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NITROGEN TRANSFER IN *E. COLI* GLUCOSAMINE-6P SYNTHASE. INVESTIGATIONS USING SUBSTRATE AND BISUBSTRATE ANALOGS.

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Abstract. The compound 2-amino-2-deoxyglucitol-6P competitively inhibits *E. coli* glucosamine-6P synthase with respect to fructose-6P whereas glutamate γ-semialdehyde is a competitive inhibitor with respect to glutamine. These compounds, which exhibit good inhibitory properties (K_m/K_i= 16 and 1000 respectively), were used in the synthesis of multisubstrate analogs.

INTRODUCTION

The enzymes responsible for the utilisation of the amide nitrogen of glutamine are known under the generic term of amidotransferases: they all catalyze the transfer of nitrogen from glutamine to a great variety of acceptors including amino acids, nucleotides and carbohydrates[1]. They consist of a glutamine amide transfer (GAT) domain and a synthetase (or synthase) domain. Glutamine hydrolysis is catalyzed by the GAT domain and the resulting ammonia is used by the synthase domain in the biosynthetic reaction. The 16 amidotransferases listed thus far have been divided into two families known as F-type and G-type depending on the location of the GAT domain on the primary sequence[1]. The F-type class includes phosphoribosyl pyrophosphate (PRPP) amidotransferase[2,3], glutamate synthase[4], asparagine synthetase[5,6] and glucosamine-6P synthase[7] (GlmS). The last enzyme is capable of catalyzing the following reactions:

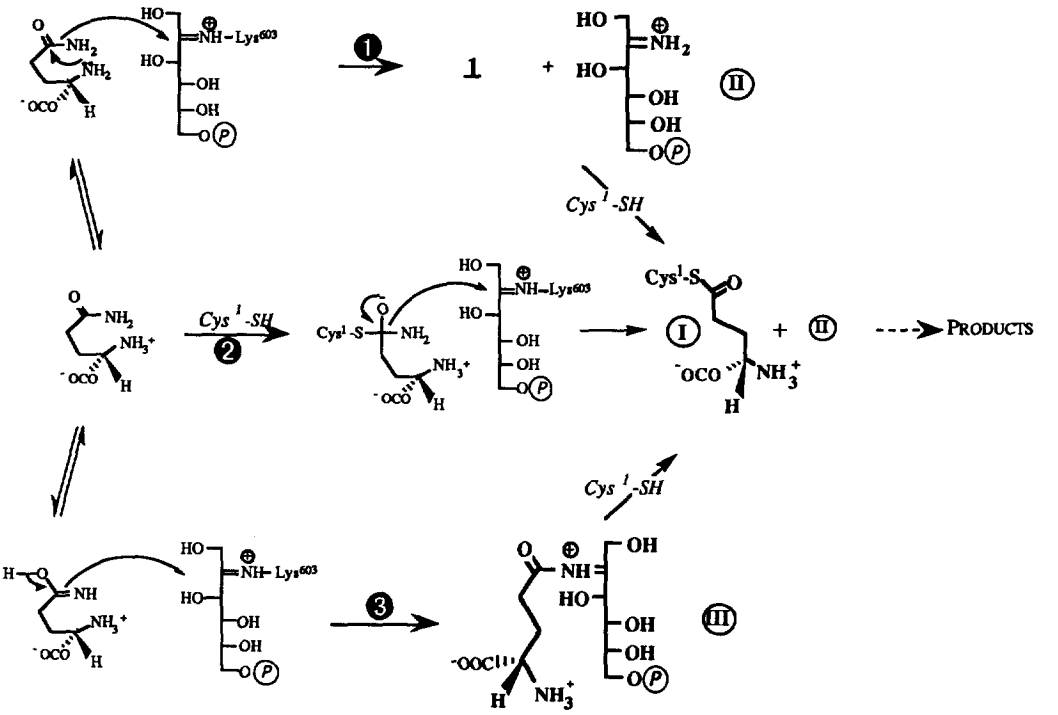
(*reaction 1*)	D-Fru-6P	+	L-Gln →	L-Glu	+	D-GlcN-6P
(*reaction 2*)	L-Gln	+	H_2O →	L-Glu		

These two reactions can be considered either as part of a single mechanism where the acceptor (fructose-6P), when lacking, is replaced by a water molecule or as two different reactions where reaction 2 can be seen as a vestige of the thiol protease mechanism. The major questions relating to the mechanism of action of this family of proteins which have emerged from the investigations performed by various groups over the last few years[8-13] can be summarized by the following: how is the nitrogen transferred from the glutamine to the corresponding acceptor?

The three most plausible mechanisms are (Scheme 1):

1) Nitrogen amide labilization as a result of cyclization of glutamine into pyroglutamic acid (mechanism ❶). Although not considered in the past in the amidotransferase mechanism, formation of this cyclic residue occurs quite frequently in amino acid chemistry[14].

2) Nitrogen labilization as a result of amide bond cleavage mediated by an enzyme nucleophile (the N-terminal cysteine, essential for activity[7], is a candidate) in a manner similar to that known for proteolytic enzymes (mechanism ❷).



Scheme 1: Proposed mechanisms for the nitrogen transfer from glutamine to fructose-6P. In the different possibilities considered here, the acceptor is assumed to be the Schiff base between fructose-6P and lysine 603 as deduced from trapping experiments[27].

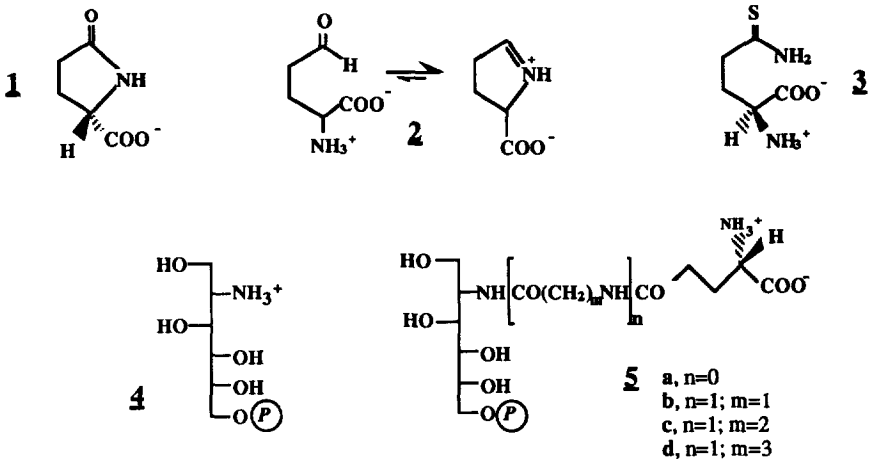
3) Direct nucleophilic attack of the amide nitrogen on an electrophilic site of the acceptor (mechanism ❸). This mechanism recently proposed for asparagine synthetase[11] is also well precedented in peptide chemistry.

In this paper we have used two glutamine analogs (glutamic γ-semialdehyde **2** and 5-thio-L-glutamine, **3**), a fructose-6P analog (2-amino-2-deoxy-D-glucitol-6P, **4**) and bisubstrate analogs (structures **5**) to test the proposed mechanisms.

RESULTS AND DISCUSSION

Neither pyroglutamate 1 nor 5-thioglutamine 3 are substrates of GlmS.

Pyroglutamate behaved neither as substrate nor inhibitor of GlmS when tested at concentrations as high as 100 mM. This fact may be taken as an argument disfavoring mechanism ❶.



We then synthesized the previously unknown sulfur analog of the substrate, 5-thioglutamine, by thionation of Boc-glutamine *t*-butyl ester with Lawesson's reagent, a more efficient route than the reaction of hydrogen sulfide with the corresponding nitrile previously used in the synthesis of thioasparagine[15]. This glutamine analog is prone to cyclization giving the corresponding thioxoproline fairly easily, even at pH 6 ($t_{1/2}$= 50 min. in phosphate buffer). Among a large number of buffers tested, bis-trispropane gave the best compromise between thioglutamine stability ($t_{1/2}$ = 3 hr in 20 mM bistrispropane pH 7.2) and enzyme catalysis. Under these conditions 5-thioglutamine 3 was not a GlmS substrate at concentrations up to 10mM. This observation also argues against mechanism ❶ (Scheme 1) since the labilization of nitrogen is greatly enhanced in the thioamide derivative compared to the normal substrate. Incubation of GlmS (0.3 mg/ml) with millimolar concentrations of 5-thioglutamine, in the presence of fructose-6P or not, did not affect the UV-visible spectra of the native enzyme. These conditions should favour the formation of a dithioester intermediate which would have been easily detected by its 310 nm absorption (logε ~ 4) as exemplified from similar experiments with papain and thioester analogs[16]. The absence of modification in the GlmS spectrum demonstrated that no accumulation of a γ-glutamyl dithioester occured. This observation is however not sufficient to rule out the intervention of mechanism ❷ or mechanism ❸ since trapping the putative intermediate I in GlmS has never been possible by the methods reported to be efficient for other amidotransferases (trichloroacetic acid or hydroxylamine).

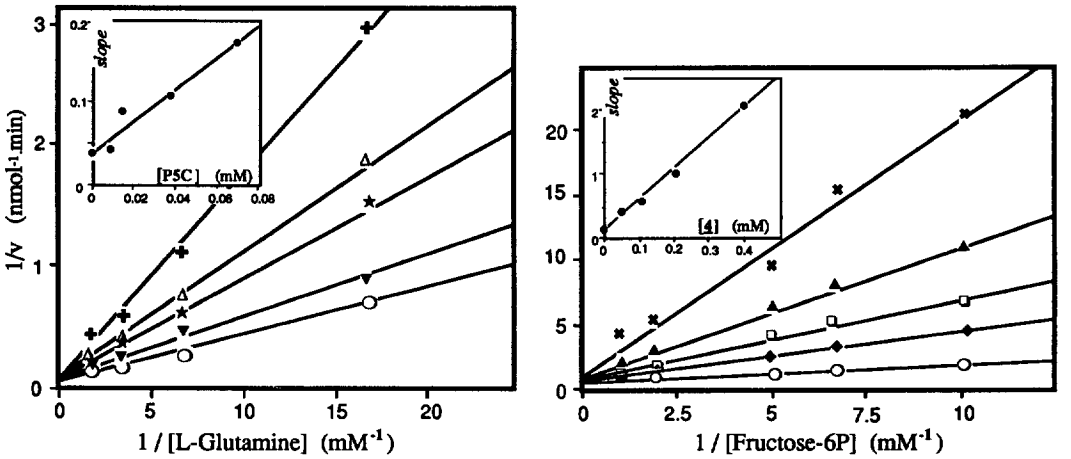
Compound 2 is a potent enzyme inhibitor.

Glutamate γ-semi aldehyde exists in neutral solution primarily as its cyclized form Δ-1 pyrroline-5-carboxylate (P5C) which can be quantitatively converted into proline by the NADPH-dependent P5C reductase[17]. D,L-P5C competitively inhibited GlmS with respect to glutamine (K_i = 23 μM, Figure 1). The complete lack of inhibition observed with proline or pyrrolidone carboxylic acid indicated that glutamate semi

aldehyde may be responsible for the observed inhibition. Assuming an upper value of 10^{-2} for the equilibrium constant P5C ⇄ glutamate γ-semi-aldehyde[17], the observed dissociation constant underestimated the binding affinity by at least two orders of magnitude ranking the aldehyde among the most potent inhibitors of glucosamine-6P synthase with anticapsin (K_i = 0.22 μM) and fumaroyldiaminopropionate (K_i = 0.15 μM)[18].

2-amino 2-deoxyglucitol-6P 4 is a potent inhibitor of GlmS.

Sodium borohydride reduction of glucosamine-6P gave the hexitol in 80% yield[19]. This compound behaves as a potent competitive inhibitor (Figure 2) *vs* fructose-6P with a K_i = 25 μM. This value (which compares favorably with the Michaelis constant of 0.4 mM for fructose-6P) must be more correctly compared with the real value of 8.8 μM which takes into account the 2.2% of the open form present in a solution of fructose-6P[20]. The potency of **4** which can be considered as a tetrahedral analog of the Schiff base II between fructose-6P and ammonia (or of the cis-enolamine resulting from H_1 proton abstraction), validates our initial hypothesis that the reaction goes via the imine II intermediate[21]. This molecule was used as the fructose-6P binding moiety in the construction of bisubstrates analogs.



Figures: 1 (left) Competitive Inhibition of P5C *vs* glutamine.The enzyme activity was analyzed using saturating concentration (10 mM) of fructose-6P and variable concentrations (0.06, 0.15, 0.3, and 0.6 mM) of glutamine in the absence (○) or in the presence of 7(▼), 14 (★), 35(Δ) and 70 (✚)mM of inhibitor. Insert: replot slope *vs* concentration of P5C.
2 (right) Competitive Inhibition of 2-amino 2-deoxy-D-glucitol-6P *vs* fructose-6P. The enzyme activity was analyzed using saturating concentration (6 mM) of glutamine and variable concentrations (0.1, 0.15, 0.2, 0.5 and 1 mM) of fructose-6P in the absence (○) or in the presence of 50(◆), 100 (❑), 200(▲) and 400 (✖)mM of inhibitor. Insert: replot slope *vs* concentration of **4**.

Synthesis and analysis of bisubstrate analogs 5.

Bisubstrate analogs were synthesized by coupling compound **4** to the γ-carboxyl function of L-glutamic acid. Starting from structure **5a** which closely mimics the bisubstrate state III of mechanism ❶, higher homologs with an increased distance between the two pseudo-substrates moieties were obtained by insertion of either glycine, β-alanine or γ-amino butyric acid. These derivatives might bring additional informations on the distance between the two substrate binding sites of the enzyme during the catalysis. For reasons of convenience the chemistry was performed using non-protected **4** and protected glutamate (as Z-Glu-OBn) activated at the γ-position with N-hydroxysuccinimide. The coupling product was purified by HPLC and deprotected by

hydrogenolysis. None of these derivatives exhibited significant inhibition up to 3 mM which may be due to 1) the high flexibility of these bisubstrate analogs preventing a correct orientation needed to enter the binding pockets and 2) the substantial conformational change occurring in the protein upon binding of its susbtrates (as demonstrated by half-of-the site reactivity behavior observed with the affinity label 6-diazo-5-oxo-L-Norleucine[7]).

CONCLUSION

The total incapacity of GlmS to accept 5-thioglutamine, a molecule structurally very similar to glutamine and more prone to cyclization argues against mechanism ❶. The high efficiency of Δ-1 pyrroline-5-carboxylic acid as a competitive inhibitor vs glutamine might suggest the participation of cysteine 1 in the formation of a hemithio acetal with glutamate γ-semialdehyde. The potent inhibitory effect of 2-amino 2-deoxy glucitol-6P can be taken as evidence that fructosimine serves as substrate of the keto/aldose isomerization. However the results obtained with the bisubstrate analogs synthesized from this structure were inconclusive. Glucosamine-6P synthase could catalyze both reactions (1) and (2) by the single mechanism ❷. Alternatively it could use two different mechanisms, i.e. mechanism ❷ (with water as the acceptor) and mechanism ❸ (with fructose-6P as the acceptor) accounting for respectively glutaminase and glucosamine synthase reactions. The fact that the K603R mutation[22] strongly decreases glucosamine synthase activity without affecting glutaminase activity can be taken as an evidence that there are two different mechanisms for the two substrates water and fructose-6P.

EXPERIMENTAL

Preparation of DL-glutamate γ-semialdehyde

γ-acetamido-γ,γ dicarbethoxybutyraldehyde ethyl enol ether (300 mg, 1 mmole)[23] was heated to 100°C in 8 ml 6N HCl for 35 minutes. After dilution (100 ml water), the product was adsorbed on Dowex 50W-X8 (2.5x16cm) and eluted with 1N HCl. The concentration of the compound in the fractions was estimated with ninhydrin ($\varepsilon = 4600\ M^{-1}cm^{-1}$; yield 64%)[17]. It was identified from its 2,4-dinitrophenylhydrazone (F=176-7°C, F_{litt}= 176-179°C)[24].

Preparation of 5-thio-L-glutamine **3**

A mixture of *N*-Boc-L-glutamine (9.84 g, 40 mmol), dicyclohexylcarbodiimide (9.08 g, 44 mmol), 2-methyl-2-propanol (38 ml, 400 mmol) and 4-(dimethylamino)pyridine (488 mg, 4 mmol) in 120 ml THF was stirred at 0°C for 5 h. The solid was filtered off and the filtrate was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2, washed with 5% acetic acid, water, dried ($MgSO_4$) and concentrated *in vacuo* before purification by column chromatography on silica gel (6:4 heptane:acetone). A mixture of this ester (5.0 g, 16.54 mmol) and Lawesson's reagent (3.35 g, 8.28 mmol) in 100 ml anhydrous dioxane was heated at 50°C for 1h30, then cooled, filtered on celite and concentrated *in vacuo* before purification by column chromatography on silica gel (1:1 heptane:ethyl acetate). This thioamide (2.0 g, 6.28 mmol) was dissolved in 40 ml dioxane saturated with hydrogen chloride at 0°C under argon. After stirring (3 hrs) the amino acid hydrochloride was precipitated by diethyl ether and purified by reverse phase HPLC (100% water) to give 0.62 g (20 % overall yield) of the desired compound. All compounds gave satisfactory analytical results (NMR, mass spectrometry, elementary analysis).

Preparation of 2-amino 2-deoxy D-glucitol-6P **4**

To a solution of D-Glucosamine-6P sodium salt (2 g, 7.7 mmol) in 115 ml ice-cooled water, sodium borohydride (583 mg, 15.4 mmol) was added portionwise over 2 hours while maintaining the pH to 8-9 (1N acetic acid). The aqueous solution was kept for 15 hours at room temperature, and the pH was adjusted to 4-5 before charging on Dowex 50W-X8 (H^+ ; 0.7 meq, 200-400 Mesh). Elution with water provided 1.6 g (6.1 mmol, 80%) of a white solid analyzed by NMR and MS (FAB).

Preparation of bisubstrate analogs **5**

N-hydroxysuccinimide esters of Z-L-glutamic acid, Z-L-glutamyl-glycine, Z-L-glutamyl-β-alanine and Z-L-glutamyl-γ-aminobutyric acid were coupled with **4** (1 eq) under standard conditions[25]. Products were purified by reverse-phase HPLC before deprotection (10% Pd/C in 4:1 MeOH:H_2O). The solution was then centrifuged, filtered and concentrated *in vacuo* to give the expected compounds (confirmed by NMR and FAB MS). Total synthesis yields ranged from 6 to 56%.

Enzyme assay

Overproduced GlmS from *E. coli* was purified as described[26]. Activity assay was performed by continuous quantitation of glutamate by glutamate dehydrogenase[7].

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