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Isomerase Activity of the C-terminal Fructose-6-phosphate Binding Domain of Glucosamine-6-phosphate Synthase from *Escherichia coli*

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The isomerase activity of the C-terminal fructose-6P binding domain (residues 241–608) of glucosamine-6-phosphate synthase from *Escherichia coli* has been studied. The equilibrium constant of the C-terminal domain k_{eq} ([glucose-6P]/[fructose-6-P]) = 5.0. A non-competitive product inhibition of the isomerase activity by the reaction product glucose-6-P has been detected. The existence of more than one binding and reaction sites for the substrate fructose-6P on the molecule of glucosamine-6-phosphate synthase can be expected. The fructose-6P binding domain possibly includes a regulatory site, different from the catalytic center of the enzyme.

Keywords: Glucosamine-6-phosphate synthase; Product inhibition; Isomerase activity; Regulatory site

Abbreviations: GlmS, glucosamine-6-phosphate synthase; PGI, glucose-6-phosphate isomerase; Fru-6P, fructose-6-phosphate; Glu-6P, glucose-6-phosphate; CT1, C-terminus, C-terminal domain of GlmS

INTRODUCTION

The enzyme glucosamine-6-phosphate synthase (GlmS) from *Escherichia coli* catalyzes the first reaction in the hexosamine biosynthesis:

L-GLUTAMINE + D-FRUCTOSE-6-PHOSPHATE

= D-GLUCOSAMINE-6-PHOSPHATE + L-GLUTAMATE (1)

This amidotransferase utilizes the amide of glutamine for the biosynthesis of coenzymes, amino acids and nucleotides.[1,2] The product of the reaction D-glucosamine-6-phosphate undergoes transformations that lead to the formation of uridine diphospho-*N*-acteyl glucosamine, an intermediate in the biosynthesis of all amino sugar-containing macromolecules. *N*-acetylglucosamine is a building block of the bacterial and fungal cell wall chitin and the enzyme GlmS is a potential target for antibacterial and antifungal agents.[3]

The molecule of GlmS from *E. coli* is a dimer, composed from two identical subunits,[4] with a

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molecular mass of 66.7 kDa for the monomer. The dimer of glucosamine-6-phosphate synthase from *E. coli* can be separated by limited chymotryptic proteolysis by two domains, a *N*-terminal glutamine buinding domain (residues 1–240) with the same capacity to hydrolyze glutamine into glutamate as the native protein and a C-terminal domain (residues 241–608) that retains the ability to bind fructose-6-phosphate,[5] and is responsible for sugar–phosphate isomerization. The C-terminal domain (CT1) of GlmS possesses glucose-6P synthase activity (phosphoglucose–isomerase-like activity).[5]

The crystal structure of the glutaminase domain has been determined at 1.8 Å resolution both complexed with L-glutamate as substrate and with a competitive inhibitor glu-hydroxamate.[6] The fructose-6-phosphate binding domain has been crystallized in the presence of substrate fructose-6-phosphate (Fru-6P) and the reaction product glucose-6-phosphate (Glu-6P). The crystal structure has been determined at 1.57 Å resolution.[7,8]

Here is reported the catalytic activity of the C-terminal fructose-6-phosphate binding domain of glucosamine-6-phosphate synthase from *E. coli*. A mechanism for the non-competitive product inhibition is proposed.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the experiments were of analytical grade (Sigma or Boehringer). The reagents of the culture medium Bacto agar, Trypton and yeast extract were from Difco.

Enzymes

The enzymes used in the study were: glucose-6P dehydrogenase from *Leuconostoc Mesenteroides* (Sigma), glutamate dehydrogenase (Sigma), fructose-6P kinase from rabbit muscle (Sigma), glycerol-6P dehydrogenase from rabbit muscle (Boehringer), triosephosphate isomerase from rabbit muscle (Boehringer), aldolase from rabbit muscle (Boehringer).

Purification of the C-terminus

The procedure of purification of C-terminus has been followed according to the literature[9,10] with modifications.

The cells of *E. coli* HB101 strain were transformed with the recombinant plasmid pMA200 (Amp^r), containing the DNA fragment, encoding the C-terminus of GlmS and the biomass was grown in LB medium at 37°C for 16 h. The centrifuged cells were destroyed by sonication in buffer A, containing 20 mM Bis–Tris propane, 2 mM EDTA, 2 mM DTT, pH 7.2, the cell extract was clarified by centrifugation and the supernatant was loaded on a Q-sepharose phast flow column (5 × 15 cm) at 150 ml h^{-1}. The column was pre-washed with 150 ml of starting buffer A and a linear gradient of 0–0.5 M NaCl in buffer A was applied. The fractions containing the enzyme were concentrated by centrifugation in Filtron (30 kDa) at 4500 rpm and 4°C. The concentrated proteins were diluted (2×) with buffer A and purified on MonoQ HR10/10 in linear gradient of 0–1 M NaCl in buffer A and 4 ml min^{-1}. The protein was concentrated to 20 mg ml^{-1} using Filtron (30 kDa) and loaded on Superose12 10/30 equilibrated with buffer B (50 mM potassium phosphate, 2 mM EDTA, 2 mM DTT, 150 mM NaCl, pH 7.2). Following the procedure of purification, described above about 30 mg of pure enzyme per liter of bacterial culture has been obtained. The purified enzyme was stored in 20% glycerol at concentration 20–50 mg ml^{-1} at −80°C.

Protein Determination

The protein concentrations were determined both using the colorimetric test of Bradford,[11]

and spectrophotometrically using $\varepsilon_{280} = 0.77\,cm^{-1}\,mg^{-1}\,ml$.

Determination of the Catalytic Activity of CT1 in the Forward Reaction (conversion of Fru-6P to Glu-6P)

The catalytic activity of CT1 was studied in 50 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM DTT, pH 7.2 and various concentrations, saturated and non-saturated, of Fru-6P. The reaction was initiated by adding of 2 mg CT1 into the reaction mixture. The reaction has been incubated for 12 min at 37°C and has been stopped by ultrafiltration in Centricon (12 kDa) at 4°C. The formation of Glu-6P was detected using the enzyme glucose-6P-dehydrogenase (3.8 U) and NADP as substrate at 340 nm ($\Delta\varepsilon = 62.220\,M^{-1}\,cm^{-1}$).[12] The reaction mixture was preincubated at 30°C and the reaction was initiated by the addition of 50 µl ultrafiltrate.

Determination of the Catalytic Activity of CT1 in the Reverse Reaction (conversion of Glu-6P to Fru-6P)

Fru-6-phosphate formed by the C-terminal domain in the reverse reaction during the incubation with the product of the reaction Glu-6P for 30 min at 37°C in the same conditions as described for the forward reaction, was detected by the multistep coupled assays using the enzymes fructose-6 phosphate kinase (1 unit), triosephosphate isomerase (17 units), glycerol-3-phosphate dehydrogenase (0.85 units) and aldolase (1.1 units). The NADH used in the reaction was measured at 25°C at 340 nm for 5 min ($\Delta\varepsilon = 62.220\,M^{-1}\,cm^{-1}$).[11]

RESULTS AND DISCUSSION

The experimental values for the rates of enzymatic reactions were referred to 1 mg of enzyme CT1 [v (nmol min^{-1})]. The enzyme preparation of CT1 used in the kinetic measurements has been tested to be free from contaminating enzymes that could affect either the substrate or the product of the reaction. Control essays for the purity of the enzyme have been performed, including all the components of the reaction and without each of them in every set of experiments. Contamination from glucosamine-6-phosphate deaminase, PGI or other enzymes of the glucosamine catabolism has not been detected.

The C-terminal domain of GlmS has been used in the catalytic reaction of isomerization of Fru-6P to Glu-6P and the reverse reaction. The reaction has been considered to be a single-substrate reaction following the mechanism of Michaelis–Menten in the both directions. The enzyme does not require cofactors or metal ions for activity.

Forward Reaction: Isomerization of Fru-6P to Glu-6P Catalyzed by CT1

The kinetic parameters in the direction of isomerization of Fru-6P to Glu-6P were determined from the Lineweaver-Burk plot. By the reciprocals of the reaction rate and substrate concentrations at various concentrations of Fru-6P (Fig. 1) it has been obtained $K_m = 3.8$ mM. K_{cat} for the forward reaction has been calculated from the maximal rate of the reaction during 12 min incubation with CT1 and has been found to be equal to 0.238 min^{-1}.

Reverse Reaction: Isomerization of Glu-6P to Fru-6P, Catalyzed by CT1

The reverse reaction of isomerization of Glu-6P to Fru-6P by the C-terminus has been studied at different concentrations of product Glu-6P (Fig. 2). From the plot of reciprocals of the rate of production of Fru-6P and the concentration of Glu-6P in the reaction it has been found $K_m =$

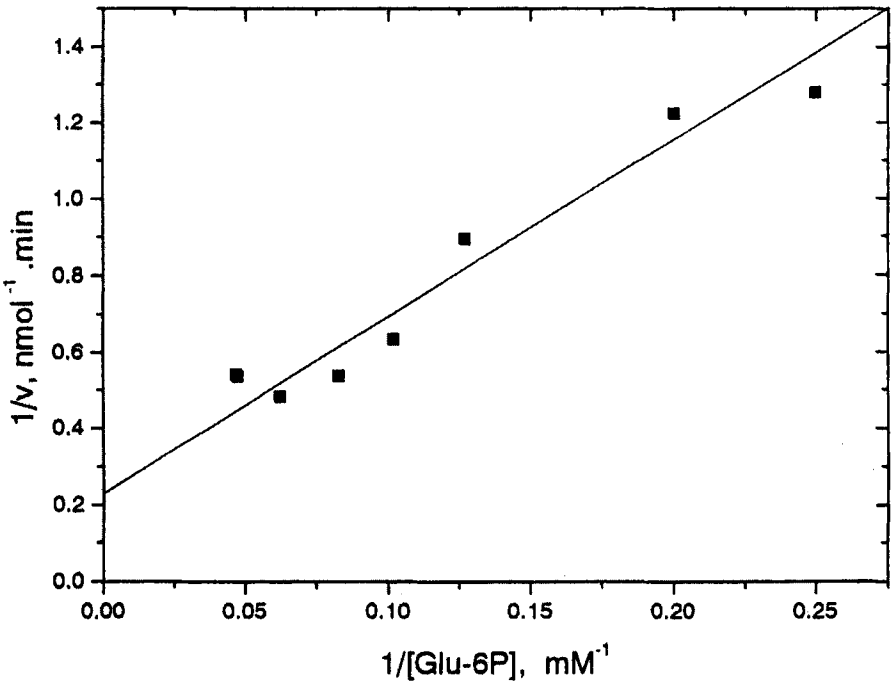


FIGURE 1 Forward reaction. Double reciprocal plot of the production rate of Glu-6P ($1/v$) versus the concentration of the substrate Fru-6P ($1/s$). Incubation of 2 mg CT1 in 50 mM K_iPO_4, 0.5 mM EDTA, 0.5 mM DTT, pH 7.2 for 12 min at 37°C; registration of the NADP formation using glucose-6P-dehydrogenase.

16.0 mM. K_{cat} has been determined by detection of the maximal rate of the reaction for 30 min of incubation with CT1 and found to be equal to 0.204 min^{-1}.

Determination of the Constant of the Equilibrium K_{eq} by the Haldane Equation

The equation of Haldane,[13]

$$K_{eq} = [(k_{cat}/K_m)_S/(k_{cat}/K_m)_P] \quad (2)$$

is valid for the equilibrium constant of the reaction of isomerization of Fru-6P to Glu-6P {$[K_{eq} = [Glu - 6P]/[Fru - 6P]$}. Substituting the experimental values for the kinetic parameters it has been found $K_{eq} = 5.0$. The calculated equilibrium constant for the isomerization of Glu-6P to Fru-6P catalyzed by the enzyme glucose-6 phosphate isomerase (PGI) in similar conditions is $K_{eq} = [Fru - 6P]/[Glu - 6P] = 0.27$.[9]

Product Inhibition of the Reaction of Isomerization of Fru-6P to Glu-6P

The product inhibition has been studied at different concentrations of the product Glu-6P and substrate Fru-6P, at saturated (20–40 mM Fru-6P) and non-saturated (2–5 mM Fru-6P) concentrations (Fig. 3). The intercept of the curves with fixed concentration of Glu-6P on the plot of reciprocals of the reaction rate ($1/v$) against the substrate concentrations ($1/s$) is on the negative abscissa in the point, corresponding to $K_m = 3.74$ mM, consistent with the mechanism of non-competitive inhibition described by the equation:

$$1/v = (1 + I/K_i)/V_{max}, \quad \text{where } I = P = \text{Glu-6P} \quad (3)$$

Such data suggest the mechanism of participation of intermediates in the reaction.

The effect of product inhibition of the enzymatic reaction has been found to be

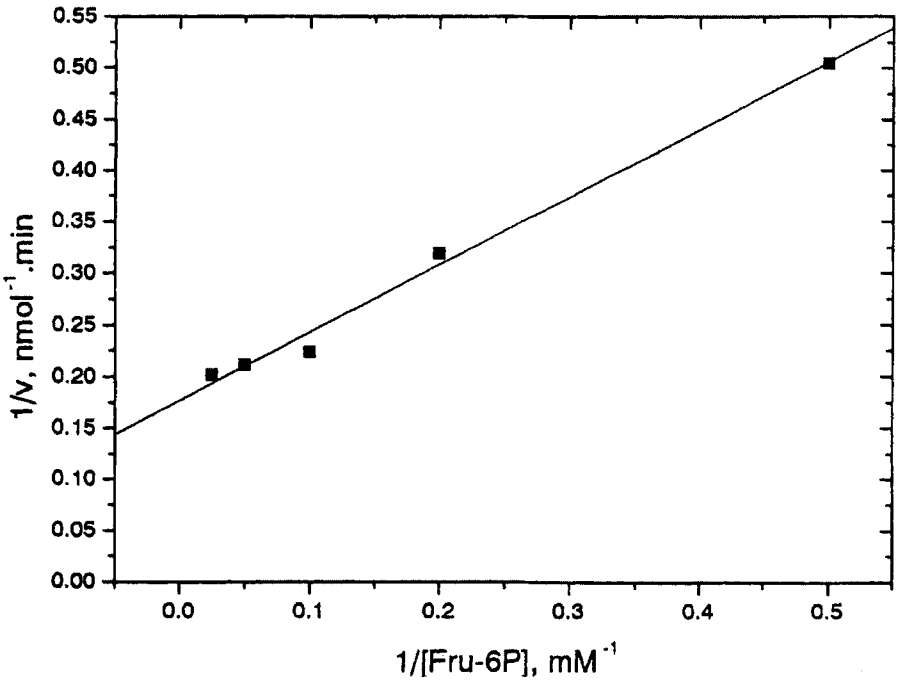
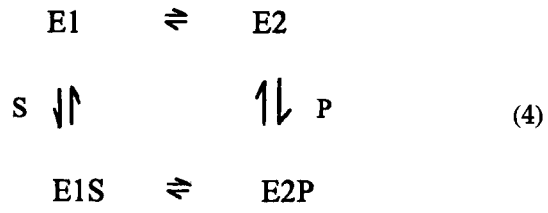


FIGURE 2 Reverse reaction. Double reciprocal plot of the production rate of Fru-6P ($1/v$) versus the concentration of Glu-6P ($1/P$). Incubation of 2 mg CT1 in 50 mM K_iPO_4, 0.5 mM EDTA, 0.5 mM DTT, pH 7.2 for 30 min at 37°C; registration of the NADH used in the reaction by multistep coupled assays including the enzymes fructose-6P kinase, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase and aldolase.

concentration dependent for non-saturation concentrations of substrate Fru-6P and reach a maximum at about 2 mM concentration of Glu-6P. For concentration of Glu-6P higher than 2 mM an effect of activation (data not shown) has been detected on the rate of the reaction, probably due to the saturation of the binding center of CT1 for Glu-6P. A possible explanation of this behavior is the existence of two centers of binding for the substrate and the product on the dimeric molecule of CT1. This is consistent with the mechanism of noncompetitive product inhibition,[14] that can be described by the scheme:



(4)

where E1 and E2 are the forms of the enzyme, binding substrate S and product P, and E1S and E2P correspond to the complex of E1 with the substrate and E2 with the product. The limiting rate of the enzyme-catalyzed reaction is the rate of return of the free enzyme from the form that liberate product back to the form that bind substrate. The reaction of product inhibition has been studied under reversible conditions. The sum of the concentrations of substrate and product remains constant when the system proceeds at equilibrium. The enzyme converts substrate to product under conditions where the concentrations of S and P are close to their equilibrium values. In the glycolytic pathway almost of the enzymes (9 from 12) proceed under reversible conditions and only three of them are displaced from their equilibrium values. This mechanism is similar to the mechanism, proposed for the enzyme proline racemase.[15] The

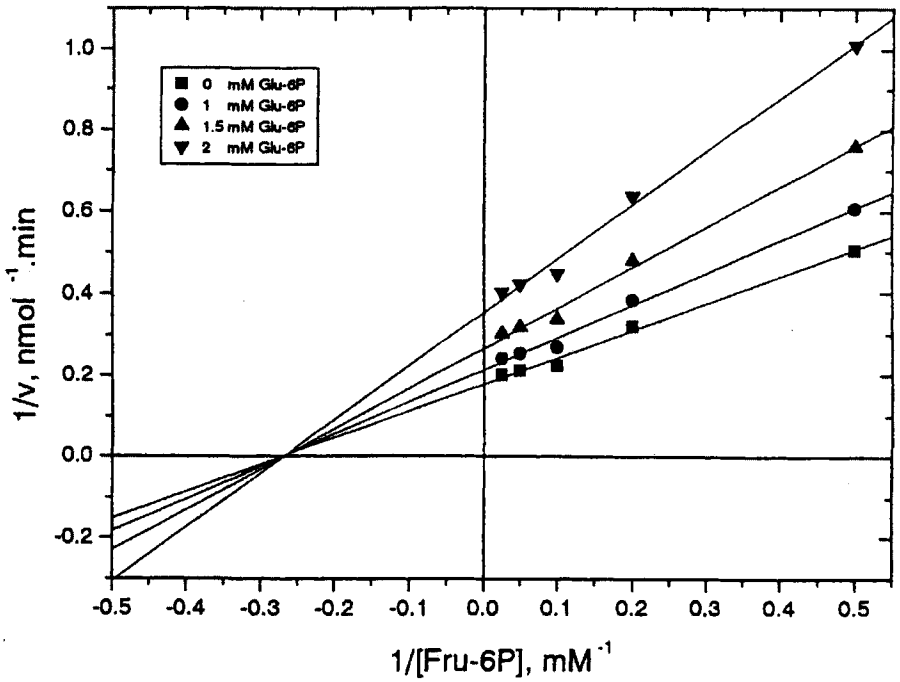


FIGURE 3 Product inhibition of CT1 from Glu-6P. Double reciprocal plot of the production rate of Glu-6P ($1/v$) versus the concentrations of the substrate Fru-6P at 0, 1, 1.5 and 2 mM Glu-6P. Incubation of 2 mg CT1 in 50 mM K_iPO_4, 0.5 mM EDTA, 0.5 mM DTT, pH 7.2 for 12 min at 37°C; registration of the formation of NADP using glucose-6P-dehydrogenase.

proposed scheme is the minimal for the mechanism of action of un enzyme, catalyzing the interconversion of a single substrate and a single product.

By this mechanism two equivalent enzymatic catalytic groups have to exist. Two lysines, one per monomer in the dimeric structure of CT1 and localized symmetrically, may be responsible for the catalyzed isomerization of the substrate- and product-bound forms of the enzyme. By site-directed mutagenesis studies it has been found that Lys603 participates in the catalytic reaction and it has been proposed mechanism, by which the open form of Fru-6P binds to the enzyme.[5] GlmS is thought to may follow two reaction pathways corresponding to the "synthetic" and "isomerase" activities in which Lys603 plays a regulatory role suppressing the glutamine-independent isomerase activity of the enzyme.[16]

The non-competitive mechanism is associated with the existence of two functional centers in the enzyme, one catalytic and other possible associated with allosteric binding. It can be expected an allosteric regulation of the enzyme by sugars.

The value of the dissociation constant of the GlmS. Fru-6P complex, determined from the competitive protection experiments $K_P = 3.3 \times 10^{-7}$M,[17] is 3 orders of magnitude less than the determined from the initial velocity data $K_{ia} = 0.54$ mM.[17] One explanation is that K_P reflects the affinity of Fru-6P for a site other than the active site. The affinity of GlmS for Fru-6P can decrease in the presence of glutamine. It is possible that the enzyme may bind a molecule of glutamine at an allosteric site or one of two active sites present on the homodimeric enzyme, lowering the affinity for Fru-6P. A synergistic

effect of Fru-6P is detected on the rate of inactivation of *Salmonella typhimurium* enzyme by FMDP [N^3-(4-Methoxyfumaroyl)-L-2,3 diaminopropionic acid] involving a saturable enzyme-inhibitor complex.[18] FMDP is an inhibitor also of GLMS from *E. coli*.

The enzymes possessing the same enzymatic activity (PGI) or similar mechanism (non-competitive) of product inhibition in one-substrate reaction (proline racemase) have dimeric structure with a single substrate-binding site for every two identical subunits. The essays on gel-filtration (data not shown) suggest that the C-terminal part of the enzyme possesses dimeric form in solution. The native enzyme GlmS from *E. coli* and from all other organisms is considered to possess a dimeric form, composed from two identical subunits.[4] The C-terminal part of the enzyme may be involved in the dimerization of the molecule in which each subunit provides one functional group at the active site. The crystal structure of the C-terminal domain of glucose-6-phosphate isomerase from *E. coli* reveals some similarity to glucose-6P isomerase,[8,19] and glucose-6P deaminase.[20] All three proteins have a nucleotide-binding fold. The catalytic residues of CT1 belong to different polypeptide chains unlikely to the most of ketol isomerases. The catalytic residues glutamate/aspartate, histidine and lysine are part of the catalytic center in triose phosphate isomerase (TIM), xylose isomerase and glucose-6P deaminase, oligomeric by structure, where the catalytic residues are located on the one of the subunits. It has been suggested that GlmS has evolved through a gene-duplication step and the two halves of the structure differentiate improving the binding of substrates or allowing the second binding site to be converted into a regulatory site. It has been assumed that the isomerase domain contains only one active site, but another putative anion binding site has been detected in the structure.[8]

The expression of gene *glmS* is reduced fourfold by the *nag* regulation genes, coding for proteins involved in the metabolism of *N*-acetylglucosamine.[21] The eucaryotic enzymes are allosterically inhibited by UDP-*N*-acetyl-D-glucosamine. The activity of glucosamine-6P deaminase, catalyzing the isomerization-deamination of glucosamine-6 phosphate to form fructose-6 phosphate and ammonia, is regulated by the binding of the allosteric activator *N*-acetylglucosamine-6 phosphate, also corepressor of glucosamine-6P synthase.[22] The functional enzyme is hexameric (266 residues per monomer) and is an genetic regulator of aminosugar metabolism. The allosteric changes of the hexameric molecule are accompanied by distortions to the tertiary structure of each individual monomer.

A biological effect of the product inhibition of glucosamine-6-phosphate synthase on the activity of the enzymes involved in the regulation of all the metabolic glycolytic and glutaminase pathways can be expected. The inhibition of GlmS might have important implications in antibacterial/antifungal therapy,[3] or treatment of diabets as the mammalian enzyme is essential for the insulin-responsive glucose-transport system.[23]

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