INHIBITION OF GLUCOSAMINE-6-PHOSPHATE SYNTHETASE FROM BACTERIA BY ANTICAPSIN

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(Received for publication February 7, 1984)

On the basis of kinetic studies on glucosamine-6-phosphate synthetase (EC 5.3.1.19) from six bacteria sources it has been shown that the epoxyamino acid anticapsin, a glutamine analog, is a competitive inhibitor of the enzyme in regard to glutamine with *Km* value of 10^{-4} M and *Ki* varying from 10^{-7} to 10^{-6} M. Unlike other glutamine analogs like 6-diazo-5-oxo-L-norleucine, chloropentanoic acid, L-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid or albizziin, anticapsin is not generally inhibitory to various amidotransferases. It does not inhibit xanthosine 5′-monophosphate amidotransferase, glutaminase or γ-glutamyltranspeptidase.

The key enzyme responsible for the synthesis of aminosugars is 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring) (EC 5.3.1.19) which catalyzes the formation of glucosamine-6-phosphate from D-fructose-6-phosphate and L-glutamine[1]. This enzyme is first in the pathway leading to the formation of UDP-*N*-acetylglucosamine, the major intermediate in the biosynthesis of all aminosugar containing macromolecules both in prokaryotic[2,3] and eukaryotic cells[4,5]. This concerns also the cell-wall macromolecules and therefore the inhibition of this enzyme has some important implications for chemotherapy. This enzyme, is one of eleven amidotransferases utilizing glutamine as a source of amino group[6]. It has been reported that glucosamine-6-phosphate synthetase is inhibited by numerous compounds including non-specific thiol-group reagents or alkylating agents and more specific glutamine analogs such as 6-diazo-5-oxo-L-norleucine, azaserine and albizziin[7]. Hitherto kinetic constants for such inhibition have not been reported. Of particular interest are glutamine analogs.

These compounds, similarly to the substrate glutamine, are attached to the active site of the enzyme through the α-amino and α-carboxyl groups[6]. In the case of glutamine and glucosamine-6-phosphate synthetase, the carbonyl and amide group are ideally positioned at the enzyme site to undergo a nucleophilic attack by the cysteine sulfhydryl group, displacing the amide group and thus effecting its transfer to the ketone function of D-fructose-6-phosphate.

KENIG *et al.*[8] reported that bacilysin, L-alanyl-L-β-(2,3-epoxycyclohexyl-4-on)alanine, a dipeptide antibiotic produced by *Bacillus subtilis* A 14, strongly inhibits glucosamine-6-phosphate synthetase in cell free extract from *Staphylococcus aureus* NCTC 6571.

This antibiotic is synonymous with bacillin[9] produced by *Bacillus* sp. KM 208 and the earlier isolated antibiotic tetaine produced by *Bacillus pumilus* theta[10,11]. The mode of action envisaged for L-alanyl-L-β-(2,3-epoxycyclohexyl-4-on)alanine (tetaine) involves rapid transport into the cell by a peptide carrier[12~14], followed by immediate hydrolysis to alanine and the *C*-terminal epoxyamino acid anticapsin by the action of cytoplasmic peptidases[13,15]. The epoxyamino acid liberated anticapsin inhibits strongly the glucosamine-6-phosphate synthetase resulting in the cessation in microorganisms of synthesis of all aminosugar containing macromolecules[12,15].

Recently, we have reported the inhibition by anticapsin of glucosamine-6-phosphate synthetase in cell-free extracts from a large number of bacteria as well as from yeasts[15]. The nature of the inhibition has not been elucidated yet. The lack of kinetic data, especially for bacteria, prompted us to perform the experiments which are the subject of the present paper. Preliminary experiments showed that anticapsin acts as a glutamine analog, and in the absence of this amino acid it irreversibly inactivates partially purified glucosamine-6-phosphate synthetase from bacteria (CHMARA and ZÄHNER, unpublished data). At high concentration of anticapsin, over 1 mM, complete inactivation of the enzyme could be achieved within 5 minutes. In the present paper we present evidence of the inhibition of glucosamine-6-phosphate synthetase by anticapsin in the presence of both substrates: L-glutamine and D-fructose-6-phosphate. Kinetics of inhibition of the enzyme from several bacteria are also compared.

Materials and Methods

Microorganisms

Escherichia coli K-12 W 945, *Salmonella minnesota* R 3 SF 117, *Pseudomonas aeruginosa* PAO 3, *Bacillus thuringiensis* HD 1, *Arthrobacter crystallopoietes* ATCC 15281 and *Corynebacterium* sp. 168 were obtained from the collection of the Institute für Biologie II Universität Tübingen, Federal Republic of Germany.

Growth Conditions and Preparation of Crude Cell-free Extract

The bacteria were grown in 15-liter fermenter in 10 liters of media containing in g/liter: Bactopeptone (Difco) 1.5 g, Lab-Lemco 1.5 g, yeast-extract (Difco) 5 g, NaCl 3.5 g, K_2HPO_4 3.68 g, KH_2PO_4 1.32 g and glucose 2 g. Glucose was sterilized separately and added to the medium. Final pH of the medium after sterilization was 7.4. Growth with vigorous aeration at 37°C was continued approximately 18 hours. Cells were chilled and harvested by centrifugation at 4°C, washed twice with buffer A and resuspended in 100 ml of buffer B and stored at −26°C until used.

Frozen cells were thawed and then subjected to sonic disruption with Branson sonifier accompanied with intensive chilling. The broken cell suspension was diluted with buffer B to volume of 200 ml and the suspension was centrifuged at 50,000×*g* for 90 minutes.

Glucosamine-6-phosphate Synthetase Assay

Standard assay mixture contained in 1 ml; 15 mM D-fructose-6-phosphate, 10 mM L-glutamine, 1 mM dithiothreitol, 1 mM ethyleneglycol-bis(2-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) and 25 mM potassium phosphate buffer pH 7.5. Incubation at 37°C started by the addition of the enzyme and terminated after 30 minutes by immersing the assay tubes in boiling water for 1 minute. The formation of glucosamine-6-phosphate was determined by previously described procedure[5].

γ-Glutamyl Transpeptidase Assay

Standard assay mixture contained 100 mM potassium phosphate buffer pH 7.5, 1 mM γ-glutamyl-*p*-nitroanilide, 20 mM glycylglycine, 2.5 μg of the enzyme in a solution of 100 μl and various concentration of anticapsin. The reaction mixture was incubated at 37°C for 15 minutes; the rate of *p*-nitroaniline formation was recorded at 410 nm.

Xanthosine 5′-Monophosphate-amidotransferase Assay

Xanthosine 5′-monophosphate-amidotransferase was partially purified according to PATEL[16] from *Escherichia coli* K-12 W 945 to the specific activity of 9.6 μmol of guanosine-5′-monophosphate formed in 1 minute per mg of protein.

The enzymatic assay were performed according to PATEL *et al.*[17].

L-Glutaminase Assay

0.25 ml of 0.08 M L-glutamine in 0.1 M sodium acetate buffer pH 4.9, 0.2 ml of 0.1 M sodium acetate buffer pH 4.9, approximately 5 units/ml of enzyme and inhibitor at different concentration were present in the reaction mixture. After 30-minute incubation at 37°C the ammonia formed was determined with

Nessler reagent.

Protein Determination

Protein was determined by the method of LOWRY *et al.* as reported by LAYNE[15] using bovine serum albumin as standard.

Purification of Glucosamine-6-phosphate Synthetase

All purification steps were conducted at +5°C. Step. 1: The crude cell-free extract, approximately 180 ml, was treated with 75 ml of 2% polymyxin sulfate in buffer A. After being stirred for 15 minutes, the mixture was centrifuged, and the residue discarded. Step. 2: First Cellex D chromatography. The supernatant after centrifugation from step 1 was diluted three-fold with buffer B. The solution was applied to a column (2.5×25 cm) of Cellex D (OH^-) equilibrated with buffer B. The column was washed with a stepwise gradient of 200-ml portions of buffer B containing of 50, 100, 150, 200, 250 mM KCl and finally 300 ml of buffer B containing 300 mM KCl. Those fractions containing enzyme activity were combined. Step. 3: Second Cellex D chromatography. Combined fractions after first Cellex D were diluted four-fold with buffer B and applied to a column (2.5×20 cm) of Cellex D (OH^-) previously equilibrated with buffer B. The column was washed by stepwise addition of 100, 150, 200, 250 and 300 mM KCl. Active fractions were collected.

The partially purified enzyme preparations in buffer B could be stored at +5°C for a few weeks without decrease of catalytic activity. For the change of the buffer especially for experiments for the determination of kinetic constants, a sample of enzyme in buffer B was filtered through a Bio-Gel P-2 column (2.5×25 cm) previously equilibrated with appropriate buffer.

MICHAELIS-MENTEN constants were determined by standard LINEWEAVER-BURK graphic method, the inhibition constant (*Ki*) was determined graphically according to the method recommended by SEGEL[10].

Buffers

Buffer A: 25 mM potassium phosphate buffer pH 7.6, 1 mM EGTA, 10 mM KCl, 5 mM dithiothreitol.

Buffer B: 25 mM potassium phosphate buffer pH 7.5, 1 mM EGTA, 1 mM dithiothreitol, 10 mM L-glutamine and 500 mM sucrose.

For kinetic constant determination, the composition of the buffers was generally as in the buffer B but without glutamine and at fixed concentration of D-fructose-6-phosphate of 15 mM; for *Km* determination in relation to fructose-6-phosphate the composition was as in buffer B but at fixed concentration of 5 mM L-glutamine.

After third step of purification the enzyme preparations exhibited following specific activities:
E. coli K-12 W 945; 4.54 μmol GlcN-6-P/mg of protein (purification factor 38).
S. minnesota R 3 SF 117: 2.66 μmol GlcN-6-P/mg of protein (purification factor 24).
P. aeruginosa PAO 3: 1.24 μmol GlcN-6-P/mg of protein (purification factor 28).
B. thuringiensis HD 1: 3.6 μmol GlcN-6-P/mg protein (purification factor 32).
A. crystallopoietes ATCC 15281: 6.0 μmol GlcN-6-P/mg of protein (purification factor 44).
Corynebacterium sp. 168: 20.5 μmol GlcN-6-P/mg of protein (purification factor 53.8).

Reagents

Fructose-6-phosphate dipotassium salt, L-glutamine, dithiothreitol, EGTA, 6-diazo-5-oxo-L-norleucine, L-γ-glutamyl-*p*-nitroanilide, glycylglycine, γ-glutamyltranspeptidase, glutaminase, adenosine 5′-triphosphate, xanthosine 5′-monophosphate and sucrose were from Sigma, St. Louis. All other biochemicals were from Serva, Heidelberg. Cellex D and Bio-Gel P-2 were from Bio-Rad, Richmond. Anticapsin was a gift from Dr. NEUSS, Eli Lilly, Indianapolis. Tetaine was obtained in our laboratory.

Results

Inhibition of Glucosamine-6-phosphate Synthetase by Anticapsin and Tetaine

The data given in Table 1 summarize the effects of the action of anticapsin and tetaine on partially

purified glucosamine-6-phosphate synthetase from a number of bacterial sources under standard conditions. Anticapsin produced marked inhibition of the enzyme.

The assay was conducted in the presence of both substrates: D-fructose-6-phosphate and L-glutamine. At concentration of 0.7 μM anticapsin inhibits 50% the enzyme activity from *E. coli*. The inhibition by tetaine requires somewhat higher concentration of the antibiotic because the active inhibitor is not tetaine but anticapsin[5].

The release of anticapsin from the tetaine upon the action of peptidases is a time dependent process. Thus the lower inhibitory effect of tetaine on the partially purified enzyme is perhaps due to the low concentration of dipeptidases in the enzyme preparations after three steps of puri-

Table 1. Effects of anticapsin and tetaine on glucosamine-6-phosphate synthetase activity.

Enzyme source	50% inhibition caused by*	
	Anticapsin (μM)	Tetaine (μM)
Escherichia coli K-12 W 945	0.7	60
Salmonella minnesota R 3 SF 117	2.2	56
Pseudomonas aeruginosa PAO 3	3.5	65
Bacillus thuringiensis HD-1	6.5	55
Arthrobacter crystallopoietes ATCC 15281	4.0	55
Corynebacterium sp. 168	0.75	30

* The anticapsin and tetaine were added to the standard assay mixture as described in Materials and Methods, and reaction was started by the addition of the enzyme. After incubation at 37°C for 30 minutes the formation of glucosamine-6-phosphate was measured.

Fig. 1. Competitive inhibition of glucosamine-6-phosphate synthetase.
A. *E. coli* K-12 W 945, B. *S. minnesota* R 3 SF 117, C. *P. aeruginosa* PAO 3, D. *B. thuringiensis* HD-1, E. *A. crystallopoietes* ATCC 15281 and F. *Corynebacterium* sp. 168 by anticapsin which concentration are given in μM.

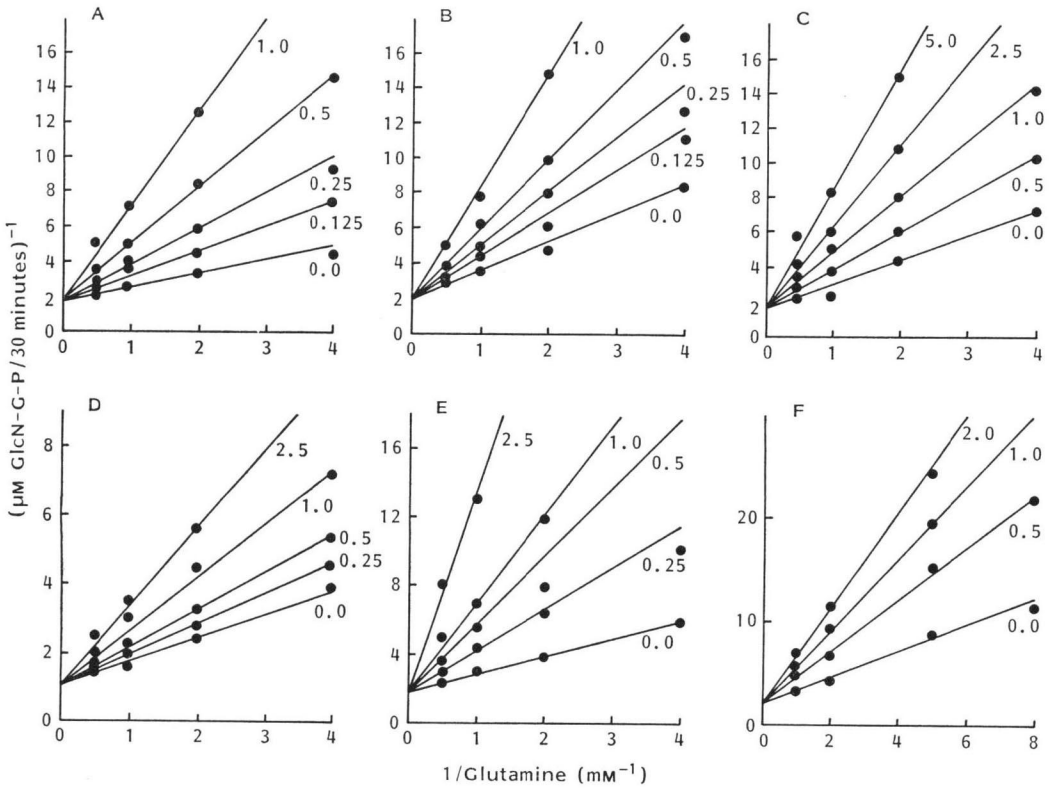


Table 2. Kinetic constants of the inhibition of glucosamine-6-phosphate synthetase by anticapsin.

Enzyme source	*Km* for Gln (M)	*Ki* (M)	*Ki*/*Km*
E. coli K-12 W 945	4×10^{-4}	1×10^{-7}	2.5×10^{-4}
S. minnesota R3 SF 117	8.3×10^{-4}	3.7×10^{-7}	4.4×10^{-4}
P. aeruginosa PAO 3	8.5×10^{-4}	13.7×10^{-7}	16.1×10^{-4}
B. thuringiensis HD-1	2.25×10^{-4}	6.4×10^{-7}	18.4×10^{-4}
A. crystallopoietes ATCC 15281	4.4×10^{-4}	2.0×10^{-7}	4.5×10^{-4}
Corynebacterium sp. 168	5.7×10^{-4}	9.5×10^{-7}	16.6×10^{-4}

Fig. 2. Competitive inhibition of glucosamine-6-phosphate synthetase from *A. crystallopoietes* by 6-diazo-5-oxo-L-norleucine.

The concentration of 6-diazo-5-oxo-L-norleucine is given in μM.

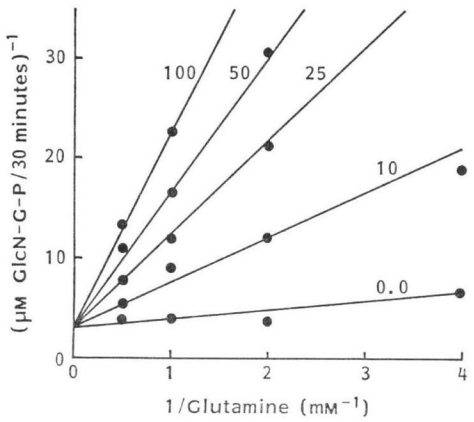
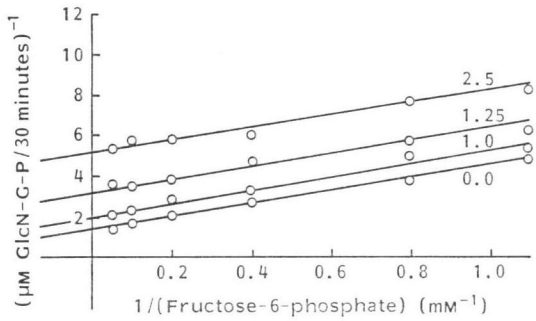


Fig. 3. Uncompetitive inhibition of glucosamine-6-phosphate synthetase from *E. coli* K-12 by anticapsin.

The concentration of anticapsin is given in μM. Experiment was performed at fixed concentration of glutamine of 5 mM.



fication.

Competitive Inhibition

When the initial rate of glucosamine-6-phosphate synthetase activity was measured as a function of glutamine in the absence and presence of various concentrations of anticapsin this inhibitor behaved as pure competitive inhibitor of the enzyme in all tested bacteria (Fig. 1, A~F). We have postulated earlier that anticapsin acts on the glucosamine-6-phosphate synthetase as a glutamine analog[15]. A second plot[10] of anticapsin concentration against the apparent *Km* for glutamine (data not shown) yields a true value of *Km* for glutamine and inhibition constant *Ki*. The inhibition data of anticapsin for enzyme from all presented bacteria are summarized in Table 2. It should be noted that the observed *Km* values in regard to glutamine remained between 2.25×10^{-4} M (*B. thuringiensis*) and 8.5×10^{-4} (*P. aeruginosa*) the *Ki* values are mainly at the same level of 10^{-7} M. As shown in Table 2 the ratio of *Ki* to *Km* for the enzyme from indicated bacteria demonstrated that the inhibitor had a very high affinity for the glutamine binding site, 10^3 to 10^4 higher than that of natural substrate, glutamine. Also KENIG *et al.*[8] reported that the action of anticapsin against glucosamine-6-phosphate synthetase activity in crude extract *Staphylococcus aureus* NCTC 6571 is pure competitive in relation to glutamine and has a value of ratio *Ki*/*Km* of 7×10^{-4} M.

As shown in Fig. 2, a classic[6] glutamine analog, 6-diazo-5-oxo-L-norleucine, exhibited a pure competitive inhibition on the enzyme from *A. crystallopoietes* in regard to glutamine with determined *Ki* value

of 18×10^{-6} M, and has a value of ratio Ki/Km of 2.5×10^{-3}.

Uncompetitive Inhibition

As shown in Fig. 3, in experiment performed with the enzyme from *E. coli* K-12 W 945, anticapsin appears to be a linear uncompetitive inhibitor with respect to the second substrate fructose-6-phosphate. These results indicate that anticapsin binds predominantly to enzyme-fructose-6-phosphate complexes. This behavior is indicative for ordered sequential mechanism of binding[20].

Xanthosine 5'-Monophosphate Synthetase, γ-Glutamyltransferase and Glutaminase

In a broad range of concentrations from 1 μM to 1,000 μM anticapsin does not exert any inhibitory effect of above mentioned enzymes.

Discussion

Evaluating the results of the inhibition of enzymatic reaction two different phenomena, namely enzyme inhibition and inactivation, should be clearly distinguished. This concerns particularly di- and poly-substrate reactions with inhibitor (inactivator) being an analog of one of substrates. Such is the case with glucosamine-6-phosphate synthetase catalyzing a two substrate reaction with glutamine and fructose-6-phosphate. In the absence of glutamine, the glutamine analog anticapsin inhibits the enzymatic reaction as a result of enzyme inactivation (CHMARA & ZÄHNER, unpublished data). On the other hand when both substrates are present the inhibition by anticapsin of enzymatic reaction is more complex and includes both enzyme inhibition and inactivation, with the extent of both phenomena depending on the concentration ratio and the time of the reaction. In a short time and high concentration of glutamine the pure enzyme inhibition effect occurs. This is in accordance with the concept of KITZ and WILSON[21], and MELOCHE[22] of protective effect of a substrate. Such protection enables the phenomenon of enzyme inhibition. The presented results indicate that anticapsin is strong competitive inhibitor of glucosamine-6-phosphate synthetase from various bacterial sources with inhibition constants varying from 10^{-6} to 10^{-7} M. These constant are among the lowest ones for glutamine analogs which inhibit the transfer of amino group from glutamine to a number of acceptors catalyzed by eleven known amidotransferases including glucosamine-6-phosphate synthetase[6]. It should be also stressed that Ki/Km values ranging from 10^{-3} to 10^{-4} indicate very high affinity of anticapsin to the glutamine binding site. This value is the lowest one as compared to other glutamine analogs inhibiting various amidotransferases[6,23]. Anticapsin distinguished itself also from other glutamine analogs by the high degree of selectivity. The glutamine analogs usually exhibit rather pleiotropic inhibitory action in regard to various amidotransferases[6,23]. The experiments described indicate that anticapsin does not inhibit some selected model enzymes of this group: γ-glutamyltranspeptidase, xanthosine 5'-amidotransferase and glutaminase, while remaining a strong inhibitor of glucosamine-6-phosphate synthetase.

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