Characterization of a Phosphoglucose Isomerase-like Activity Associated with the Carboxy-Terminal Domain of *Escherichia coli* Glucosamine-6-phosphate Synthase

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Glucosamine-6-phosphate (-6P) synthase (GlmS), the key enzyme for hexosamine biosynthesis, catalyzes the conversion of L-glutamine and D-fructose-6P into glutamate and glucosamine-6P. The latter compound is believed to result from isomerization of fructosimine-6P generated by transimination of the Schiff base formed between the substrate and the enzyme by the amide nitrogen of glutamine.[1] While the hydrolysis of this amide bond is characteristic of all glutamine-dependent amidotransferases[2] and the *pro-R* stereospecificity of H_1 proton abstraction[3] is the signature of all 2*R*-keto/aldose isomerases, the mechanism of nitrogen migration has not been elucidated.[4,5] We describe here the characterization in native GlmS of a new glucose-6P synthase activity [phosphoglucose isomerase (PGI)-like activity], which was associated with the isolated carboxy-terminal domain of the protein.

To physically characterize the Schiff base between fructose-6P and lysine-603 by ^{13}C-NMR, [2-^{13}C]fructose-6P was prepared by hexokinase-mediated phosphorylation of commercially available [2-^{13}C]fructose.[6] Incubation of *Escherichia coli* GlmS (0.1 mM) with labeled fructose-6P at room temperature for 15 h in phosphate buffer (pH 7.2) resulted in the almost total disappearance (Figure 1a) of the starting peaks at 105.9 and 102.6 ppm, corresponding to α and *β* anomers, respectively, with the simultaneous appearance of two singlets with resonances at 73.3 and 76.0 ppm (Figure 1b).

Complete conversion occurred when the temperature was increased to 37 °C (data not shown). Proton decoupling revealed a coupling constant of 145 Hz for both signals (Figure 1c), indicative of tertiary carbons, which are consistent with the presence of glucose-6P but not with the presence of glucosamine-6P. When glutamine was added at 20 mM in the incubation mixture, a resonance signal at 76 ppm corresponding to glucosamine-6P was the only signal detectable (data not shown). In the absence of glutamine, the synthesis of glucose-6P was confirmed and quantified by enzymatic analysis using

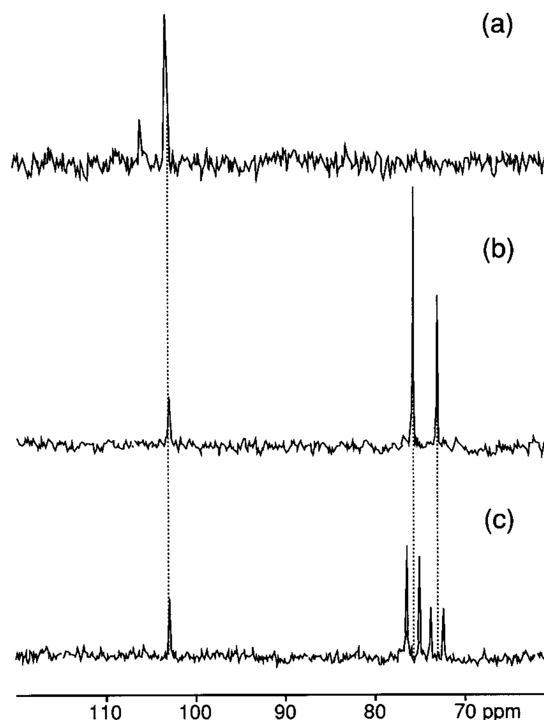

Figure 1. 400 MHz ^{13}C-NMR spectrum of [2-^{13}C]fructose-6P (2 mM) in 50 mM KPO_4 buffer (pH 7.2), 20 °C, alone (a) or after 15 h of incubation (NS = 15 630) with 0.1 mM GlmS with (b) or without (c) decoupling.

glucose-6P dehydrogenase and NADP$^+$.[7] This allowed the determination of the kinetic parameters for this PGI-like activity (K_m = 7.6 mM, k_{cat} = 0.2 min^{-1}).

The isomerization of fructose-6P to glucose-6P is known to reach an equilibrium when catalyzed by PGI under standard conditions (K_{eq} = 0.27).[8] The low efficiency of GlmS to perform the reverse reaction, together with the hydrolysis of fructose-6P upon prolonged incubation at 37 °C, prevented the determination of the amounts of the respective sugars at equilibrium. From the amounts of fructose-6P formed by incubation of GlmS with millimolar concentrations of glucose-6P,[9] the k_{cat} in the reverse reaction was estimated to be 0.082 min^{-1}. Since the position of the equilibrium is not affected by the enzyme (*i.e.*, K_{eq} = 0.27), the Michaelis constant for glucose-6P was calculated from the Haldane equation [$(k_{cat}/K_m)_{Fru\text{-}6P}/(k_{cat}/K_m)_{Glc\text{-}6P} = K_{eq}$], giving $K_{m\ Glc\text{-}6P} \approx 0.9$ mM.

These observations proved, in addition, that glucose-6P formation was not a result of contamination of the GlmS sample by PGI.

The strong commitment of GlmS for glucosamine-6P synthesis (K_m = 0.43 mM, k_{cat} = 931 min^{-1})[10] in the presence of glutamine compared to its PGI-like activity in its absence (ratio k_{cat}/K_m of 8.2×10^4 in favor of the former) likely explains why this activity was not detected before. This side activity was insensitive (data not shown) to millimolar concentrations of 6-diazo-5-oxo-L-norleucine (DON), a highly specific affinity label of the N-terminal cysteine of GlmS in the glutamine

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(6) Phosphorylation was carried out according to Chenault and Mandes (1994). The solution (3 mM product) in 38 mM ammonium bicarbonate (pH 7.5) was adjusted to pH 9 and applied to a column of Dowex (AG 3-X4A Bio-Rad, 200−400 mesh, 2 g, 5.6 mequiv) (bicarbonate) anion exchange resin. The column was rinsed with water and eluted with 220 mM ammonium bicarbonate (pH 7.5) to give [2-^{13}-C]fructose-6P (diammonium salt) in 50% yield based on titration using fructose-6P kinase (1 unit), ATP (1 mM), $MgCl_2$ (1.5 mM), aldolase (1.1 units), triosephosphate isomerase (17 units), α-glycerophosphate dehydrogenase (0.85 unit), and NADH (0.2 mM) in triethanolamine buffer (100 mM, pH 7.6). Chenault, H. K.; Mandes, R. F. *Bioorg. Med. Chem.* **1994**, *2*, 627−629.

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(9) GlmS (5 mg, 0.1 mM) was incubated with glucose-6P (20 and 50 mM) at 37 °C in phosphate buffer (pH 7.2). Titration of fructose-6P within 4 h was performed according to footnote 6. The slope of the linear results from [P] = *f*(*t*) was used to assign V_m.

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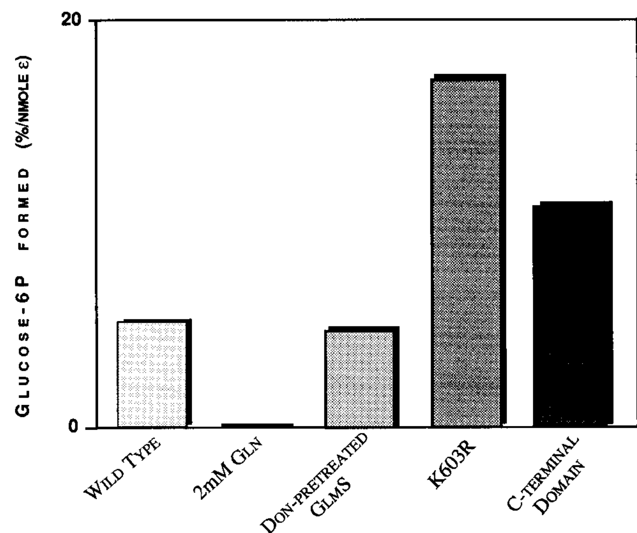
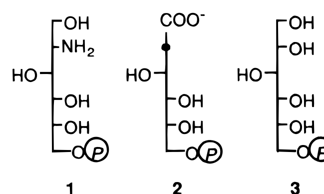


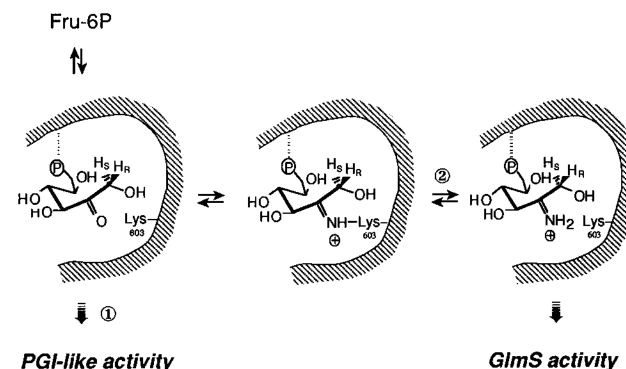
Figure 2. Glucose-6P synthase (PGI-like) activity of native GlmS (alone, in the presence of 2 mM glutamine, pretreated with 2 mM DON, the K603R mutant, and the overexpressed C-terminal domain). The enzyme (5 mg) in 0.5 mL of KPO_4 buffer (pH 7.2) was incubated at 37 °C with 2 mM fructose-6P. Glucose-6P was quantified enzymatically.[7]

binding domain (K_i = 3 μM),[11] suggesting that the PGI-like activity was located, not surprisingly, on the C-terminal fructose-6P binding domain. This was confirmed by the behavior of the overexpressed carboxy-terminal domain (residues 241−608[12]) which, despite the lack of glucosamine-6P synthesizing activity,[13] exhibited a 2.5-fold higher efficiency in synthesizing glucose-6P (K_m = 9.6 mM, k_{cat} = 0.48 min^{-1}) than the native enzyme itself. Moreover, the K603R mutation in native GlmS, which produces a 40-fold decrease in the normal enzyme activity, resulted in a 3-fold increase in the PGI-like reaction (Figure 2). The distinct substrate binding features apparently required for the two activities are emphasized by the behavior of the inhibitors depicted in Chart 1. The PGI-like activity was unaffected by 2-amino-2-deoxyglucitol-6P (**1**), a potent inhibitor of glucosamine synthesizing activity (K_i = 25 μM, K_m/K_i = 17),[4] whereas glucosamine-6P synthesis was insensitive to 2-deoxy-6-phosphogluconate (**2**), which is a good inhibitor of

Chart 1



Scheme 1



glucose synthesizing activity (K_i = 700 μM, K_m/K_i = 11). Glucitol-6P (**3**), another known inhibitor of phosphoglucose isomerase,[14] exhibited a similar K_i (2.4 mM) for both activities but was shown from the ratios of K_m/K_i to be 17 times more efficient at inhibiting the PGI-like activity.

A possible mechanism accounting for the two activities of GlmS is shown in Scheme 1. The enzyme may bind to the open form of fructose-6P, in possible equilibrium with the lysine-603 Schiff base. The absence of the glutamine-driven reaction to form fructosimine-6P along pathway 2 would then favor the PGI-like activity along pathway 1.

Although such a mechanism explains the conservation of PGI-like activity in the K603R mutant as well as the specific inhibition of pathway 1 by compounds **2** and **3** and of pathway 2 by compound **1**, detailed knowledge of the residues involved in each reaction will require additional investigations.

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