Structural Basis for Morpheein-type Allosteric Regulation of Escherichia coli Glucosamine-6-phosphate Synthase

EQUILIBRIUM BETWEEN INACTIVE HEXAMER AND ACTIVE DIMER

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The amino-terminal cysteine of glucosamine-6-phosphate synthase (GlmS) acts as a nucleophile to release and transfer ammonia from glutamine to fructose 6-phosphate through a channel. The crystal structure of the C1A mutant of Escherichia coli GlmS, solved at 2.5 Å resolution, is organized as a hexamer, with glutaminase domains adopting an inactive conformation. Although the wild-type enzyme is active as a dimer, size exclusion chromatography, dynamic and quasi-elastic light scattering, native polyacrylamide gel electrophoresis, and ultra-centrifugation data show that the dimer is in equilibrium with a hexameric state, in vitro and in cellulo. The previously determined structures of the wild-type enzyme, alone or in complex with glucosamine 6-phosphate, are also consistent with a hexameric assembly that is catalytically inactive because the ammonia channel is not formed. The shift of the equilibrium toward the hexameric form in the presence of cyclic glucosamine 6-phosphate, together with the decrease of the specific activity with increasing enzyme concentration, strongly supports product inhibition through hexamer stabilization. Altogether, our data allow us to propose a morpheein model, in which the active dimer can rearrange into a transiently stable form, which has the propensity to form an inactive hexamer. This would account for a physiologically relevant allosteric regulation of E. coli GlmS. Finally, in addition to cyclic glucose 6-phosphate bound at the active site, the hexameric organization of E. coli GlmS enables the binding of another linear sugar molecule. Targeting this sugar-binding site to stabilize the inactive hexameric state is therefore suggested for the development of specific antibacterial inhibitors.

Results: The dimer is in equilibrium with a hexameric state that is catalytically inactive in vitro. The glucosamine 6-phosphate product completely shifts the equilibrium toward the hexamer.

Conclusion: This accounts for a morpheein-type allosteric regulation of E. coli GlmS activity.

Significance: The inactive hexamer is a target for developing specific antibacterial agents.

Glucosamine-6-phosphate synthase (GlmS) (EC 2.6.1.16) catalyzes the first and rate-limiting step of hexosamine metabolism, the conversion of fructose 6-phosphate (Fru6P) into d-glucosamine 6-phosphate (GlcN6P) in the presence of glutamine (Gln) (1). Because the end product of the pathway, UDP-N-acetylglucosamine (UDP-GlcNAc), is an important building block of bacterial and fungal cell walls, GlmS is a potential target for antibacterial and antifungal drugs (2, 3). Escherichia coli GlmS is composed of two structurally and functionally distinct globular domains connected by a linker (supplemental Fig. S1). The glutaminase domain (residues 1–239) generates ammonia from glutamine, which is transferred through an 18-Å-long channel to the synthase domain (residues 249–608), where Fru6P is aminated and isomerized to GlcN6P or simply isomerized to d-glucose 6-phosphate (Glc6P).

The crystal structures of the isolated domains have revealed the overall architecture of the domains and identified the catalytically important residues (4–6). The synthase domain is organized as a dimer (supplemental Fig. S1A). One synthase monomer consists of two topologically identical subdomains with a flavodoxin fold, suggesting that this domain has evolved through gene duplication (5, 7). The glutaminase domain fold is characteristic of the amino-terminal nucleophile hydrolase superfamily (4). Accordingly, the amino-terminal cysteine residue has a crucial role in catalysis, as demonstrated by results of chemical modification by specific covalent inhibitors (8, 9) and the complete lack of activity of the C1A-GlmS mutant (10, 11).

* This work was supported by CNRS.
** This article contains supplemental Tables S1–S4 and Figs. S1–S8.

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Thus, the first catalytic step at the glutaminase site involves the nucleophilic attack of the thiol group of Cys1 on the carboxamide group of glutamine to release ammonia and yield a γ-glutamyl-thioester intermediate.

The structures of full-length *E. coli* GlmS alone, in complex with Fru6P, with Glc6P and 6-diazo-5-oxo-l-norleucine (DON, a glutamine analog), or with GlcN6P have previously been determined (12–15). The comparison of these structures (16, 17) together with molecular dynamics simulations (18, 19) has shed light on the conformational changes required for sugar isomerization as well as for the formation and opening of the ammonia channel. In the absence of ligands, the ammonia channel is not shaped. Fru6P binding leads to the formation of the channel, which remains closed until glutamine binds. Two particular loops in the synthase domain are crucial for catalysis: the C-loop (residues 600–608) and the His-loop from the synthase domain of the second monomer (residues 503*–505*); the former closes the synthase site once Fru6P is bound and is a major building block of the channel, whereas in the latter, Lys-503* is another constituent of the channel, and His-504* is responsible for sugar isomerization (supplemental Fig. S1). In the glutaminase domain, the Q-loop (residues 73–81) closes the glutaminase site upon glutamine binding and bears Trp-74, which acts as the gate of the channel (13). The GlmS-Fru6P and GlmS-Glc6P-DON crystal structures (13, 14) together with biochemical studies (8) indicate that the active form of the protein is the 134-kDa dimer. The synthase active site is actually located at the dimer interface formed by two synthase domains (supplemental Fig. S1A). The two glutaminase domains are located on opposite sides of the synthase dimer. Because the glutamine mimic DON has one extra carbon compared with the true substrate, the GlmS-Glc6P-DON structure represented only an approximation of the active GlmS form in complex with its two substrates (13). In an attempt to more closely approach this complex, we have determined the structure of the inactive C1A mutant of *E. coli* GlmS (C1A-GlmS), crystallized in the presence of Fru6P and Gln. Unexpectedly, the C1A-GlmS structure is organized as a hexamer, which led us to investigate the quaternary structure of *E. coli* wild-type GlmS in solution. We show that the enzyme is regulated by a morpheein-type allosteric mechanism, in which functional dimeric GlmS is in equilibrium with the inactive hexamer.

**Experimental Procedures**

**Protein Preparation and Crystallization**—The C1A mutant of GlmS was purified as described previously for the wild type protein (13). After gel filtration on a Superdex 200 column, the protein was followed by SDS-PAGE, and the fractions corresponding to the main peak were pooled. The Bradford method was used to quantify the protein concentration. Crystals were grown at 18 °C in hanging drops by vapor diffusion. 5 µl of a solution containing 20 mg/ml protein, 10 mM Fru6P, and 10 mM glutamine in 20 mM HEPES, pH 7.2, 0.3 mM NaCl was mixed with 5 µl of a 1-ml reservoir solution (22% PEG 3350, 0.2 ammonium acetate, 0.1 mM HEPES, pH 7.0). Crystals grew to a size of 0.5 × 0.5 × 0.5 mm in a few days. After soaking for a few min in the same solution containing 28% PEG 3350 and 10% PEG 400, crystals were flash frozen in a cold nitrogen stream at 100 K.

The DON-GlmS covalent complex was formed by incubating GlmS (9 mg/ml) with 10 mM DON and 2 mM Fru6P for 1 h in 50 mM HEPES, pH 7.2, at room temperature. The removal of DON by dialysis at 4 °C against 50 mM MES, pH 6.0, 50 mM NaCl was monitored by following the 280 nm absorbance of the dialyzing buffer. Electrospray ionization mass spectrometry analysis indicates that all of the protein is modified in these conditions (data not shown).

**Data Collection and Structure Determination**—Diffraction data were collected on beamline ID14-EH2 at the European Synchrotron Radiation Facility (Grenoble) using a MarCCD detector. Data were processed with MOSFLM (20) and SCALA (21) (supplemental Table S1). The structure was solved by molecular replacement (supplemental Table S2), and refinement was carried out with PHENIX (22) using tight noncrystallographic symmetry restraints. The hinge regions (residues 240–248) of the different monomers appear to be very flexible and were not fully modeled.

**Dynamic Light Scattering**—The hydrodynamic radius was measured with a Wyatt DynaPro NanoStar. Various concentrations of wild-type GlmS in 50 mM MES, pH 6.0, 50 mM NaCl, 1 mM DTT were centrifuged at 15,000 × g for 30 min prior to measurements to remove dust and large aggregates. Samples (10 µl) were then pipetted into a 1-µl quartz cuvette. Measurements were performed at 25 °C in triplicate with at least three readings and an acquisition time of 8 s. The viscosity and refractive index of the solvent were set to 1.02 and 1.33, respectively. The hydrodynamic radius and polydispersity indexes were calculated by regularization using the averaged correlation functions in the Dynamics software (version 7.0.3.12) and size distribution by mass. Similar hydrodynamic radius (Rg) values were obtained in 50 mM Bistris propane, pH 7.0, 50 mM NaCl, 1 mM DTT.

**Static Light Scattering**—Wild type GlmS at various concentrations was analyzed by multilange laser light scattering coupled (MALLS) with size exclusion chromatography (SEC) using a SEC-MALLS/UV/RI/quasi-elastic light scattering Wyatt instrument. 30 µl of protein solution was injected, and separation was performed using a Shimadzu liquid chromatography system (Prominence) equipped with a Bioseps 3000 column (Phenomenex) before light-scattering measurements. Isocratic elution was performed at a flow rate of 0.5 ml/min in 50 mM MES, pH 6.0, 50 mM NaCl, 1 mM DTT at 25 °C. Light scattering was monitored using a mini DAWN™ TREOS system equipped with a quasi-elastic light scattering module (Wyatt Technology, Santa Barbara, CA) and refractometer (Optilab rEX, Wyatt Technology). The concentration of the molecule in solution was determined using a specific refractive index increment (dn/dc) of 0.183 at 658 nm. The absolute refractive index of the solution was measured using the refractive index detector. The light scattering detector was calibrated with toluene. The detector was controlled and the data were analyzed using the ASTRA V software (version 5.3.4.20; Wyatt Technology).

**Analytical Centrifugation**—Sedimentation velocity and equilibrium measurements were carried out using a Beckman Optima XL-A ultracentrifuge equipped with a UV-visible detection system using an AN60-Ti four-hole rotor and cells with two-channel 12-mm or 3-mm path length centerpieces.
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For sedimentation velocity measurements, 400 µl of protein was spun at 35,000 rpm (89,000 × g) and at 15 °C. Absorbance (at 280 or 290 nm) displacement profiles were recorded every 5 min. Data were analyzed with the program SEDFIT (model C(S)) (23).

Native PAGE and Western Blot Analysis—Blue native polyacrylamide gel electrophoresis was performed using Native-PAGE™ gels (Invitrogen) and dark blue or light blue cathode buffer containing 0.02 or 0.002% G-250, respectively (24). Proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore) for 1 h (Mini Trans-blot, Bio-Rad). The membrane was saturated with TTBS (Tris-buffered saline, 0.1% Tween), 10% low fat milk overnight at 4 °C and then was soaked for 1 h with monoclonal antibody (0.5 µg/ml) in TTBS, 10% low fat milk at room temperature. After washing with TTBS, the membrane was incubated for 1 h at room temperature with a goat antiserum to rat IgG-HRP conjugated (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TTBS, 10% low fat milk (1:5000 dilution). The Western blot was revealed after a 5-min incubation in a solution containing the two substrates at a 1:1 ratio (Supersignal West Pico chemiluminescent substrate, Thermo Scientific) before exposure to x-ray film.

Activity Measurements—Enzyme activity was measured following two established protocols (8). One enzyme unit is defined as 1 µmol of Glu or GlcN6P produced/min. GlmS (0.007–7.15 µM) was incubated at 15 °C in 100 mM potassium phosphate, pH 7.2, 1 mM EDTA, 50 mM KCl, 70 mM glutamine, 70 mM Fru6P for 10 min before GlcN6P detection. The reaction was initiated by adding GlmS. The quantity of GlcN6P formed was measured using the colorimetric microplate Morgan-Elson assay. In addition, the amount of Glu produced was followed spectrophotometrically at 365 nm at 37 °C on an aliquot of the incubation mixture using the glutamate dehydrogenase-catalyzed appearance of acetylpyridine adenine dinucleotide (an NADH analog). The assay mixture contained 50 mM potassium phosphate, pH 7.2, 1 mM EDTA, 50 mM KCl, 1 mM APAD, 13 units of glutamate dehydrogenase, 70 mM Fru6P, 70 mM Glu.

RESULTS

Although the E. coli C1A-GlmS mutant was crystallized in the presence of Fru6P and Gln, cyclic Glc6P and Glu were observed at the synthase and glutaminase catalytic sites, respectively (supplemental Fig. S2). The C1A-GlmS mutant crystallized in space group H32 with huge unit cell parameters (a = b = 247 Å, c = 630 Å) and eight copies of the 67-kDa monomer in the asymmetric unit (supplemental Table S1). Therefore, the positioning of 16 domains (eight synthase and eight glutaminase domains) of GlmS (supplemental Table S2) as well as structure refinement were laborious tasks.

The C1A-GlmS Structure Crystallizes as a Hexamer—The asymmetric unit of the C1A-GlmS structure contains three GlmS dimers (AB, CD, and GH) that are related by a noncrystallographic 3-fold symmetry and form one hexamer. In addition, another GlmS dimer (EF) forms, in turn, a hexamer with the equivalent dimers in two neighboring asymmetric units (Fig. 1A). The interface corresponding to the specific hexameric contacts covers 1170–1250 Å², representing half of the GlmS dimer surface area (supplemental Table S3). This value agrees with that for oligomeric proteins, which ranges from 700 to 10,000 Å² (25). Therefore, the C1A-GlmS structure contains two copies of a hexamer constituted by trimers of dimers. The solvation free energy gains upon formation of the two hexamers, and the free energies of hexamer dissociation were estimated from the solvent-accessible surface area with the program PISA (Protein Interfaces, Surfaces, and Assemblies), which is appropriate to identify oligomeric states (supplemental Table S3) (26). Although more than 10% errors in PISA results at free energies lower than 30 kcal/mol are expected (27), the large negative solvation free energy gain upon formation of the hexameric assembly and the positive free energy difference between the dissociated and associated states suggest that the hexameric state exists in solution. The calculated free energy of dissociation of the dimeric assembly is similar whether the glutaminase domains are taken into account or not, but that of the hexameric assembly becomes negative when they are not included in the calculation (supplemental Table S3). Indeed, the dimeric contacts are formed almost exclusively by the synthase domains, whereas the glutaminase domains are involved in the stabilization of the hexamer (Fig. 1B). The region preceding the C-loop (residues 589–599), the linker (residues 245–250), and Arg-539 at the extremity of helix CF (residues 520–540) are crucial elements involved in the formation of the hexameric contacts (Fig. 1, C and D).

Wild-type GlmS Also Crystallizes as a Hexamer—Wild-type GlmS in complex with Fru6P or in complex with Glc6P and DON crystallized as a dimer (13). However, both the wild-type full-length protein, either alone or in complex with cyclic Glc6P (15), and the isolated synthase domain (5, 6) were shown to crystallize in the H32 space group but with different cell parameters compared with the C1A-GlmS structure. In these cases, the cell parameters (a = b = 144 Å, c = 173 Å) were very similar, and the asymmetric unit contained one GlmS monomer. Although not pointed out previously, a hexamer can be formed in these structures by packing six monomers belonging to neighboring asymmetric units using the crystallographic symmetry. The hexamer formed by the synthase domains in these structures of wild-type GlmS superimposes well with that of the C1A-GlmS structure, especially when region 417–429, which contains a flexible loop, is omitted (Fig. 2A).

Helix CF Is Shifted and the Ammonia Channel Is Not Formed in the Hexameric Structures—Superposition of one synthase domain of one monomer of the hexameric C1A-GlmS structure with one of the dimeric GlmS:Fru6P structure reveals that helix CF adopts different conformations in the two structures (Fig. 2B). The shift of helix CF by one helix turn in the C1A-GlmS structure, compared with the dimeric GlmS:Fru6P structure, was previously observed in the structures of the wild-type enzyme crystallized in space group H32 (15). It is accompanied by a major conformational change of Lys-503* from the His-loop, whose side chain constitutes one of the building blocks of the ammonia channel in the dimeric structure (Fig. 2C). Thus, because of the shift of helix CF, the ammonia channel is not formed in the structures of GlmS alone or in complex with Glc6P; nor is it formed in the C1A-mutant structure.
Interestingly, the active dimer of wild-type GlmS, as observed in the dimeric GlmS-Fru6P structure, cannot assemble as a hexamer because of steric hindrance between the glutaminase domains (Fig. 2D). However, the synthase domains of the active dimer can be superimposed on the hexamer without overlapping, which indicates that the synthase domains with
helix CF in both positions are able to form a hexamer. The glutaminase domains appear to be highly dynamic in the hexameric structures, as shown by the absence of electron density corresponding to these domains in the structures of wild-type GlmS alone or in complex with GlcN6P (15). In the C1A-GlmS structure, the mobility of the glutaminase domains is revealed by their higher average B-factors (55.6 Å²) compared with those of the synthase dimers (36.5 Å²).

In the C1A-GlmS Structure, the Glutaminase Domains Adopt an Unexpected Conformation—The four C1A-GlmS synthase dimers in the asymmetric unit are very similar (supplemental Fig. S3). The two glutaminase domains on both sides of one synthase dimer adopt distinct orientations, which are not comparable with those previously observed in the GlmS-Fru6P and GlmS-Glc6P-DON structures (Fig. 3) (13). The relative orientations of the glutaminase domains relative to the synthase dimer in the C1A-GlmS structure are not compatible with catalysis because the ammonia channel is not formed. In particular, the distance between the synthase and glutaminase sites is 33.2 Å in the C1A-GlmS structure instead of 16.6 Å in the Fru6P structure, where the channel is formed, albeit closed by Trp-74.

The Shift of Helix CF Creates a Second Sugar-binding Site—The position of helix CF in the hexameric C1A-GlmS structure creates a pocket that is filled by an elongated molecule (Fig. 4). Given the presence of substantial electron density at a contour of 6σ of the difference map (Fig. 4B) and the composition of the crystallization drop, we assigned the electron density to a linear hexose phosphate. This new sugar-binding site is located at the interface between two monomers that form one functional synthase dimer, in the second synthase subdomain topologically equivalent to the catalytic synthase subdomain (supplemental Fig. S1, A and B and Fig. 4) (7). The sugar at this site was modeled with full occupancy but with a high B-factor (102 Å²), in agreement with the presence of numerous surrounding water molecules (Fig. 4C). Interestingly, this site was occupied by a MES buffer molecule in the structure of the synthase domain of GlmS in complex with GlcN6P or 2-amino-2-deoxyglucitol 6-phosphate (5) (supplemental Fig. S4). However, the pocket is quite large, and the MES and hexose phosphate ligands do not occupy the same position, which indicates a potentially wide specificity for ligand binding.

In Solution, GlmS Is in Equilibrium between Dimeric and Hexameric Forms—The existence of a dimer or hexamer in different crystal forms of E. coli GlmS prompted us to examine the quaternary structure of wild-type GlmS in solution. The protein was initially characterized as dimeric by SEC (8). However, preliminary sedimentation velocity ultracentrifugation experiments showed the existence of an equilibrium between dimeric GlmS and a higher molecular weight oligomer (11). The question was whether this oligomer could be a hexamer, as observed in the crystal structures.

Dynamic light scattering (DLS), which measures the fluctuations of light scattering intensity due to diffusion of molecules, permits us to determine their $R_h$ value. The theoretical hydrodynamic parameters for the dimer and hexamer of GlmS were calculated with the program HYDROPRO (version 10) (28), using the crystallographic coordinates (supplemental Table S4A). Because DLS distinguishes only molecules that have a radius that differs by at least a factor of 3–4, the elongated dimer and the globular hexamer are not expected to be resolved. However, this technique was used to monitor the average size of the protein at different concentrations. The hydrodynamic radius of GlmS was found to be dependent on protein concentration between 1 and 10 mg/ml (7.5 and 75 μM dimer concentration), indicating a change in the oligomerization of the protein (Fig. 5A). At 0.5 mg/ml, the amount of dimer and hexamer is similar, whereas only the hexamer form is present at a protein concentration above 5 mg/ml.

The oligomerization state of GlmS in solution was further investigated by MALLS, a technique that does not rely on the calibration of standards and assumptions of the globular state of the protein (Fig. 5B). Static light scattering, which measures the intensity of the scattered light as a function of angle, can be used to determine the absolute molecular mass using the Zimm formalism. At low concentrations of GlmS, the chromatogram contains a major species eluting at 8.25 ml on a Phenomenex Biosep s3000 column. The MALLS/UV/RI analysis yielded a measured mass of 143 kDa for the main peak of the sample at 4 mg/ml. This mass is in reasonable agreement with the theoretical mass of 134 kDa expected for the GlmS dimer. The polydispersity (1.004) of this peak is low, with an $R_h$ value of 4.6 ± 0.2 nm. At higher protein concentrations, the RI elution profiles display an asymmetric peak, and the molar mass during peak elution decreases, indicating the existence of a mixture of oligomeric forms in rapid equilibrium on the time scale of the experiment. For the injection at 38 mg/ml, the determined masses cover the 150–310 kDa range, indicating the presence of oligomeric species between a dimer and an order of more than 4. The mass of 150 kDa is substantially higher than the 134 kDa expected for the dimer. This is probably due to the fact that high order oligomers trailing on the column contaminate the

![Image](https://example.com/image1.png)

**FIGURE 1. The C1A-GlmS hexamer. A, arrangement of the 16 domains present in the asymmetric unit of the C1A-GlmS structure. The asymmetric unit contains one C1A-GlmS hexamer (ABCDGH) and one protein dimer (EF) that forms an hexamer with two other dimers belonging to neighboring asymmetric units (EFEF**). The synthase domains, which form one hexamer in the asymmetric unit, are colored dark and light blue, dark and light green, orange, and yellow. In addition, two synthase domains colored magenta and pink interact with other molecules of neighboring asymmetric units to form a second hexamer. The glutaminase domains have pale colors corresponding to that of the synthase domain to which they are linked. The buried surface areas (in Å²) between synthase domains belonging to the same catalytic dimer are indicated in red, and areas between those from neighboring dimers are shown in black. B, stereoview of one C1A-GlmS hexamer. The Glc6P and Glu ligands at the active sites are shown as blue and orange sticks, respectively, and Glc6P at the secondary sugar binding site is shown as green sticks. The C-loop and the preceding region (residues 589–608), which is involved in the hexameric contacts, are indicated in red. C, orthogonal stereoview to highlight the hexameric interface. Besides the region preceding the C-loop (in red), Arg-539 from helix CF (magenta, with Arg-539 shown as sticks) and the linker (residues 245–250 in green) are the main elements involved in hexamer formation. D, details of the hexameric contacts. Tyr-491 from the synthase domain also contributes to the hexameric interface as well as residues 9–11, 14–15, 18, 21–22, 184–186, 197–201, 217, and 235 from the glutaminase domains. Salt bridges between Arg-10 and Glu-186, Glu-14 and Arg-217, Arg-201 and Glu-235, and Arg-249/His-250 and Asp-594 stabilize the hexameric assembly. Among these residues, Arg-201, Arg-217, and Glu-235 are not replaced by equivalent residues in eukaryotes (supplemental Fig. S1B).
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FIGURE 2. Comparison of the GlmS hexamer and the GlmS-Fru6P dimer. A, superposition of the hexameric structure of the synthase domains of wild-type GlmS (PDB code 2VF4) generated using the crystallographic symmetry (in yellow) with the two different hexamers present in the asymmetric unit of the C1A-GlmS structure (in blue and cyan). The two synthase hexamers of C1A-GlmS superpose with a root mean square deviation of 0.42 Å² over 1837 Ca atoms, and the hexamer of the GlmS structure superposes on the noncrystallographic hexamer of C1A-GlmS with a root mean square deviation of 1.14 Å² over 1798 Ca atoms and a root mean square deviation of 0.70 Å² over 1720 Ca atoms, excluding residues 417–429. B, stereoview of the superposition of one synthase domain of the C1A-GlmS (blue) and GlmS-Fru6P (PDB code 2BPL, purple) structures. The two regions (417–429 and 520–540 that correspond to helix CF) that differ significantly between the two structures are indicated in pink for the GlmS-Fru6P structure. The sugar at the synthase sites of the GlmS-Fru6P and C1A-GlmS structures are shown as green sticks. C, comparison of the synthase sites of the GlmS (PDB code 2VF4) and GlmS-Fru6P (PDB code 2BPL) structures. Upon Fru6P binding, ordering of the C-loop and the glutaminase domains leads to the anchoring of Trp-74 on the C-loop. This induces a conformational change of the His-loop, including a huge rearrangement of Lys-503* and a shift of helix CF. An asterisk denotes that the residue belongs to the adjacent GlmS monomer. D, artificial hexamer assembled by superimposing three dimers of the GlmS-Fru6P structure (colored as in Fig. 1A) onto the hexameric C1A-GlmS structure (not shown). Several clashes indicate that the glutaminase domains cannot adopt the catalytic orientation in the hexameric form.

signal of the dimer, leading to more light scattering and, thus, masses higher than expected. This observation is consistent with the fact that, at 19 mg/ml, the determined masses cover the narrower 132–310 kDa range.

We then investigated the dynamic nature of the distribution of the GlmS quaternary structure assemblies by sedimentation velocity experiments. Four different enzyme concentrations (1.4, 2.7, 3.8, and 10 mg/ml, corresponding to a dimer concentration of 10.5, 20.2, 28.5, and 75 μM, respectively) were subjected to ultracentrifugation at 35,000 rpm (Fig. 5C and supplemental Table S4B). Data analysis with the program SEDFIT (model C(S)) shows the presence of the dimer ($s_{20,w} = 7.5$ S, $R_h = 4.1$ nm, friction coefficient $f/f_0 = 1.21$) and a higher molecular weight oligomer. The calculated $R_h$ value of the slow sedimenting species (4.1 nm) is consistent with the $R_h$ value determined by DLS at low enzyme concentrations (4.6 nm). Between 0 and 3.8 mg/ml, the sedimentation coefficient $s_{20,w}$ of the higher molecular weight oligomer increased with protein concentration, and the relative proportion of the slow sedimenting species decreased. This behavior indicates that the dimer and the other oligomer are in equilibrium. The sedimentation coefficient $s_{20,w}$ of the second population reached a plateau at 12.08 S. A hexameric protein with such a sedimentation coefficient would have an $f/f_0$ of 1.56 and an $R_h$ of 7.6 nm, which is consistent with the $R_h$ value determined by DLS (7.4 nm).

Blue native PAGE using the anionic dye Coomassie G-250 to shift the protein charges, which provides native molecular masses (24), also indicated that the higher molecular weight species is a hexamer (Fig. 6A). This technique was also used to monitor the dimer/hexamer ratio with enzyme concentration (Fig. 6, B and C). At 0.8 mg/ml, only 27% of the protein formed the hexamer, in contrast to the DLS results that indicated almost complete formation of the hexamer at this concentration (Fig. 5A). Thus, partial dissociation of the hexamer appears to occur under native PAGE conditions. The fast equilibrium between dimer and hexamer was further confirmed by SEC and sedimentation equilibrium centrifugation (see supplemental Results and Figs. S5 and S6).

GlcN6P Shifts the Equilibrium toward the Hexamer—The quaternary structure of GlmS in the presence of the Gln substrate and that of GlmS covalently modified by the Gln analog DON were monitored by sedimentation velocity experiments to examine whether occupancy of the glutaminase site could shift the oligomer equilibrium (Fig. 5D and supplemental Table S4C). Whereas 20 mM Gln did not modify the oligomeric mixture of GlmS, the equilibrium was shifted toward the dimer for the DON-modified protein, as shown by its higher proportion and by the decrease of the sedimentation coefficient of the high molecular weight oligomer ($s_{20,w} = 8.79$ S). However, the addition of 20 mM Fru6P did not modify this trend further. To know whether the Fru6P, Glc6P, or GlcN6P sugars could shift the oligomer equilibrium, the distribution of the GlmS quaternary of the His-loop, including a huge rearrangement of Lys-503*, and a shift of helix CF. An asterisk denotes that the residue belongs to the adjacent GlmS monomer. D, artificial hexamer assembled by superimposing three dimers of the GlmS-Fru6P structure (colored as in Fig. 1A) onto the hexameric C1A-GlmS structure (not shown). Several clashes indicate that the glutaminase domains cannot adopt the catalytic orientation in the hexameric form.
structure assemblies was examined in the presence of the sugars by sedimentation velocity experiments (Fig. 5E and supplemental Table S4C). Ultracentrifugation data analysis shows that, in the absence of ligands, the dimer ($s_{20,w} = 7.03$ S) is in fast equilibrium with another species ($s_{20,w} = 9.84$ S). In the presence of 20 mM Fru6P or Glc6P, the equilibrium is slightly shifted toward the higher molecular weight oligomer, whose sedimentation coefficient increases ($s_{20,w} = 10.74$ S). Unexpectedly, in the presence of 20 mM GlcN6P, the equilibrium is completely shifted toward one oligomer with an $s_{20,w}$ of 13.31 S, in close agreement with the theoretical value for the hexamer (supplemental Table S4A). DLS experiments with GlmS at 0.5 mg/ml showed that the hydrodynamic radius of GlmS varied non-linearly with GlcN6P concentrations, indicating that GlcN6P displaces the equilibrium between oligomers toward the hexamer, with a complete shift at 100 $\mu$M GlcN6P (Fig. 5F).

The Hexamer Exists in Cellulo and Is Catalytically Inactive in Vitro—To know whether the oligomer equilibrium could regulate the activity of *E. coli* GlmS, the profile of the specific activity as a function of enzyme concentration was examined (Fig. 7A). It shows that the enzyme does not obey Michaelis-Menten kinetics and that the specific activity of the enzyme unexpectedly decreases with protein concentration. The quantity and quaternary structure of GlmS in *cellulo* were also estimated by Western blot analysis. The concentration of GlmS in non-recombinant *E. coli* cells is estimated to be around 2.5 $\mu$M (sup-
To probe the existence of the hexamer, 20 mM GlcN6P was added either during cell growth or before cell disruption. The hexamer could be detected when 20 mM GlcN6P was added to the crude extract before loading onto the native PAGE gel (Fig. 7B, lane c).

**DISCUSSION**

Several Crystal Forms of E. coli GlmS Are Organized as Hexamers—The C1A-GlmS mutant in complex with Glc6P and Glu was found to form hexamers constituted of trimers of tightly bound dimers, in contrast to the active homodimeric forms observed in the crystals of GlmS in complex with linear Fru6P or in complex with linear Glc6P and a glutamine analog (13, 14). The hexameric form was also observed in crystals of wild-type E. coli GlmS, where the glutaminase domains were either absent (as in the isolated synthase domain in complex with cyclic Glc6P, cyclic GlcN6P, and 2-amino-2-deoxyglucitol 6-phosphate (5, 6)) or mobile (as in the structure of GlmS alone or in complex with cyclic GlcN6P (15)), although the hexameric assembly could not be detected with the program *PISA*. The existence of the same hexameric architecture of GlmS in different crystal systems (same space group H32 but very different cell parameters) indicates that this packing does not result from a crystallization artifact but rather corresponds to a biologically relevant state of the protein. This idea is consistent with the extended buried surface area for the hexamer interface. In some cases, crystal packing has permitted the visualization of weaker but biologically relevant snapshots of biological macromolecular assemblies that are difficult to detect using other methods (29–31). Because the glutaminase domains cannot adopt the catalytically competent orientation necessary for ammonia channel formation in the hexameric form, hexamer formation is expected to lead to enzyme inactivation.

Existence of the Hexameric State of GlmS in Solution—In solution, E. coli GlmS was previously shown to co-exist as a dimer as well as a higher molecular weight oligomer (11). Our biophysical experiments indicate that the dimer of E. coli GlmS is in fast equilibrium with a hexamer. In vitro, the hexamer and dimer co-exist at equimolar concentrations around 0.5 mg/ml GlmS, which is very close to the concentration of GlmS in non-recombinant E. coli cells (supplemental Fig. S7). Thus, a dimer to hexamer shift with enzyme concentration is physiologically likely. Covalent modification of GlmS by the Gln analog DON clearly increases the proportion of the dimer. The presence of 20 mM Fru6P or Glc6P shifts only slightly the equilibrium toward the hexamer. On the contrary, a concentration of GlcN6P of 0.1 mM, 1 order of magnitude below the *in vivo* concentration (32), completely shifts the equilibrium toward the hexamer. Accordingly, the synthase domains and full-
length wild-type GlmS in complex with cyclic GlcN6P are organized as hexamers (5, 6, 15). However, the metastability of the hexamer is indicated by the negative free energy of dissociation of the hexamer formed by the association of the synthase domains of C1A-GlmS alone, in agreement with the fact that the PISA program could not predict a hexamer assembly from the monomer present in the H32 crystals of the wild-type enzyme. The crystallographic data indicate that the glutaminase domains, as well as the linker between the two domains, are involved in the stabilization of the hexameric assembly of the C1A mutant. It is likely that the intrinsic mobility of the glutaminase domains could help to promote disassembly/assembly of the hexamer and that the linker is a main player in this conformational change, as observed for other proteins (33).

In contrast to Bacterial GlmS, Eukaryotic Glucosamine-6-phosphate Synthases Are Organized as Tetramers—In contrast to the hexameric state of E. coli GlmS, the eukaryotic glucosamine-6-phosphate synthases from Candida albicans (34), rat...
liver (35), and humans (36) exist as homotetramers in solution, and the tetramer was also observed in the crystals (37, 38) (PDB code 2V4M) (supplemental Fig. S8). For the human enzyme, the tetramer (not the dimer) can be reconstituted from the monomer present in the asymmetric unit using the crystallographic symmetry (38). Thus, there are no indications to date that the dimer could be a biologically relevant state in eukaryotes. The question can be raised whether the active form of the eukaryotic enzyme could be the dimer (like the E. coli enzyme), whereas the tetrameric form would be biologically relevant but catalytically inactive. This hypothesis relies on the observation that only the synthase domains of the eukaryotic glucosamine-6-phosphate synthases (and not the full-length enzymes) have been crystallized yet, and the isolated synthase domain of E. coli GlmS is hexameric although the active form is a dimer.

The residues responsible for the tetramerization of eukaryotic glucosamine-6-phosphate synthases are conserved only among eukaryotic sequences (supplemental Fig. S1) (37). This suggests that the bacterial GlmS enzymes would not adopt the same architecture. Indeed, the superposition of the E. coli GlmS dimer, as observed in the GlmS-Fru6P structure, on the tetramer formed by the synthase domains of C. albicans Gfat1 (supplemental Fig. S8) indicates a lack of interactions and several bad contacts at the tetrameric interface. Thus, if a tetrameric form of E. coli GlmS exists in the cell, its organization should be different from that of the eukaryotic tetramer.

**Biological Relevance of the Hexamer: Morphein-type Allosteric Regulation Mechanism of E. coli GlmS**

The existence of functionally distinct quaternary structure assemblies for GlmS, observed by X-ray crystallography (inactive hexamer, active dimer), together with the strong effect of the GlcN6P sugar that shifts the oligomer equilibrium toward the hexamer, suggests a morphein-type allosteric regulation mechanism (Fig. 7C). In the case of the prototype enzyme of this type of regulation, porphobilinogen synthase, different mutations were associated with an increased propensity of the protein to exist as the inactive hexameric assembly compared with the high activity octamer (39, 40). This regulation model involves the existence of distinct conformations of the protein monomer, each dictating a different quaternary structure with alternative functionality (41, 42). In the hexameric form, the GlmS dimer adopts a conformation very different from that observed in the active dimer because of the position of the two glutaminase domains (Figs. 3 and 7C). Moreover, the formation of the hexamer is prevented when both glutaminase domains adopt the active conformation because of steric hindrance (Fig. 2D). Therefore, the simplest equilibrium of morphein forms is 2-mer (active) ⇄ 2-mer* (transiently stable) ⇄ 6-mer* (inactive).

In normal cellular conditions, where the GlcN6P concentration is in the millimolar range (32), its synthesis would not be necessary, and GlmS would exist as the inactive hexamer. Under conditions of low GlcN6P concentrations, the enzyme would be in equilibrium between the dimeric and hexameric states. Upon Fru6P binding, the functional orientation of the glutaminase domains would lead to the anchoring of Trp-74 on the synthase His-loop (Fig. 2C). This would trigger a conformational change of Lys-503*, due to steric hindrance, allowing formation of a closed ammonia channel. The concomitant shift of helix CF would induce the repositioning of His-504* to open the sugar ring and initiate catalysis. Disso-
Association of the hexamer following the opening of the sugar ring is supported by the crystal structures of full-length GlmS, which are hexameric in the presence of cyclic GlcN6P and dimeric in the presence of linear Fru6P. Alternatively, the quaternary change of GlmS from hexamer to dimer could occur after binding of the second substrate, glutamine, as...
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suggested by the shift of the oligomer equilibrium toward the dimer upon modification of the protein by the glutamine analog DON. The fact that 20 mM Gln did not shift the oligomer equilibrium probably results from the requirement that the synthase site must be occupied before the glutaminase domains can adopt the catalytic conformation.

Product Inhibition through Hexamer Stabilization—Blue native PAGE indicates that adding 20 mM GlcN6P to the crude extract of non-recombinant cells induces hexamer formation, although this technique underestimates the amount of hexamer because of its partial dissociation. The hexamer was not observed when cells were grown in the presence of GlcN6P because the latter cannot cross the cellular membrane because only non-phosphorylated exogenous sugars are actively transported by glucose transporters. Moreover, the specific activity decreases over a concentration range of GlmS (0.5–500 μg/ml; Fig. 7A) that is lower than that where the shift from dimer to hexamer of GlmS alone was observed (0.5–5 mg/ml; Fig. 5A). In fact, the decrease of activity with protein concentration can be explained qualitatively by the equilibrium shift toward the inactive hexameric state, which results not only from the higher concentration of enzyme but also from the formation of increasing amounts of the GlcN6P product formed during the assay. The presence of the Fru6P substrate, which is used at high concentration in the enzyme assay and which maintains the equilibrium between the dimer and hexamer, as shown by the ultracentrifugation results, should also be taken into account. Therefore, it is not possible to directly relate enzyme inactivity with the amount of hexamer that is present using this experiment. Nevertheless, our data strongly support product inhibition through hexamer stabilization.

The conformational equilibrium between the dimeric and hexameric forms of E. coli GlmS appears to regulate the metabolic function of the bacterial enzyme at the post-translational level in a way complementary to post-transcriptional regulation. Indeed, GlmS synthesis is also activated through concomitant expression of the glmUS operon by a conserved small RNA in several Gram-negative bacteria, including E. coli (43). In contrast, in Gram-positive bacteria, post-transcriptional regulation is performed by the glmS ribozyme (44, 45), whereas a more classical post-translational regulation resulting from UDP feedback inhibition or enzyme phosphorylation was reported for the eukaryotic enzymes from C. albicans (34, 46), Drosophila (47), and humans (48, 49).

Inhibition Strategy Targeting the Hexameric Structure of GlmS—The position of helix CF in the C1A-GlmS mutant has created a new binding site that accommodates a linear phosphate sugar. Interestingly, in the tetrameric eukaryotic enzymes, the CF helix has a very different orientation compared with that in hexameric E. coli GlmS (37). Thus, in eukaryotic enzymes, there is no sugar-binding site located at a position similar to the new sugar-binding site unveiled by this study. Because the presence of a sugar in this pocket is not compatible with the conformational change to the dimer necessary for the enzyme to become catalytically active, we propose to design specific inhibitors targeting this site as a new inhibition strategy of E. coli GlmS in the search for antibacterial agents. Indeed, these inhibitors would act by trapping GlmS in its inactive hexameric form. The previous discovery of an inhibitor that binds to the allosteric site specific to the inactive hexamer of porphobilinogen synthase illustrates the great potential to exploit the structural differences between morpheein assemblies for drug development (50, 51). The extremely important thermodynamic mechanism of allosteric regulation, which is becoming increasingly prevalent in drug discovery, highlights how conformational flexibility is closely linked to protein function, including enzyme catalysis (52, 53).

CONCLUSION

The hexameric crystal forms of the E. coli C1A-GlmS mutant and the wild type protein raised the question of the functional significance of this oligomeric state. SEC, DLS, MALD, analytical centrifugation analyses, and native PAGE indicated that, in solution, the dimer is in equilibrium with a hexamer, which increases with enzyme concentration. Whereas the dimer is the catalytically active species, the hexamer is an inactive form, and the cyclic GlcN6P product shifts the equilibrium toward the hexamer. Therefore, E. coli GlmS appears to follow a morpheein-type allosteric regulation mechanism. Because this type of regulation is difficult to highlight experimentally, there are only very few established examples to date of this type of regulation, besides the well studied porphobilinogen synthase. Nevertheless, a list of several proteins for which data suggest a morpheein-type mechanism has recently been published (42). The absence of GlmS in this list shows the difficulty of identifying putative morpheeins from the literature. The location of a secondary sugar-binding pocket at the dimer interface in the hexameric state of GlmS might help in the design of specific inhibitors directed to stabilize the inactive hexameric state. Therefore, it appears that the exploitation of the differences in the oligomeric assemblies between hexameric bacterial and tetrameric eukaryotic glucosamine-6-phosphate synthases is a promising strategy to develop specific inhibitors of this enzyme, which is present in all known organisms.

FIGURE 7. The hexameric form of GlmS is an inactive form that regulates the activity of GlmS. A, profile of the specific activity (units of enzyme activity/protein mass) versus protein concentration. The reaction was followed by the Morgan Elson assay (filled circles) and glutamate dehydrogenase assay (open circles). The various concentrations of the GlmS dimer (0.65, 3.57, 7.15, 35.75, 71.5, 715, and 3575 nm) correspond to 0.13, 0.24, 0.32, 1.2, 2.3, 16, and 33% conversion, respectively. B, 3–12% BisTris blue native gel of native GlmS revealed by Western blot using monoclonal antibody 505.1, which preferentially binds the native glucaminase domain (54). Three cultures (a, b, and c) of HB101 E. coli strain were grown at 37 °C in 2YT medium until A600 of buffer (20 mM sodium phosphate, pH 7.2, 1 mM EDTA, 2 mM DTT, and anti-protease mixture). 20 mM GlcN6P was included in the buffer for pellet c. After cell disruption, 7.5 μl of crude extract was loaded onto a NativePAGEgel run using Light Blue cathode buffer. 2 μM GlcN6P was added to the anode and cathode buffers. Lane d contains 0.1 μg of purified recombinant GlmS. A lane including native weight standards apoferritin band 2 (480 kDa), β-phycocerythrin (242 kDa), lactate dehydrogenase (146 kDa), and bovine serum albumin (66 kDa) was included in the gel. After electrophoresis, the membrane was stained with Ponceau S, and the position of the standard markers, hand-marked on the exposed film by transparency, is indicated on the left of the gel. C, scheme illustrating the regulation of E. coli GlmS. The inactive dimer 2-mer is a transient form that is not stable in solution. The morpheein model is 2-mer (active) ⇄ 2-mer* (transiently stable) ⇄ 6-mer* (inactive).
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