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

THE ROLE OF THE 2-AMINO FUNCTION IN CATALYSIS*

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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-d-glucitol 6-phosphate and 2-amino-2-deoxy-d-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-d-glucose 6-phosphate and d-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-d-glucitol 6-phosphate, respectively.

Glucosamine-6-phosphate synthase (L-glutamine: D-fructose-6-phosphate aminotransferase (GlmS,1 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-acetylglucosamine from which other amino sugar-containing molecules are derived. One of these products, N-acetylglucosamine, 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 aminotransferases, GlmS is organized into two domains: the NH2-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 NH2-terminal cysteine thiol, which forms a γ-glutamyl thioester intermediate during the reaction. This catalytic role was confirmed by conversion of the NH2-terminal cysteine to alanine using site-directed mutagenesis which abolished enzymatic activity (2). In general, glutamine aminotransferases are inactivated by glutamine affinity analogues such as 6-diazo-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 NH2-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 phosphoglucomoserase 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-d-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-d-glucose 6-phosphate (d-glucoamine 6-phosphate), 2-deoxy-d-glucose 6-phosphate (dGluc-6-P), d-Fru-6-P, and d-ribose 5-phosphate were purchased

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1 The abbreviations used are: GlmS, glucosamine-6-phosphate synthase; Fru-6-P, d-fructose 6-phosphate; GlcN-6-P, d-glucosamine 6-phosphate; GlcNol-6-P, 2-amino-2-deoxy-d-glucitol 6-phosphate; dGlc-6-P, 2-deoxy-d-glucitol 6-phosphate; dGlc-6-P, 2-deoxy-d-glucose 6-phosphate.
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.

2-Deoxy-d-glucitol 6-Phosphate—2-Deoxy-d-glucitol 6-phosphate (dGlc-6-P) was synthesized by reduction of 2-deoxy-D-glucose 6-phosphate (101.26 MHz, D2O) (dGlc-6-P) was synthesized by reduction of 2-deoxy-D-glucose 6-phosphate, followed 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 on cellulose (99% EtOH, water. Fractions containing product were identified by thin layer chromatography on cellulose (99% EtOH, water).

2-Deoxy-d-glucitol 6-phosphate (dGlc-6-P) was synthesized by reduction of 2-deoxy-D-glucose 6-phosphate, followed 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 on cellulose (99% EtOH, water. Fractions containing product were identified by thin layer chromatography on cellulose (99% EtOH, water.)

Fractions containing dGlcol-6-P were combined and lyophilized yielding a disodium salt, formulated as C2H12O8N2P2. The disodium salt was converted to the less hygroscopic monocy-clohexylammonium salt using Dowex 50 (cyclohexylammonium form). Elemental analysis was as follows.

C6H11O2N2P (345.38)
Calculated: C 41.73% H 8.12% N 4.06% P 8.97%
Found: C 40.63% H 8.12% N 3.79% P 8.96%

**ANALYSIS 1**

**d-Arabinose Oxime 5-Phosphate—d-Arabinose oxime was prepared by treating d-arabinose with hydroxylamine as described by Finch and Merchant (20, 21) and Hockett et al. (22, 23). The oxime was then converted to its corresponding 5-phosphate by hexokinase-catalyzed phosphorylation as described by Finch and Merchant (20). The proton-coupled 31P NMR spectrum of the enzymatically synthesized and isolated d-arabinose oxime 5-phosphate showed broad triplets centered at 5.74 ppm (δH = 7.3 Hz) and 4.90 ppm (δH = 4.4 Hz). These signals were assigned to the phosphorus nuclei coupled to H-5a,5b in the E and Z oxime phosphate, respectively, similar to the 31P NMR assignments reported by Finch and Merchant (20). There were also five very minor peaks (≤ 7%) present at 3.58, −4.74, −9.29, −9.07, and −9.29 possibly due to contaminating phosphoenolpyruvate or nucleotides. The actual concentration of d-arabinose oxime 5-phosphate used in the inhibition studies was determined by integration of the 1H NMR spectrum. The well resolved doublets at 7.59 ppm and 6.95 ppm arising from H-1 of the E and Z oxime phosphate, respectively, was found to be relatively insensitive to changes in ionic strength.

The ratio of GlmS (6.7, 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 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 on cellulose (99% EtOH, water. Fractions containing product were identified by thin layer chromatography on cellulose (99% EtOH, water.)

**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 were 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 ω-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 N2-fumaryl-yl-2,3-diaminopropanoate derivatives (26–29) and 2-amino-
Glucosamine-6-phosphate Synthase Inhibition

**TABLE I**

| Intermediate Analogue | $K_i$ (M) |
|------------------------|----------|
| 2-amino-2-deoxy-D-glucitol 6-phosphate | $1.90 \times 10^{-3}$ |
| D-arabinose 5-phosphate | $3.22 \times 10^{-3}$ |
| D-ribose 5-phosphate | $1.2 \times 10^{-3}$ |
| 2-deoxy-D-glucose 6-phosphate | $2.4 \times 10^{-4}$ |
| D-glucosamine 6-phosphate | $3.8 \times 10^{-4}$ |

* Inhibition constant from Bearne (12). This value is similar to the value of the inhibitor dissociation constant ($K_i = 25 \mu M$) determined independently by Badet-Denis et al. (11).

**Inhibition by Reactive Intermediate Analogues**—The structures of several analogues of the putative cis-enolamine intermediate and their corresponding inhibition constants ($K_i$) 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 $K_i$ value for GlcNol-6-P (9 $\mu M$) was shown to be very similar to the dissociation constant of 34 (±7) $\mu M$ 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. 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 discrimination.

The values shown are the apparent $K_i$ values for the combination of all carbohydrate species present in solution (cyclc, acyclic, free aldehyde, and hydrate forms) as outlined under “Results and Discussion.”

3-((N-halomethyl)amino)propanoate derivatives (30, 31). Although the enzyme is believed to follow an ordered Bi Bi kinetic mechanism (27), glutamine-like inactivators bind and inactivate GlmS effectively even in the absence of the first substrate Fru-6-P (26, 27).

Recently, there has been an effort to identify carbohydrate-based inhibitors specific for GlmS (10–13). One approach to developing potent enzyme inhibitors is to design compounds that are analogues of either the transition states or reactive intermediates that are formed during catalysis (15–18). The mechanisms of phosphorylated sugar isomerases that catalyze the interconversion of an aldose and a ketose have been shown to involve proton abstraction from either substrate to give an enzyme-bound cis-enediol intermediate (32, 33). Virtually all of these enzymes show activity in the absence of metal ions and have exchange (to solvent) to intramolecular hydrogen transfer ratios greater than 1 (33, 34). As expected, analogues of the cis-enediol are potent inhibitors of such isomerases (18). For example, triose-phosphate isomerase is inhibited by 2-phosphoglycerate and 2-phosphoglycerolhydroxamate (35, 36), glucose-6-phosphate isomerase is inhibited by 5-phosphoarabinonate (37), and both arabinose-5-phosphate isomerase and ribose-5-phosphate epimerase are inhibited by 4-phosphoethonate (38, 39). To the extent that the transition state for the rate-determining step resembles an enediol, these analogues may be considered transition state analogues. A similar mechanism has been described for both glucosamine-6-phosphate deaminase (40) and GlmS (5) where proton abstraction from the substrate yields an enzyme-bound cis-enolamine intermediate (Scheme 2). In accord with this mechanism, both the deaminase (40) and the synthase (5) have exchange (to solvent) to intramolecular hydrogen transfer ratios greater than 1, similar to the phosphosugar isomerases discussed above. In addition, GlcNol-6-P, an analogue of the cis-enolamine intermediate, is a potent inhibitor of both enzymes (11, 12, 40). In fact, GlcNol-6-P is the tightest binding reversible carbohydrate-based inhibitor reported for GlmS. The present work describes the inhibition of *Escherichia coli* GlmS by several analogues of the cis-enolamine intermediate and the energetic contribution that the 2-amino function makes to ligand binding.

Inhibition of GlmS by analogues of the putative cis-enolamine intermediate. The $K_i$ values shown are the apparent $K_i$ values for the combination of all carbohydrate species present in solution (cyclic, acyclic, free aldehyde, and hydrate forms) as outlined under “Results and Discussion.”
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 aldehyde 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 C1. 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 $k_{cat} / K_m$ 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-α-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 ($\Delta G_{\text{binding}}$) 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 ($\Delta G_{\text{binding}}$) (27). Thus the 2-amino function contributes $-3.0 ± 0.1$ kcal/mol ($\Delta G = \Delta G_{\text{GlcN-6-P}} - \Delta G_{\text{GlcNol-6-P}}$) 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 ($\Delta G_{\text{GlcNol-6-P}}$) 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 ($\Delta G_{\text{GlcNol-6-P}}$) (12). Thus the 2-amino function contributes $-4.1 ± 0.1$ kcal/mol ($\Delta G = \Delta G_{\text{GlcNol-6-P}} - \Delta G_{\text{GlcNol-6-P}}$) 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 isoelucyl-tRNA synthetase (57), respectively. For the aminoglycoside 3’-phosphotransferases, 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 ($\Delta G = -RT \ln (k_{cat} (K_m)^{\text{NH}_2} / (k_{cat} (K_m)^{\text{NH}_3}))$) (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-
hydroxyl groups are displayed and labeled. The 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.

Thus, 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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