Structures of the *Bacillus subtilis* Glutamine Synthetase Dodecamer Reveal Large Intersubunit Catalytic Conformational Changes Linked to a Unique Feedback Inhibition Mechanism*

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Glutamine synthetase (GS), which catalyzes the production of glutamine, plays essential roles in nitrogen metabolism. There are two main bacterial GS isoenzymes, GSI-α and GSI-β. GSI-α enzymes, which have not been structurally characterized, are uniquely feedback-inhibited by Gln. To gain insight into GSI-α function, we performed biochemical and cellular studies and obtained structures for all GSI-α catalytic and regulatory states. GSI-α forms a massive 600-kDa dodecameric machine. Unlike other characterized GS, the *Bacillus subtilis* enzyme undergoes dramatic intersubunit conformational alterations during formation of the transition state. Remarkably, these changes are required for active site construction. Feedback inhibition arises from a hydrogen bond network between Gln, the catalytic glutamate, and the GSI-α-specific residue, Arg62, from an adjacent subunit. Notably, Arg62 must be ejected for proper active site reorganization. Consistent with these findings, an R62A mutation abrogates Gln feedback inhibition but does not affect catalysis. Thus, these data reveal a heretofore unseen active site restructuring that is linked to a novel feedback regulatory mechanism. This GSI-α-specific regulatory network could be exploited for inhibitor design against Gram-positive pathogens.

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Glutamine is a key metabolite in nitrogen assimilation because it is not only incorporated into proteins but also serves as the direct nitrogen donor for the synthesis of nearly 25% of all nitrogen-containing compounds (1). Glutamine is synthesized from ammonium, ATP, and glutamate by the enzyme glutamine synthetase (GS). Underscoring its essential role in nitrogen metabolism and assimilation, GS is found in all organisms and is tightly regulated (2–3). GS enzymes are all large homooligomeric machines, in which each active site is formed between subunit interfaces (3–13). There are three different classes of GS enzymes, categorized as GSI, GSII, and GSIII (14). GSII enzymes are specific to prokaryotes and archaea and have thus far all been shown to be dodecamers (3, 7–14). GSIII enzymes are decamers consisting of two stacked pentamer rings and are found in eukaryotes and soil-dwelling bacteria (4, 5). GSIII proteins are harbored in certain anaerobic bacteria and cyanobacteria and, like GSI enzymes, are dodecamers but are formed from hexamer-hexamer interactions different from those that form GSI oligomers (6).

Despite their distinct oligomeric arrangements, the active site and residues involved in catalysis are conserved in GS proteins, suggesting similar overall mechanisms. This enzymatic reaction can be divided into two steps, which are facilitated by either Mg²⁺ or Mn²⁺ (3, 15). In the first step, the ε-oxygen of the substrate glutamate attacks the γ-phosphorus of ATP to form a γ-glutamyl phosphate intermediate ([16]). In the second step, ammonia, formed by the deprotonation of an ammonium ion, attacks the reaction intermediate to produce glutamine (Fig. 1A). Because of their central role in metabolism, GSs have emerged as important chemotherapeutic targets. For example, the herbicide glufosinate-ammonium, which inhibits plant GS, has recently become commercially available, and research is under way for the development of compounds that selectively target the *Mycobacterium tuberculosis* GS (22). The
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TABLE 1
Selected crystallographic data for GS structures

| Structure                      | Apo-GS          | GS-glutamate-AMPPCP | GS-transition state |
|-------------------------------|-----------------|---------------------|---------------------|
| Resolution (Å)                | 120.2–3.10      | 120.4–2.87          | 117.1–2.58          |
| Space group                   | P1              | C222, P1            | 110.0               |
| Cell constants (Å)            | a = 110.0       | a = 138.9           | a = 110.0           |
|                              | b = 138.4       | b = 240.9           | b = 141.6           |
|                              | c = 138.7       | c = 207.6           | c = 142.1           |
| Cell constants (degrees)      | α = 119.8       | α = 90.0            | α = 60.3            |
|                              | β = 90.2        | β = 90.0            | β = 67.4            |
|                              | γ = 93.9        | γ = 90.0            | γ = 76.2            |
| Overall Rsym (%)              | 11.5 (43.6)     | 5.0 (30.9)          | 5.76 (20.1)         |
| Overall I/σ(I)                | 12.1 (1.8)      | 13.8 (2.5)          | 12.2 (3.4)          |
| No. of total reflections      | 186475          | 239473              | 364805              |
| % Complete                    | 97.8 (98.2)     | 92.1 (92.0)         | 90.0 (90.4)         |

| Refinement statistics         |                 |                     |                     |
|-------------------------------|-----------------|---------------------|---------------------|
| Rwork/Rfree (%)               | 21.7/26.7       | 19.5/23.6           | 20.3/23.2           |
| r.m.s. deviation              | 1.30            | 1.33                | 1.12                |
| Bond angles (degrees)         | 0.008           | 0.010               | 0.022               |
| Bond lengths (Å)              | 138.7           | 141.6               | 119.8               |

* Rsym = Σ[|Fo| – |Fc|]/Σ|Fo|, where |Fo| is observed intensity, and |Fc| is the final average value of intensity.
* Values in parentheses are for the highest resolution shell.
* Rwork = Σ|Fo| – |Fc|)/Σ|Fo| and Rfree = Σ|Fo| – |Fc|)/Σ|Fc|, where all reflections belong to a test set of 5% randomly selected data.

**EXPERIMENTAL PROCEDURES**

Protein Purification, Crystallization, and Structure Determination—An artificial gene (codon optimized for E. coli expression) encoding the B. subtilis GS was obtained from GenScript Corp. (Piscataway, NJ) and subcloned into pET15b for protein expression. Gel filtration of GS species indicated a dodecameric oligomer. The His tag was removed for all structural and biochemical studies. Crystals were grown via hanging drop vapor diffusion at room temperature. B. subtilis apo-GS crystals were obtained by mixing the protein (40 mg/ml) at a 1:1 ratio with 40% 4-methyl-2,4-pentanediol and 200 mM MgSO4 and inverting the drop over the reservoir solution. These crystals contain a dodecamer in the crystallographic asymmetric unit (ASU). To produce GS-glutamate-AMPPCP crystals, glutamate and AMPPCP were added to GS (at 40 mg/ml) to final concentrations of 5 mM, and the solution was mixed 1:1 with a reservoir of 15% PEG 8000, 0.1 M Hepes, pH 7.5, and 10 mM MgCl₂. These crystals contain a hexamer in the ASU, and the dodecamer is generated via symmetry.

GS-Met-Sox-P-ADP was produced by mixing GS with 5 mM MgCl₂, 5 mM ATP, and 5 mM L-methionine-S-sulfoximine (Met-Sox) for 1 h. This solution was then combined 1:1 with a crystallization reagent of 10% PEG 4000, 0.1 M Hepes, pH 7.5. These crystals contain a dodecamer in the ASU. Two crystal forms of GS-Gln were obtained. Form 1 was grown by mixing the GS-glutamine complex 1:1 with 40% 4-methyl-2,4-pentanediol and 100 mM MgCl₂. Form 2 was obtained using 10% PEG 8000, 0.1 M Tris, pH 8.0, 5 mM MgCl₂ as a crystallization solution. Form 1 contains a dodecamer in the ASU, whereas form 2 contains a hexamer (with the dodecamer generated by crystal symmetry). All x-ray intensity data were collected at 100 K at the Advanced Light Source beamline 8.3.1. The space group and cell parameters of each crystal are shown in Tables 1 and 2. Phases were determined first for the apo-GS form by molecular replacement, using the M. tuberculosis GS dodecamer as the search model. Several nonconserved loops were removed from the search model before molecular replacement. Refinement was carried out using the Crystallography and NMR Software pack-
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TABLE 2
Selected crystallographic data for GS-Gln structures

|                      | Form 1 (P1) | Form 2 (C2) |
|----------------------|-------------|-------------|
| Resolution (Å)       | 119.0–2.95  | 61.9–2.90   |
| Space group          | P1          | C2          |
| Cell constants (Å)   | 112.0       | 209.0       |
|                      | 136.5       | 138.9       |
|                      | 137.7       | 144.7       |
| Cell constants (degrees) | 119.8  | 90.0        |
|                      | 90.2        | 125.7       |
|                      | 9.3         | 9.0         |
| Overall Rwork (%)    | 10.3 (37.3) | 8.0 (37.7)  |
| Overall Rfree (%)    | 12.2 (2.0)  | 13.3 (2.1)  |
| No. of total reflections | 274,737     | 175,345     |
| Percentage complete  | 96.1 (96.0) | 98.8 (91.6) |

Refinement statistics

|                      | Rwork/Rfree (%) |
|----------------------|-----------------|
| r.m.s. deviation     | 20.0/25.8       |
| Bond angles (degrees)| 1.12/1.20       |
| Bond lengths (Å)     | 0.022/0.009     |

Note:
- Rwork = Σ||Fobs|−|Fcalc||/Σ|Fobs|, where |Fobs| is the observed structure factor and |Fcalc| is the calculated structure factor.
- Rfree = Σ||Fobs|−|Fcalc||/Σ|Fobs|, where |Fobs| is the observed structure factor and |Fcalc| is the calculated structure factor for a test set of 5% randomly selected data.

- Values in parentheses are for the highest-resolution shell.

Structure Determinations of B. subtilis GS and Its Complexes

To deduce the structural mechanism of GS-α catalysis and its regulation, de novo crystals were obtained of the apo-, substrate-bound, and transition state complex (Tables 1 and 2). The transition state structure was obtained by reacting GS with ATP and Met-Sox (see “Experimental Procedures”). GS phosphorylates Met-Sox in the presence of ATP to form the transition state analog, Met-Sox-P, which causes irreversible inhibition of the enzyme (44). The B. subtilis apo-GS structure was solved first by molecular replacement using the M. tuberculosis GSI-β structure, which is 41% identical to B. subtilis GS (see “Experimental Procedures”). The structure, refined to Rwork/Rfree values of 21.7%/26.7% 3.1 Å (Table 1),
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FIGURE 1. Structure and enzymatic mechanism of B. subtilis GSI-α. A, top, a scheme depicting the GS reaction mechanism. Bottom left, secondary structural elements of the GSI-α subunit. For reference, the Glu flap is colored blue, and the Tyr loop is colored magenta. Ribbon diagrams figures were made using PyMOL (47). Bottom right, the GS active site is illustrated as a bifunnel with entrance and exit portals. The glutamate and ATP substrates enter and bind at opposite ends of the bifunnel, and the respective products leave via the same portals. B, a hexamer of the dodecameric B. subtilis GS viewed down the molecular 6-fold axis. C, the GS dodecamer rotated by 90° relative to Fig. 18. In this figure, the “bottom” hexamer is shown as a surface to highlight the interhexamer contacting α14–α15 helices. D, close-up view of an active site created by neighboring subunits. Key active site loops that line the active site are labeled and colored differently. A bound glutamate is shown in CPK. E, comparison of GS dodecamers of the B. subtilis GSI-α and S. typhimurium GSI-β proteins. Shown in different colors (B. subtilis in blue and S. typhimurium in red) are the active site Tyr179 loops that line the dodecameric pore (labeled). F, superimposition of a B. subtilis subunit (blue) onto a S. typhimurium subunit (red), highlighting their strong structural correspondence except for the Tyr179 loop. The location of the pore is labeled in B and E for reference.

contains 444 residues/subunit and is composed of 19β-strands and 15α-helices. The subunit can be divided by helix α3 into a larger C-terminal domain and a smaller, N-terminal region (Fig. 1A). Two hexameric rings are formed from six GS subunits and stack to form a dodecamer (Fig. 1, B and C). The hexameric rings are held together primarily by interactions between C-terminal helices α14 and α15 and α14’ and α15’ (where a prime indicates the neighboring subunit).

Each subunit has the classical GS bifunnel structure, whereby key residues that mediate catalysis are located on loops that encapsulate the active site (3, 45, 46) (Fig. 1A). The active site regions are mainly located in the C-terminal domain, with the N-terminal domain of the adjacent subunit contributing one loop. Previous studies on GSI-β enzymes led to the designations of key active site loops (8). Based on homology with GSI-β proteins, the corresponding GSI-α active site loops within the C-domain are the Glu flap (B. subtilis residues 301–306), the Tyr loop (residues 365–373), the Asn loop (residues 231–242), and the Tyr179 loop (residues 148–158). The N-terminal domain provides the Asp50’ loop (also called the “latch”), which contributes a key catalytic aspartic acid. In B. subtilis, this loop consists of residues 52–66, and the catalytic aspartic acid corresponds to Asp53’ (Fig. 1D).

Analysis of the Apo-GS Structure—The Glu flap and latch are arguably the most critical GS active site loops because they contribute residues that are central for catalysis. The aspartic acid from the Asp50’ loop abstracts a proton from ammonium to create ammonia, and the Glu flap harbors the catalytic base (Glu304 in B. subtilis GS), which abstracts a proton from the transition state to form glutamine and ADP. In the apo-GSI-α structure, the Glu flap is almost entirely disordered; only residues 301 and 306 are visible, and they display B-factors (average) > 100 Å² (Table 3). Glu flap flexibility is important because it permits facile substrate entry and product release. The Tyr loop, which contains the tyrosine that is adenylated in GSI-β enzymes, is also disordered. A corresponding tyrosine (Tyr373) is found in the B. subtilis GSI-α; however, studies have shown that it is not adenylated, consistent with a different GSI-α regulatory mechanism (23). Aside from the Glu flap and Tyr loop, clear density is observed for the other active site regions in the GSI-α apo-structure, and with the exception of the Tyr179 loop, the catalytic regions of the GSI-α enzyme are similar in length to GSI-β proteins. In GSI-β enzymes, the Tyr179 loop contains 36 residues and extends from the active site into the central dodecamer channel, whereas in the GSI-α structure, this region consists of a short, 11-residue loop (Fig. 1, E and F) (3). When the Tyr179 region is excluded, the Ca atoms of the B. subtilis GS subunit can be superimposed onto those of S. typhimurium (Protein Data Bank entry 1f52) and M. tuberculosis (Protein Data Bank entry 2bvc) with root mean square
(r.m.s.) deviations of 1.3 and 1.5 Å, respectively. The altered structures of the Tyr179 loop in GSI-α compared with GSI-β lead to different pore structures for these enzymes. The function(s), if any, of the pores, however, is not known (Fig. 1E).

GSI-α-Substrate and Transition State Structures Reveal That Large Conformational Changes Are Required for Catalysis—To gain insight into the enzymatic mechanism employed by GSI-α enzymes, we determined the structure of GSI-α bound to the substrates glutamate and AMPPCP (which is a non-hydrolyzable ATP analog) to 2.87 Å (Table 1). The substrate glutamates and AMPPCP molecules bind at each end of the bifunnel. The glutamate substrates are located under the Glu flap. Although better ordered than the apo-structure the Glu flap residues in this structure, as well as the glutamate substrates, display high B-factors (B-factors (average) = 86.7 Å² for the glutamate substrates and 85.0 Å² for Glu flap residues) (Table 3). This suggests that the active site may become protected by Glu flap closure only during catalysis. The glutamate substrate peptide carboxyl and amide groups interact with Arg398 and His245, whereas its side chain carboxyl interacts with a Mg²⁺ ion. The adenine moiety of the AMPPCP is sandwiched between the side chains of Arg331 and Tyr201, whereas hydrogen bonds to the adenine N6, N1, and N7 atoms are provided by the carbonyl oxygen of Ile328 and the side chains of Ser249 and Ser329. Interestingly, the phosphate groups of AMPPCP point away from the active site and are only contacted weakly by Lys44. In fact, density is only observed for the GSI-α and GSI-β-phosphates of the AMPPCP molecules. Hence, AMPPCP does not appear to bind GS productively, which is consonant with ITC data showing that GS binds AMPPCP with 100-fold lower affinity than AMP (Fig. 2). Comparisons of the substrate-bound and apo-GSI-α structures revealed that not only are the subunits and their corresponding active sites essentially unchanged upon substrate binding, but the dodecameric arrangement remains unaffected as well (superimposition of Cα atoms = 0.50–0.60 Å/subunit and 0.60 Å for all Cα atoms of the dodecamers) (Fig. 3, A–D).

Key active site residues are conserved in all classes of GS, suggesting a similar overall catalytic mechanism. According to this reaction scheme, ATP and glutamate first bind in the active site at each end of the bifunnel. The bound ATP γ-phosphate is polarized by the metal ions and an arginine residue (corresponding to B. subtilis GSI-α Arg316), which facilitates nucleophilic attack by the bound glutamate substrate to yield γ-glutamyl phosphate (Fig. 3A). The next step in the reaction involves proton abstraction of ammonium by the Asp50 aspartic acid to create the ammonia. The ammonia then reacts with the γ-glutamyl phosphate to form the tetrahedral transition state. The final step is dissociation of the phosphate from the intermediate and concomitant abstraction of the proton from the γ-amino group of the intermediate by the Glu flap glutamate (B. subtilis Glu304).

GSI-β structures have defined the residues and specific active site architecture optimal for catalysis (3). At first glance, the minimal shifts in the active site regions of the apo- and substrate-bound B. subtilis GS structures appear to suggest that GSI-α catalysis may also involve only moderate active site loop movements. However, close examination of the apo- and substrate-bound GSI-α active sites reveal that they lack an optimal

**TABLE 3**

| Structure        | Glu flap B (av) | Substrate B (av) |
|------------------|----------------|------------------|
| Apo              | >100           | NA               |
| Glutamate-bound  | 85             | 86.7             |
| Transition state | 24.0           | 20.1             |
| Glutamine-bound  | 35.9           | 33.0             |

*Not applicable.*

**FIGURE 2.** Isothermal titration calorimetry thermograms of AMP and AMPPCP binding to GS. A, AMP binding to GS, $K_d = 7.5 \pm 0.3 \mu M$. B, AMPPCP binding to GS, $K_d = 667 \pm 21 \mu M$. 
organization of catalytic residues. This is largely due to the deep insertion of the latch, in particular residues Arg62 and Glu65, into the neighboring active site of each subunit. Arg62 blocks and prevents the correct positioning of the catalytic Asp53 as well as Ser56, which stabilizes the Glu flap. Moreover, the side chain of Glu65 overlaps the location of the key catalytic arginine, Arg316, which assists in phosphoryl transfer. In fact, in the apo- and substrate-bound GSI-α structures, the side chain of Arg316 is located entirely outside the active site.

The non-optimal active site organization in the apo- and substrate-bound GSI-α structures suggests that either a different catalytic mechanism may be at play in GSI-α and GSI-β proteins or that significant structural changes must occur during catalysis. To address this question, we generated a GSI-α transition state complex by reacting the protein with Met-Sox, ATP, and Mg²⁺ and crystallized and solved the structure to 

\[ R_{work}/R_{free} = 20.3\%/23.2\% \] to 2.58 Å (see “Experimental Procedures”) (Table 1). Met-Sox-P mimics the GS transition state as its methyl group occupies the ammonium substrate binding site and forms a stable complex with the enzyme that precludes the last step, which is release of the phosphate from the intermediate and concomitant abstraction of the proton from the intermediate γ-amino group by Glu flap residue Glu304. The structure revealed that the expected catalysis had occurred, leading to Met-Sox-P and ADP creation (Fig. 3B).
GS enzymes all utilize two Mg$^{2+}$ ions to aid substrate binding, transfer of the phosphate group, and structural stability. In the GSI-α-transition state structure, a third metal ion is found, which probably functions in neutralizing the negative charge arising from the intermediate. A similarly located metal ion was identified in the M. tuberculosis GSI-β-Met-Sox-P and maize GSI-phosphinothricin structures (5, 8). Contacts to the adenine and ribose moieties of ADP in the transition state complex are essentially identical to those made to AMPPPCP. In the transition state complex, however, the phosphate groups are rotated into the active site near the Met-Sox-P and are contacted by Arg$^{321}$ and Arg$^{331}$ as well as a Mg$^{2+}$ ion. The β-phosphate lies close to the phosphate transferred to Met-Sox to form Met-Sox-P. There are numerous contacts from GSI to the Met-Sox-P. Arg$^{398}$ interacts with the carboxyl moiety, and the Met-Sox-P amide nitrogen interacts with the Glu$^{134}$ side chain as well as the carbonyl of Gly$^{311}$. The Met-Sox-P phosphate moiety is surrounded by multiple basic residues, including Arg$^{316}$, Arg$^{321}$, Arg$^{335}$, and His$^{245}$ (Figs. 3, C–E, and 4). Glu flap residue Glu$^{304}$ contacts Asp$^{53}$ and is within close distance to the Met-Sox-P methyl group. The Glu$^{304}$–Asp$^{53}$ interaction probably helps to stabilize and shield the Met-Sox-P from attack by bulk solvent as well as facilitating proton abstraction of the ammonium to form ammonia. The resultant ammonia is then in an ideal position to attack the γ-glutamyl phosphate intermediate. Hence, the GSI-α-Met-Sox-P-ADP complex mimics the tetrahedral adduct before the generation of glutamine and inorganic phosphate products.

Comparison of the transition state structure with the apo- and substrate-bound forms revealed dramatic conformational changes, consonant with the finding that the residues in the transition state structure are optimally positioned for catalysis. The largest structural changes are located on the active site loop regions (r.m.s. deviations of apo- /substrate-bound versus Met-Sox-bound subunits = 1.8–2.0 Å, whereas the r.m.s. deviations are 0.8 Å when the active site loops are not included). Moreover, superimposition of the transition state dodecamer onto the apo- or substrate-bound dodecamers results in r.m.s. deviations of 2.5–3.0 Å, indicating that the catalytically induced structural changes are transmitted between subunits, leading to significant alterations in the overall dodecamer structure (Fig. 3D). Although all of the GSI-α active site loops are altered upon transition state formation, residues in the Asp$^{501}$ loop undergo the most dramatic structural rearrangements (r.m.s. deviations = 3.7–4.0 Å for superimposition of the Asp$^{501}$ loops of the transition state and glutamate-bound structures). As noted, the Asp$^{501}$ loop is wedged into the active site in apo- and substrate-bound structures, preventing an optimal configuration of catalytic residues. The restructuring of the active site that takes place during catalysis results in a functional arrangement of catalytic residues (Fig. 3, C and E). These extensive structural changes include the unfolding of one turn of the helix from residue 53 to 59. The structural changes in the Asp$^{501}$ loop lead to the expulsion of Glu$^{65*}$ and Arg$^{621}$ more than 10 Å from the active site to the solvent-exposed surface of the dodecamer (Fig. 3E). Concomitantly, the Arg$^{316}$ loop moves by ~10 Å, resulting in relocation of the Arg$^{316}$ side chain into the position vacated by Glu$^{65*}$. These alterations create an active site organization that allows GSI-α to employ the same general catalytic mechanism used by all characterized GS proteins (3).

**Demonstration that B. subtilis GS Is Cooperative Using Mixed Oligomers**—The structural changes that take place during GSI-α catalysis are found in all the active sites and result in an altered dodecamer structure, suggesting that the enzyme may be cooperative, whereby binding and catalysis at one active site is affected by adjacent subunits. Such cooperativity has been indicated by previous studies revealing active site coupling with E. coli GS (45). However, to directly assess GSI-α cooperativity, we developed a mixed dodecamer assay. Previous studies showed that single E304A and A305G mutations abrogated GS activity (40). Thus, in this assay, WT GS subunits were mixed with inactive, double GS(E304A/A305G) mutant subunits in the presence of 2 M urea, allowing subunit exchange. The mixture was then dialyzed into urea free buffer resulting in the formation of mixed oligomers. 2 M urea treatment did not cause denaturation as assessed by circular dichroism but broke up subunit-subunit interactions (see “Experimental Procedures”) (Fig. 5). To assay possible effects of urea treatment on GS function, we first tested the activity of WT GS and WT GS that had undergone the identical urea treatment as the mixed oligomer. As shown in Table 4, this procedure had no effect on GS enzyme activity. If GSI-α is not cooperative, a mixed WT GS-GS(E304A/A305G)-containing dodecamer should display either WT or only slightly reduced enzyme activity. By contrast, if the dodecamer is cooperative, then incorporation of even a few GS(E304A/A305G) subunits into the dodecamer should impair or abrogate activity for a cooperative enzyme. The mixture used in the assay depicted in Fig. 5 contained between a 3- and 4-fold excess of the WT protein (lower molecular weight band) compared with the mutant. Thus, this experiment provided a strong test case of cooperativity. Even under these conditions, where there is far less mutant than WT, the activity was abrogated, supporting the hypothesis that the dodecamer is cooperative (Table 4).
Molecular Basis for GSI-α-specific Gln Feedback Inhibition—GSI-α enzymes are distinct from their GSI-β counterparts in that they are feedback-inhibited by the reaction product, Gln. Previous GSI-β-Gln structures showed no interactions between Gln and side chains of the Glu flap, and the bound glutamine and Glu flap were largely disordered in the structure (3). Indeed, this Glu flap flexibility was suggested as being important in facile product release following catalysis. The finding that GSI-α enzymes are feedback-inhibited by Gln suggests that differences must exist in GSI-Gln isoenzyme structures. It also indicates that important distinctions must be present between GSI-α-Glu and GS-α-Gln structures. Understanding the structural basis for GSI-α-Gln feedback inhibition is crucial, because this form of the enzyme serves as a key nexus in the Bacilli nitrogen regulation pathway (29–31). Thus, we next determined structures of B. subtilis GS-Gln-Mg\(^{2+}\) complexes. Two structures were obtained using different crystallization conditions (see “Experimental Procedures”) (Table 2). The two structures are identical and reveal that Gln binds within the Glu flap pocket in a similar location as the substrate glutamate (Fig. 6A). However, there are striking differences between the Gln-bound and Glu-bound states. Unlike the Glu-bound state, in the Gln-bound structure, both the side chain and backbone atoms of Glu flap residues are well ordered; the B-factors (average) for Glu flap residues in the Gln-bound structure are 35.9 Å\(^2\) compared with 85.0 Å\(^2\) for the glutamate-bound form, and the B-factors (average) for the glutamates are 24.0 Å\(^2\) for Glu flap residues and 20.0 and 20.3 Å\(^2\) for ADP and Met-Sox-P, respectively (Table 3). In fact, although not directly comparable due to their differing resolutions, the B-factors of the Glu flap residues in the Gln-bound structures are on par with those in the transition state structure (which have transition state structure (which have a key Arg residue, explaining why they are not subject to Gln feedback inhibition in the logo, residues are colored according to type, basic in blue, acidic in red, hydrophobic in black, and glycine/serine in green).

Thus, we next determined structures of GSI-α-Gln complexes. Two structures were obtained using different crystallization conditions (see “Experimental Procedures”). The two structures are identical and reveal that Gln binds within the Glu flap pocket in a similar location as the substrate glutamate (Fig. 6A). However, there are striking differences between the Gln-bound and Glu-bound states. Unlike the Glu-bound state, in the Gln-bound structure, both the side chain and backbone atoms of Glu flap residues are well ordered; the B-factors (average) for Glu flap residues in the Gln-bound structure are 35.9 Å\(^2\) compared with 85.0 Å\(^2\) for the glutamate-bound form, and the B-factors (average) for the glutamates are 24.0 Å\(^2\) for Glu flap residues and 20.0 and 20.3 Å\(^2\) for ADP and Met-Sox-P, respectively (Table 3). In fact, although not directly comparable due to their differing resolutions, the B-factors of the Glu flap residues in the Gln-bound structures are on par with those in the transition state structure (which have B-factors (average) = 33.0 Å\(^2\) for Glu flap residues and 20.0 and 20.3 Å\(^2\) for ADP and Met-Sox-P, respectively) (Table 3). In the GSI-α-Gln structures, the Asp50 loop adopts the same conformation as that observed in the apo- and substrate-bound
GSI-α forms (Figs. 6B and 7). Remarkably, however, in the GS-Gln structure, Arg$_{62}$ plays a central role in Gln binding and, hence, Gln feedback inhibition. Specifically, the Arg$_{62}$ side chain hydrogen-bonds with the key catalytic residue from the Glu flap, Glu$_{304}$ (Fig. 6B). The stabilized Glu$_{304}$ also makes a tight hydrogen bond to the glutamine Ne atom. This intimate hydrogen bond network ties the Glu flap closed, explaining its well ordered state in this structure (Fig. 6B). The vital role played by Arg$_{62}$ in Gln feedback inhibition probably explains why, unlike GSI-β enzymes, the GSI-α Asp$_{50}$ loop adopts distinct structures in different states. Asp$_{50}$ loop insertion into the active site in GSI-α is critical for Gln feedback inhibition, but it can then be readily ejected in the absence of the Gln-specific hydrogen bond network to allow catalysis. In this way, the Asp$_{50}$ loop acts as a GSI-α-specific regulatory trigger.

The finding that Glu$_{304}$ directly interacts with Gln provides a molecular explanation for previous studies showing that E304D or E304A mutants were defective in Gln feedback inhibition (40). The intricate Arg$_{62}$–Glu$_{304}$–Gln hydrogen bond linkage also explains why GSI-α, but not GSI-β, enzymes are feedback-inhibited by Gln because sequence alignments of GS proteins show that Arg$_{62}$ is completely conserved in GSI-α enzymes but not GSI-β enzymes, where this position is typically Gly or Ala (Fig. 6B). Thus, these structures demonstrate that B. subtilis GS uses a residue found only in GSI-α enzymes, Arg$_{62}$, along with a conserved catalytic residue (Glu$_{304}$) to mediate glutamine binding. The structures also reveal the basis for Gln feedback inhibition, because the Arg$_{62}^{-}$–Glu$_{304}$–Gln hydrogen bond network locks the Glu flap in a closed state, preventing Gln release, substrate binding and catalysis.

The Role of Arg$_{62}$ in GS Activity and Regulation; Kinetic, Enzymological, and in Vivo Studies—Our structures indicate that Arg$_{62}$ plays a key role in GSI-α-specific Gln feedback inhibition. The structures also suggest that Arg$_{62}$ is not involved in catalysis and, in fact, must be ejected from the active site during transition state formation. To test these structure-based hypotheses, an R62A mutant was constructed. The purified R62A enzyme had only small differences (less than 2-fold) in its steady-state kinetic constants compared with WT, indicating that it does not play a significant role in substrate binding or catalysis, consistent with our structural data (Table 5). Unlike the E304A substitution in GS, the R62A replacement did not increase the $K_m$ for ammonium (Table 5). This result is again consistent with the structural data and argues that Arg$_{62}$ is not part of the ammonium substrate binding site. In contrast to E304A, which is highly resistant to Met-Sox inhibition, the R62A enzyme has a sensitivity to Met-Sox that is similar to that of WT GS (Table 6). These data, again, support the idea that Arg$_{62}$ is not involved in the GS reaction mechanism. Strikingly, however, and consistent with our structural findings, the R62A mutant was highly resistant to Gln feedback inhibition (Table 6). The R62A substitution was also found to increase the resistance to AMP inhibition. We have argued previously that there is an interdependence between the glutamate (glutamine) and ATP (AMP) binding sites (40). The activities of the nitrogen transcription factors TnrA and GlnR are regulated by the feedback-inhibited form of GS (29–31). TnrA- and GlnR-regulated genes were shown previously to be expressed constitutively in mutants encoding feedback-resistant GS enzymes (29–31). The contribution of the GS Arg$_{62}$ residue to the in vivo regulation of TnrA and GlnR was assessed by constructing a B. subtilis mutant containing an R62A substitution in the chromosomal GS gene (glnA) (see “Experimental Procedures”) (40). Gene reg-

**TABLE 5**

Enzymatic constants for wild-type and mutant GS proteins

The kinetic constants were determined for the Mg$^{2+}$-dependent biosynthetic reaction. All assays were performed at least twice. The uncertainty is the S.E. from the nonlinear regression analysis of the data.

| Enzyme  | $K_m$ Glutamate | $K_m$ ATP | $V_{max}$ Ammonium | $V_{max}$ Hydroxylamine | $V_{max}$ Met-Sox |
|---------|----------------|-----------|---------------------|-------------------------|-------------------|
| Wild type | 27 ± 2         | 2.4 ± 0.1 | 0.18 ± 0.03         | 0.83 ± 0.07             | 3.7 ± 0.2         |
| R62A    | 14 ± 2         | 2.3 ± 0.3 | 0.34 ± 0.05         | 1.4 ± 0.2               | 2.9 ± 0.1         |
| E304A   | 3.4 ± 0.2      | 1.2 ± 0.1 | 32 ± 2              | 0.68 ± 0.09             | 1.3 ± 0.1         |

**TABLE 6**

Sensitivities of wild-type and mutant GS proteins to inhibition

The $IC_{50}$ is the inhibitor concentration that reduces enzymatic activity by 50%. The Mg$^{2+}$-dependent biosynthetic reaction was used to measure the inhibition. All values are the averages of at least two determinations. The S.E. values were less than 10% for all values. The values for the wild-type and E304A enzymes are taken from Ref. 40.

| Enzyme  | Glutamate | AMP | Met-Sox |
|---------|-----------|-----|--------|
| Wild type | 2.4       | 0.5 | 0.13   |
| R62A    | >140      | 20  | 0.19   |
| E304A   | >140      | >30 | 26     |
ululation by TnRA and GlnR was abolished in cells encoding GS with an R62A mutation (Table 7). These results indicate that the glnA(R62A) mutant has the in vivo phenotypes expected for a strain encoding for feedback-resistant GS enzyme.

In conclusion, we have captured all key catalytic and regulatory states of the B. subtilis GSI-α enzyme. Surprisingly, these structures reveal heretofore unseen large structural changes during catalysis. GSI-α enzymes are also distinct among GSs in being feedback-inhibited by the product, Gln. Remarkably, our work demonstrates that the catalytic and regulatory features of GSI-α are linked. Specifically, Gln feedback inhibition arises from a hydrogen bond network between Gln, the active site Glu304, and a GSI-α-specific residue, Arg62, from an adjacent subunit. Notably, Arg62 is ejected from the active site as part of the GSI-α active site reorganization during catalysis. These key Arg62 roles were supported by biochemical and in vivo studies; mutation of Arg62 to alanine eliminates Gln feedback inhibition but does not affect catalysis. The extensive conformational changes that take place during catalysis also suggest a cooperative catalytic mechanism, which was supported by a mixed dodecamer enzyme assay. Due to its critical role in nitrogen metabolism, GS is an attractive and validated target for drug design against pathogenic bacteria. The isoenzyme-specific glutamine interaction network uncovered here presents a promising point of intervention in the development of highly specific inhibitors in the treatment of Gram-positive pathogenic bacteria.

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