Inhibition of *Escherichia coli* Glucosamine-6-phosphate Synthase by Reactive Intermediate Analogues

**THE ROLE OF THE 2-AMINO FUNCTION IN CATALYSIS**

(Received for publication, August 24, 1999, and in revised form, October 14, 1999)

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Glucosamine-6-phosphate synthase (GlmS) catalyzes the formation of d-glucosamine 6-phosphate from d-fructose 6-phosphate using l-glutamine as the ammonia source. Because N-acetylglucosamine is an essential building block of both bacterial cell walls and fungal cell wall chitin, the enzyme is a potential target for antibacterial and antifungal agents. The most potent carbohydrate-based inhibitor of GlmS reported to date is 2-amino-2-deoxy-d-glucitol 6-phosphate, an analogue of the putative cis-enolamine intermediate formed during catalysis. The interaction of a series of structurally related cis-enolamine intermediate analogues with GlmS is described. Although arabinose oxime 5-phosphate is identified as a good competitive inhibitor of GlmS with an inhibition constant equal to 1.2 (±0.3) mM, the presence of the amino function at the 2-position is shown to be important for potent inhibition. Comparison of the binding affinities of 2-deoxy-o-glucitol 6-phosphate and 2-amino-2-deoxy-o-glucitol 6-phosphate indicates that the amino function contributes −4.1 (±0.1) kcal/mol to the free energy of inhibitor binding. Similarly, comparison of the binding affinities of 2-deoxy-o-glucose 6-phosphate and o-glucosamine 6-phosphate indicates that the amino function contributes −3.0 (±0.1) kcal/mol to the free energy of product binding. Interactions between GlmS and the 2-amino function of its ligands contribute to the uniform binding of the product and the cis-enolamine intermediate as evidenced by the similar contribution of the amino group to the free energy of binding of d-glucosamine 6-phosphate and 2-amino-2-deoxy-o-glucitol 6-phosphate, respectively.

Glucosamine-6-phosphate synthase (1-glutamine: d-fructose-6-phosphate aminotransferase, EC 2.6.1.16) catalyzes the first step in hexosamine biosynthesis, converting d-fructose 6-phosphate (Fru-6-P) into d-glucosamine 6-phosphate (GlcN-6-P) using glutamine as the ammonia source (Scheme 1) (1–3). GlcN-6-P is a precursor of uridine diphospho-N-acetylglicosamine from which other amino sugar-containing molecules are derived. One of these products, N-acetylglicosamine, is an important constituent of the peptidoglycan layer of bacterial cell walls and fungal cell wall chitin. Accordingly, GlmS offers a potential target for antibacterial and antifungal agents and has attracted the interest of several research groups (2).

GlmS catalyzes two coupled enzymatic reactions. The first is the hydrolysis of glutamine to yield glutamate and nascent ammonia, which is transferred to Fru-6-P. The second reaction is the isomerization of Fru-6-P from a ketose to an aldose, corresponding to a Heyns rearrangement (4, 5). Like other amidotransferases, GlmS is organized into two domains: the NH$_2$-terminal glutamine amidotransferase domain, which catalyzes the hydrolysis of glutamine, and the COOH-terminal synthase domain, which catalyzes the isomerization (3, 6–8). The glutamine hydrolysis reaction has been studied extensively and utilizes the NH$_2$-terminal cysteine thiol, which forms a γ-glutamyl thioester intermediate during the reaction. This catalytic role was confirmed by conversion of the NH$_2$-terminal cysteine to alanine using site-directed mutagenesis which abolished enzymatic activity (2). In general, glutamine amidotransferases are inactivated by glutamine affinity analogues such as 6-diaz-o-5-oxo-l-norleucine and 6-chloro-5-oxo-l-norleucine (chloroketone), which alkylate the essential cysteine residue (3, 7, 9). Indeed, many of the active site-directed irreversible inactivators developed for GlmS contain an electrophilic function at the γ-position of glutamate and react irreversibly with the NH$_2$-terminal cysteine residue. More recently, attempts to develop carbohydrate-based inhibitors have been made with the hope of developing more specificity (10–13).

The ketose/aldose isomerase activity of the enzyme proceeds by abstraction of the C1 pro-R hydrogen of a putative fructose-imine 6-phosphate intermediate to form a cis-enolamine reactive intermediate that, upon reprotonation at the Re face of C2, gives rise to GlcN-6-P (Scheme 2) (5). In accord with this mechanism, Badet and co-workers (14) have shown that GlmS, in the absence of glutamine, displays a low phosphoglucosomerase activity. Analogues of the cis-enolamine reactive intermediate are expected to be potent inhibitors of the enzyme (15–18) and indeed, 2-amino-2-deoxy-o-glucitol 6-phosphate (GlcNol-6-P) is the most potent carbohydrate-based inhibitor reported to date (11, 12).

Identification of those structural elements necessary for tight binding is an important part of inhibitor design. This paper describes the inhibition of GlmS by several analogues of the cis-enolamine intermediate in an attempt to probe the structural requirements for potent inhibition of this enzyme. The energetic contribution of the 2-amino group to binding of the product and the cis-enolamine intermediate is determined.

**MATERIALS AND METHODS**

d-Arabinose, d-arabinose 5-phosphate, 2-amino-2-deoxy-o-glucitol 6-phosphate (dGlc-6-P), 2-deoxy-o-glucose 6-phosphate (dGlc-6-P), 2-Fru-6-P, and d-ribose 5-phosphate were purchased...
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2-Deoxy-D-glucitol 6-Phosphate—2-Deoxy-D-glucitol 6-phosphate (dGalcol-6-P) was synthesized by reduction of 2-deoxy-D-glucose 6-phosphate (dGlc-6-P) with sodium borohydride and purified by ion-exchange chromatography following procedures similar to those outlined for the preparation of GlcNol-6-P (12). dGlc-6-P (250 mg, 0.868 mmol) was dissolved in 10 ml of water and cooled on ice for 10 min. Sodium borohydride (0.750 g, 19.83 mmol) was added to the dGlc-6-P solution by small portions over 20 min. During the NaBH₄ addition, the solution was stirred vigorously and held on ice. After the addition was complete, the solution was stirred for 1 h at room temperature. Reduction was complete as indicated by the inability of the solution to reduce Fehling’s reagent. Undissolved NaBH₄ was removed by filtration, and the filtrate was cooled on ice. The remaining NaBH₄ was destroyed by dropwise addition of 6 M acetic acid over 30 min until the final pH was approximately 4. The solution (25 ml) was allowed to come to room temperature and stirred for 1 h. This solution was then filtered, and the filtrate was applied to a Dowex 50 (H⁺ form) column (1.5 x 47 cm) and eluted with water. Fractions containing product were identified by thin layer chromatography of the eluted material and were collected. The combined fractions were pooled and lyophilized yielding 69 mg dGalcol-6-P, a yield of 8.0% of theoretical. The degradation in yield was attributed to the reaction of sodium borohydride with the terminal hydroxyl to form sodium borate. 

Elemental analyses were performed by Canadian Microanalytical Service Ltd., B.C.

2-Deoxy-D-glucitol 6-Phosphate

**Scheme 1.**

![Scheme 1](https://example.com/scheme1.png)

**Scheme 2.**

![Scheme 2](https://example.com/scheme2.png)

from Sigma Chemical Company. All other chemicals were analytical grade and used without further purification. NMR spectra (13C, 31P) were obtained using a Bruker AC 250F spectrometer. Chemical shifts (δ) for 13C and 31P spectra are reported relative to the deuterium lock signal and external H₃PO₄ (85% w/v in D₂O), respectively. Elemental analyses were performed by Canadian Microanalytical Service Ltd., B.C.

Enzyme Purification and Assays—GlmS was purified as described previously (12, 24). Inorganic and organic phosphate assays were conducted according to the procedures described by Leloir and Cardini (25).

Inhibition Studies—Assays were conducted in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The concentration of inhibitors used in the assays was as follows: D-arabinose 5-phosphate, 4.6, 9.1, and 13.7 mM; D-arabinose oxime 5-phosphate, 3.1, 6.2, and 9.4 mM; D-ribose 5-phosphate, 9.1, 18.2, and 27.3 mM; dGlc-6-P, 25.0 and 50.0 mM; and dGlc-6-p, 57.2 and 171.7 mM. In addition, the assays contained GlmS (6.7 x 10⁻³ unit/ml), l-glutamine (15 mM), and D-Fru-6-P at concentrations equal to 0.45, 0.89, 1.79, 4.47, and 8.94 mM. GlmS was found to be relatively insensitive to changes in ionic strength, and therefore no attempt was made to correct for changes in ionic strength. Complete Michaelis-Menten plots were constructed at all inhibitor concentrations using the concentrations of D-Fru-6-P given above. Kinetic data were analyzed by nonlinear regression analysis of the Michaelis-Menten plots using the program EnzymeKinetics (1990) from Trinity Software. The inhibition constants were determined in triplicate, and the average value is reported. The reported error is the S.D.

**RESULTS AND DISCUSSION**

Much attention has been focused on utilizing glutamine analogues to inhibit GlmS activity with the goal of developing antibacterial and antifungal agents. Typically, these analogues possess an electrophilic function at the γ-position of glutamate, which is believed to react irreversibly with the NH₂-terminal cysteine residue located in the glutamine amidotransferase domain. The most effective inactivators have been N³-fumaryl-L-2,3-diaminopropanoate derivatives (26–29) and 2-amino-
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Inhibition by Reactive Intermediate Analogues—The structures of several analogues of the putative cis-enolamine intermediate and their corresponding inhibition constants (Ki) are shown in Table I. In all cases, the analogues were found to be competitive inhibitors of GlmS activity with respect to Fru-6-P. The values of the inhibition constants should be accurate estimates of the dissociation constants for these inhibitors since the Ki value for GlcNol-6-P (19 μM) was shown to be very similar to the dissociation constant of 34 (±7) μM determined for this ligand using protection experiments (12). The carbonyl functions present in the open chain forms of arabinose 5-phosphate and ribose 5-phosphate mimic the double bond present in the cis-enolamine intermediate. That the enzyme displays a low affinity for both of these compounds relative to GlcNol-6-P indicates that the 2-amino and 1-hydroxyl functions are important for tight binding of GlcNol-6-P. Changing the stereochemistry at C2 causes approximately a 4-fold reduction in the binding of ribose 5-phosphate relative to arabinose 5-phosphate. This observation is not unexpected because enzymes that isomerize carbohydrate substrates generally show such stereochemical discrimination (41).

Table I shows both arabinose 5-phosphate and ribose 5-phosphate in their acyclic free carbonyl forms so that their structural similarity to the cis-enolamine intermediate is evident.

### Table I

<table>
<thead>
<tr>
<th>Intermediate Analogue</th>
<th>Ki (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,3-tris(hydroxymethyl)amino)propanoate derivatives</td>
<td>2-(N-halomethyl)amino)propanoate derivatives</td>
</tr>
<tr>
<td>3-phosphoglycerate</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>2,3-diphosphoglycerate</td>
<td>2,3-diphosphoglycerate</td>
</tr>
<tr>
<td>Phosphoenolpyruvate</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>Oxaloacetate</td>
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<tr>
<td>Aspartate</td>
<td>Aspartate</td>
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<tr>
<td>Glutamate</td>
<td>Glutamate</td>
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<tr>
<td>Lactate</td>
<td>Lactate</td>
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<tr>
<td>Malate</td>
<td>Malate</td>
</tr>
<tr>
<td>Citrate</td>
<td>Citrate</td>
</tr>
<tr>
<td>Succinate</td>
<td>Succinate</td>
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</tbody>
</table>

### Notes

* Inhibition constant from Bearne (12). This value is similar to the value of the inhibitor dissociation constant (Ki = 25 μM) determined independently by Badet-Deniset et al. (11).
* Arabinose 5-phosphate and ribose 5-phosphate are represented as the straight chain forms to highlight their structural similarity to the cis-enolamine intermediate. The Ki values shown are the apparent Ki values for the combination of all carbohydrate species present in solution (cyclic, acyclic, free aldehyde, and hydrate forms) as outlined under “Results and Discussion.”
* The syn (E) isomer is shown to highlight its structural similarity to the cis-enolamine intermediate; however, the Ki value shown is for the equilibrium mixture of anti (Z) (15%) and syn (E) (85%) isomers.
* Inhibition constant at saturating concentration of glutamine from Badet et al. (27).
However, in neutral aqueous solution, the acyclic forms of arabinose 5-phosphate and ribose 5-phosphate only comprise approximately 2.4 and 0.6% of the total concentration of species present, respectively (42). Under similar conditions, Fru-6-P exists in the β-furanose (81.1%), α-furanose (16.1%), and free carbonyl (2.2%) forms (42). Whether the cyclic or acyclic form of Fru-6-P is the actual substrate for GlmS is not known. Badet-Denisot et al. (2) have argued that there is no need for GlmS to catalyze ring opening because the spontaneous rate of ring opening of Fru-6-P (18–21 s⁻¹; Ref. 42) is similar to the catalytic rate (19–23 s⁻¹). However, other phosphosugar isomerases that proceed via an enolization mechanism such as glucose-6-phosphate isomerase (43–45), mannose-6-phosphate isomerase (46), and GlcN-6-P deaminase (47), are believed to catalyze ring opening of the cyclic carbohydrate substrate to form the corresponding straight chain species prior to deprotonation. Recently, the crystal structures of complexes of the GlmS Fru-6-P binding domain (8) with different ligands have been reported, including Glu-6-P (48), GlcN-6-P (48), and GlcNol-6-P (49). Based on these crystal structures, Teplyakov and co-workers (48, 49) have proposed that GlmS uses His-504 as a general base to catalyze ring opening of cyclic Fru-6-P. Thus it is possible that GlmS may catalyze the ring opening of arabinose 5-phosphate and ribose 5-phosphate. Arabinose 5-phosphate exists in the α-furanose (57.3%), β-furanose (40.4%), hydrate (2.2%), and free carbonyl (≤0.2%) forms in neutral aqueous solution (40). If the free aldehydes were the actual inhibitory species, adjustment of the observed inhibition constant to reflect the concentration of free carbonyl present in solution would yield an upper limit for the $K_i$ value equal to 17 μM. However, there seems to be no convincing reason to adjust the observed inhibition constant in this manner.

Arabinose oxime 5-phosphate is an analogue of the cis-enolamine that contains the double bond and the hydroxyl function but lacks the 2-amino function. GlmS binds this analogue with an apparent inhibition constant equal to 1.2 mM, approximately 7-fold less than the $K_i$ value observed for arabinose 5-phosphate. It is important to note that the inhibition mixture tested was an equilibrium mixture containing 15% of the anti (Z) form and 85% of the syn (E) form, the latter being analogous to the cis-enolamine intermediate. Adjusting the apparent $K_i$ value to reflect the concentration of the E form present in solution gives a $K_i$ value equal to 1.0 mM. The affinity that GlmS displays for the oxime is still 53-fold less than the affinity with which GlmS binds GlcNol-6-P. Is this lack of binding affinity principally due to the missing amino function? Replacement of the hydrogen on C2 of the oxime with an amino group would yield a compound that differs from the cis-enolamine intermediate by only the substitution of a nitrogen at C2. Because the amino function contributes approximately 4.1 kcal/mol to the binding affinity (see below), the expected $K_i$ value for such a compound would be approximately 1.3 μM. This value is approximately 15-fold less than the $K_i$ value for GlcNol-6-P and might serve as an estimate of the upper limit for the enzyme’s affinity for the actual cis-enolamine intermediate.

Corizzi et al. (10) have reported that the non-isosteric phosphonate analogue of Fru-6-P is a poor competitive inhibitor of GlmS with respect to Fru-6-P ($K_i = 2.5$ mM). However, the oxime of this compound was reported to have a much higher affinity for the enzyme with a $K_i$ value equal to 0.2 mM (10), which is slightly less than the inhibition constant observed for arabinose oxime 5-phosphate in the present work. This is unexpected because, unlike the arabinose derived oxime, the oxime of the phosphonate is not isosteric with the putative cis-enolamine intermediate. One explanation for this difference in binding affinities may be that the oxime of the phosphonate exists more predominately in the Z form. Unfortunately, the relative amounts of E and Z forms were not reported for the oxime of the phosphonate. Despite the difference in binding affinities, the inhibitory nature of the oximes reflects their structural similarity to the proposed cis-enolamine intermediate.

**Contribution of the Amino Group to Ligand Binding**—The interactions between an enzyme and a ligand always involve a substantial number of groups. The general approach to understanding the observed affinity has been to dissect it into the contributions of each group by measuring the change in affinity which results when one of the groups of interest is removed (50–52). This type of analysis may be conducted by removing a group from either the enzyme using site-directed mutagenesis or from the ligand (51, 53). When the latter approach is used, the $k_{on}/K_m$ values observed with the natural substrate and a modified substrate may be compared by calculating the effect of the modification on the free energy of the transition state relative to the ground state. Alternatively, transition state analogue inhibitors may be modified and the subsequent changes in binding affinity interpreted as the contribution of the removed moiety to transition state binding (54). This latter approach is used in the present work to assess the contribution that the 2-amino function makes to the binding of the product (GlcN-6-P) and the reactive intermediate analogue (GlcNol-6-P). 2-Deoxy-D-glucose 6-phosphate, which differs from GlcN-6-P by the absence of an amino function at the 2-position, is only weakly bound by the enzyme ($K_i = 46 ± 7$ mM). This corresponds to a free energy of binding ($\Delta G^{GlcN-6-P}_{\text{free}}$) equal to $-1.90 ± 0.09$ kcal/mol compared with a value of $-4.85$ kcal/mol observed for the binding free energy of GlcN-6-P ($\Delta G^{GlcN-6-P}_{\text{free}}$) (27). Thus the 2-amino function contributes $-3.0 ± 0.1$ kcal/mol ($\Delta G = \Delta G^{GlcN-6-P}_{\text{free}} - \Delta G^{GlcNol-6-P}_{\text{free}}$) to the free energy of product binding. Similarly, dGlcol-6-P, which differs from GlcNol-6-P by the absence of an amino function at the 2-position, is also only bound weakly by the enzyme ($K_i = 15 ± 2$ mM). This corresponds to a free energy of binding ($\Delta G^{GlcNol-6-P}_{\text{free}}$) equal to $-2.59 ± 0.08$ kcal/mol compared with a value of $-6.70 ± 0.02$ kcal/mol observed for the binding free energy of GlcNol-6-P ($\Delta G^{GlcNol-6-P}_{\text{free}}$) (12). Thus the 2-amino function contributes $-4.1 ± 0.1$ kcal/mol ($\Delta G = \Delta G^{GlcNol-6-P}_{\text{free}} - \Delta G^{GlcN-6-P}_{\text{free}}$) to the free energy of reactive intermediate analogue binding. These values are similar to the binding free energies reported for amino functions participating in other protein-ligand interactions. For example, $\Delta G$ values of $-3.4$ kcal/mol and $-6.7$ kcal/mol have been reported for the binding contribution of the amino function on ligands interacting with the enzymes phenylalanyl-tRNA synthetase (55, 56) and isoleucyl-tRNA synthetase (57), respectively. For the aminoglycoside 3′-phospho-transferases, types Ia and Iia, values as large as $-6$ to $-11$ kcal/mol have been reported for the energetic contribution of the amino function to the stabilization of transition state species ($\Delta G = -RTln(K^-1/K^+$)) (58).

Thus, the amino function at the 2-position contributes approximately the same amount of binding energy to the binding of the product, GlcN-6-P, as it does to the binding of the reactive intermediate analogue, GlcNol-6-P. The amino function, therefore, contributes to the uniform binding (59, 60) of both the product and the cis-enolamine intermediate. Selective sta-

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2 The free energy of binding ($\Delta G$) is calculated using the equation $\Delta G = -RT\ln K$, where $T = 310.2$ K and $R$ is the gas constant. The contribution of the amino function to binding ($\Delta G_a$) is calculated using the equation $\Delta G_a = -RT\ln(K_{a}^{enolamine}/K_{a}^{amino})$, where $K_{a}^{enolamine}$ and $K_{a}^{amino}$ represent the competitive inhibition constants for ligands lacking and containing the 2-amino function, respectively.
hydroxyl groups are displayed and labeled. The carbonyls, and water molecules relevant to binding of the amino and C1 structures 1MOQ and 1MOS (48, 49). Residue side chains, backbone carbonyls, and water molecules relevant to binding of the amino and C1 hydroxyl groups are displayed and labeled. The dotted lines indicate potential hydrogen bonds as proposed in Refs. 48 and 49.

FIG. 1. Binding of GlcN-6-P (panel A) and GlcNol-6-P (panel B) to the active site of GlmS. Stereograms of the respective enzyme bound ligands are based on the coordinates of the crystallographic structures 1MOQ and 1MOS (48, 49). Removal of the amino function from the cis-enolamine intermediate relative to the ground state must therefore be caused by another binding determinant, likely the hydroxyl function at C1. Protein Interaction with the 2-Amino Group—The structures of enzymes complexed to substrates, products, and transition state or reactive intermediate analogues are often useful in delineating the role of enzyme-ligand interactions in catalysis (61). The crystal structures of complexes of the Fru-6-P binding site or reactive intermediate analogues are often useful in delineating the role of enzyme-ligand interactions in catalysis (61). The crystal structures of complexes of the Fru-6-P binding site or reactive intermediate analogues are often useful in delineating the role of enzyme-ligand interactions in catalysis (61).

This interaction suggests that the glutamine amidotransferase domain interacting affinity for the amino function remains roughly unchanged.

The interactions between GlmS and the 2-amino function of its ligands are responsible for the uniform binding of the product and the cis-enolamine intermediate as evidenced by the similar contribution of the amino group to the free energy of binding of GlcN-6-P and GlcNol-6-P, respectively. The amino function contributes significantly to the free energy of binding both the product and the reactive intermediate analogue, indicating that the 2-amino function is an important moiety to be included in the design of carbohydrate-based GlmS inhibitors.

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doi: 10.1074/jbc.275.1.135

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