
- 16) MELOCHE, P.: Bromopyruvate inactivation of 2-keto-3-deoxy-6-phosphogluconic aldolase. I. Kinetic evidence for active site specificity. *Biochemistry* 6: 2273~2280, 1967
- 17) BUCHANAN, J. M.: The amidotransferases. *Adv. Enzymol.* 39: 91~183, 1973
- 18) TSO, J. Y.; S. G. BOWER & H. ZALKIN: Mechanism of inactivation of glutamine amidotransferase by the antitumor drug L-(α ,S,S)- α -amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid. *J. Biol. Chem.* 255: 6734~6738, 1980