Atomic Structure of Plant Glutamine Synthetase
A KEY ENZYME FOR PLANT PRODUCTIVITY*3

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Plants provide nourishment for animals and other heterotrophs as the sole primary producer in the food chain. Glutamine synthetase (GS), one of the essential enzymes for plant autotrophy catalyzes the incorporation of ammonia into glutamate to generate glutamine with concomitant hydrolysis of ATP, and plays a crucial role in the assimilation and re-assimilation of ammonia derived from a wide variety of metabolic processes during plant growth and development. Elucidation of the atomic structure of higher plant GS is important to understand its detailed reaction mechanism and to obtain further insight into plant productivity and agronomical utility. Here we report the first crystal structures of maize (Zea mays L.) GS. The structure reveals a unique decameric structure that differs significantly from the bacterial GS structure. Higher plants have several isoenzymes of GS differing in heat stability and catalytic properties for efficient responses to variation in the environment and nutrition. A key residue responsible for the heat stability was found to be Ile-161 in GS1a. The three structures in complex with substrate analogues, including phosphinothricin, a widely used herbicide, lead us to propose a mechanism for the transfer of phosphate from ATP to glutamate and to interpret the inhibitory action of phosphinothricin as a guide for the development of new potential herbicides.

Inorganic nitrogen is an essential, often limiting nutrient for plant growth and development. In most natural soils, nitrate is the major form of inorganic nitrogen. After uptake of nitrate, plants first reduce it to ammonia, and then assimilate it into an organic compound as an amide moiety of glutamine. Because glutamine synthetase (GS)7 catalyzes the very step of assimilation of inorganic nitrogen and because the amide moiety of glutamine is utilized as the donor of amino residue to synthesize a number of essential metabolites such as amino acids, nucleic acids, and amino sugars, glutamine synthetase by plant GS is the cornerstone of plant productivity and thus nitrogen nourishment of all animals on the Earth. For this reason, the importance of plant GS is comparable with that of ribulose-1,5-bisphosphate carboxylase/oxygenase, the carbon dioxide assimilating enzyme (1). Comparison of the primary structures of GSs from prokaryotes and eukaryotes, results in plant GS being categorized as type II, this type commonly occurring in eukaryotes including animals (2). In contrast, type I GS is widely found in prokaryotes (2). The regulatory mechanisms of type I GS activity such as adenyllylation and metabolite feedback have been thoroughly characterized (3). The crystal structures of GS from Mycobacterium tuberculosis (4) and Salmonella typhimurium (5) have been determined and the proteins shown to be dodecameric, with each dodecamer being composed of one identical subunit with a molecular mass of about 52 kDa. Types I and II GS are thought to share a common ancestor but to have diverged into the two types at a very early stage during molecular evolution (2). Only a faint similarity in the primary structures is appreciable between the two types of GS and it seems that no sophisticated regulatory mechanism as seen in Type I GS exists in Type II GS. As no three-dimensional structure of any plant or animal GS has yet been determined, our structural understanding of Type II GS still remains obscure.

Plant GSs are divided into two subtypes with different subcellular localization: GS1 in the cytosol and GS2 in the plastids. GS1 is encoded by a small multigene family, and the GS1 members are further categorized into two groups based on expression profile in response to external nitrogen status, enzymatic property, and physicochemical stability. Maize has five GS1s (GS1a–GS1e) (6, 7), and two representative isoforms that are well characterized namely GS1a and GS1d, show high sequence identity (86%), but show remarkable
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TABLE 1
Data collection and refinement statistics
Numbers in parentheses are for the highest shell.

| Crystal type | ADP/MetSox-P/Mn | ADP/PPT-P/Mn | AMPPNP/MetSox/Mn |
|--------------|----------------|-------------|------------------|
| Data set     | Native II      | Native I    | Hg               |
| Space group  | P2₁           | P2₁         | P2₁              |
| Unit cell dimension (Å) | | | |
| a (Å) | 95.8 | 95.1 | 95.1 | 95.8 | 96.2 | 95.8 |
| b (Å) | 191.0 | 190.6 | 190.7 | 191.0 | 191.0 | 190.9 |
| c (Å) | 118.1 | 118.1 | 118.1 | 118.2 | 118.2 | 117.9 |
| β (°) | 101.5 | 101.5 | 101.3 | 101.4 | 101.3 | 101.2 |
| Wavelength (Å) | 1.0000 | 1.5418 | 1.5418 | 1.5418 | 1.5418 | 1.5418 |
| Resolution (Å) | 50.0-2.63 | 22.0-3.2 | 30.0-3.7 | 50.0-3.3 | 50-3.8 | 50-3.5 |
| Measured | 334,295 | 165,797 | 182,850 | 336,775 | 58,501 | 146,178 |
| Resolution (Å) | 50.0-2.63 | 22.0-3.2 | 30.0-3.7 | 50.0-3.3 | 50-3.8 | 50-3.5 |
| I/σ(I) | 15.8 (2.1) | 8.4 (3.1) | 13.3 (3.3) | 16.7 (4.5) | 8.2 (3.0) | 12.0 (3.2) |
| Completeness (%) | 87.1 (65) | 98.1 (99.1) | 99.0 (100) | 99.6 (100) | 99.6 (100) | 83.1 (87.2) |
| Rsym (%) | 6.4 (38) | 9.0 (26) | 12.2 (26) | 9.3 (32) | 14.5 (35.5) | 9.5 (29.3) |

Phasing statistics

| Resolution (Å) | 26.13-2.63 | 27.36-3.81 | 33.50-3.50 | 27.36-3.81 |
| Protein atoms | 27,460 | 27,460 | 27,460 | 27,460 |
| Ligand atoms | 450 | 450 | 450 | 450 |
| Water molecule | 721 | 238 | 42 | 238 |
| Rsym (%) | 19.5 (21.4) | 18.4/21.9 | 16.5 (22.5) | 18.4/21.9 |
| Root mean square deviations |
| Bond lengths (Å) | 0.016 | 0.020 | 0.015 | 0.020 |
| Bond angles (°) | 1.669 | 1.763 | 1.051 | 1.763 |

REFINEMENT STATISTICS

| a | b | c | α | β | γ |
|---|---|---|---|---|---|
| 118.1 | 191.0 | 95.8 | 95.1 | 118.1 | 101.5 |
| 118.1 | 191.0 | 95.1 | 95.1 | 118.1 | 101.5 |
| 118.1 | 191.0 | 95.1 | 95.1 | 118.1 | 101.5 |

| Rmerge (%) | 15.8 (2.1) | 8.4 (3.1) | 13.3 (3.3) | 16.7 (4.5) |
| Rsym (%) | 6.4 (38) | 9.0 (26) | 12.2 (26) | 9.3 (32) |

Preparation of Recombinant Maize GS Proteins—Escherichia coli strain JM109 cells transformed with GS1a, GS1d, and various mutant GS genes were grown in Luria broth supplemented with 50 µg/ml ampicillin for 2 h (4 h for crystallization) at 37 °C after inoculation with an overnight seed culture at 1% volume. Isopropyl-β-D-thiogalactoside was then added to a final concentration of 1 mM, and cultivation was continued for a further 12 h. The cells were harvested by centrifugation at 4,000 × g for 10 min and stored at −30 °C until use. Maize GS1a purification for crystallization were performed as described previously (7).

Crystalization—GS crystals were grown at 20 °C in 2–3 weeks by the hanging-drop vapor diffusion method. GS crystals in complex with ADP and methionine sulfoximine phosphate (MetSox-P) were obtained by mixing 4 µl of protein solution (17.5 mg/ml) with 4 µl of reservoir solution (9% (w/v) polyethylene glycol 8000 (PEG8000, Hampton Research), 100 mM Tris-HCl (pH revealing the reaction mechanism of phosphate transfer and inhibitory mechanism of PPT used as a herbicide.

DIFFERENCES IN STABILITY (7). We chose GS1a for an initial trial for three-dimensional structure determination because of its high stability (7). We report the role of Ile-161 responsible for differences in heat stability between the two GS isoforms, and three complex structures of maize GS1a

FIGURE 1. Fₜₒ₋₁ₘᵦ omit electron density maps (blue) for ligands bound to maize GS1a and NCS averaged anomalous difference Fourier maps. a, models and electron density map of AMPNP, MetSox, and Mn²⁺ in the AMPPNP/MetSox/Mn crystal. Substrate analogues and Mn²⁺ ions were omitted in the calculation of the Fₜₒ₋₁ₘᵦ omit maps (blue). The electron density is not NCS averaged. The omit maps are contoured at 4σ. NCS averaged anomalous difference Fourier maps from CuKα radiation shows electron density for Mn²⁺ (pink spheres). The contours level of anomalous maps is at 18 σ. Carbon, oxygen, nitrogen, phosphorus, and sulfur atoms are colored gray, red, blue, salmon, and yellow, respectively. b, ADP, MetSox-P, and Mn²⁺ in the ADP/MetSox-P/Mn crystal. Graphical representation is the same as in panel a. The contour levels of omit and anomalous maps are at 5.5 σ and 8 σ, respectively. c, models and electron density map of ADP, PPT-P, and Mn²⁺ in the ADP/PPT-P/Mn crystal. Graphical representation is the same as panel a. The contour levels of omit and anomalous maps are at 5.5 σ and 8 σ, respectively. These figures were produced using the program BOBSCRIPT (31).
7.8), 5% (v/v) 2-methyl-2,4-pentanediol, 10 mM MnCl₂) containing 1 mM ATP and 1 mM MetSox. GS crystals in complex with ADP and phosphinothricin phosphate (PPT-P) and with AMPPNP and MetSox were obtained the same way as above except using a reservoir solution containing 1 mM ATP and 1 mM PPT, and 1 mM AMPPNP and 1 mM MetSox, respectively. The crystals were harvested in a solution comprising 17.5% (w/v) PEG8000, 100 mM Tris-HCl (pH 7.8), 5% (v/v) 2-methyl-2,4-pentanediol, 10 mM MnCl₂, and 1 mM substrate analogues. Heavy atom derivatives were obtained by soaking native crystals for 12 h in the harvest solution containing 1 mM K₂PtCl₄ or 1 mM HgCl₂. Each crystal was mounted in a glass capillary before data collection.

**Structure Determination**—Diffraction data, except native data set II, were collected in-house with a Rigaku FR-E Super Bright rotating anode x-ray generator at a wavelength of 1.5418 Å (CuKα) using a RAXIS VII (Rigaku) imaging plate detector. High resolution data (native II) were collected using synchrotron radiation on beamline BL5A at the Photon Factory (Tsukuba, Japan) with a CCD detector Quantum 315 (ADSC, Poway, CA). All data collections were performed at a temperature of 295 K because cryocooling of crystals degraded diffraction resolution. Indexing, integration, and scaling of all diffraction data sets were performed using the program HKL2000 (8). Data collection statistics are summarized in Table 1. Native I, mercury, and platinum data sets were used for phase calcula-
tion by multiple isomorphous replacement with anomalous scattering using the program SOLVE (9). Density modification, model building, and refinement were done with RESOLVE (10, 11), O (12), and REFMAC5 (13). In subsequent refinement, native data set I was replaced with native data set II. NCS restraints were applied among 10 subunits throughout refinement. ADP, MetSox-P, and Mn$^{2+}$ ion models were fitted into the substrate binding sites based on the difference electron density map (Fig. 1). Three Mn$^{2+}$ positions per subunit were confirmed in NCS averaged anomalous difference Fourier maps (Fig. 1) from CuK$_{α}$ radiation. Omit maps for residues 202–237 in chain A were depicted in Supplementary Fig. S1. Figs. 2, 4, and 7A were prepared with MOLSCRIPT (14) and RASTER3D (15). Fig. 3, a and b, were prepared with PyMOL. Structure-based sequence alignment with M. tuberculosis GS was performed using the program MATRAS (16).

Construction of GS1a–GS1d Chi-mera and Site-directed Mutagenesis—E. coli JM105 (supE endA sbcB15 hsdR4 rpsL thi Δ(lac-proAB) F$^{[traD36 proAB^{+} lacI^{q} lacZ^{+}] M15}$) was used for the construction of plasmids carrying GS1a–GS1d chimeric genes. Plasmid pTrcGS1a–GS1d was constructed from pTrcGS1a and pTrcGS1d (7). In pTrcGS1a–GS1d, GS1a and GS1d genes are located in tandem in the same orientation under control of the Ptrc promoter. The plasmid was linearized by digestion with KpnI and EcoRI, which are located between the two GS genes, and transformed to E. coli JM105. The transformants were incubated on a Luria-agar plate containing 50 μg/ml ampicillin. Only recombined and circularized plasmid(s) were rescued by the ampicillin selection. The colonies were randomly selected and restriction analysis of the chimeric GS gene was performed to classify the recombinant sites. Finally, nucleotide sequencing of the clones was performed to determine the recombined site.

GS mutants that have one amino acid substitution were constructed by an overlap extension method by two-step polymerase chain reaction using a combination of two terminal primers and a pair of two mutagenic primers. All mutation sites and the sequence integrity of the entire coding region of GS were confirmed by DNA sequencing.

Enzyme Assay—The preparation of crude extracts of E. coli cells was performed as described previously (7). The activities of GS were determined by the methods of Cullimore and Sims (17). One unit of GS activity was defined as the amount of enzyme that produced 1 μmol of γ-glutamylhydroxamate per min under the conditions of the assays of GS activities.

RESULTS AND DISCUSSION

Overall Structure—Three different crystal forms of maize GS1a in complex with ADP and glutamate analogues were prepared in the presence of Mn$^{2+}$, a divalent cation essential for GS activity (3). Substrate combinations were ADP and MetSox-P (18) (ADP/MetSox-P/Mn), AMPPNP and MetSox (AMPPNP/MetSox/Mn), and ADP and PPT-P (19) (ADP/PPT-P/Mn). Crystal structures of the three GS1a derivatives were determined at 2.63-, 3.50-, and 3.80-Å resolutions, respectively.
(Table 1). Electron density maps in these complex data sets were extremely clear (supplementary Fig. S1, Fig. 1) considering resolution limits, revealing features of most side chains and the detail of the substrate/substrate analogue structures for the crystals of AMPPNP/MetSox/Mn and ADP/PPT-P/Mn. High quality electron density maps in all complex data sets led to the final R<sub>free</sub> and R<sub>work</sub> values almost equal to that of high resolution crystal analysis (Table 1), hence allowing our detailed discussion of the reaction mechanism for phosphate transfer described below.

The maize GS crystal structure is decamer with dimensions of 115 Å × 115 Å × 95 Å, having 52 symmetry with five 2-fold axes perpendicular to a 5-fold axis (Fig. 2, a and b). The decameric structure is composed of two face-to-face pentameric rings of identical subunits, with a total of 10 active sites, each formed between every two neighboring subunits within each ring (Fig. 2a).

The early study indicated the octamer structure for eukaryote GS derived from electron microscope observations, which reported that one octamer structure was composed of two tetramer rings (20, 21). We previously reported a solution experiment (7), in which the gel filtration elution pattern of maize GS1a showed a single peak estimating a total molecular mass at 440 kDa in solution. The total molecular mass of decameric maize GS1a without metal ions or substrates/substrate analogues is 398 kDa, which approximately correspond to that estimated with gel filtration. The structural description only from our crystal structure analysis may raise a possibility of pentamer structure in solution, and of an artificial decameric formation from crystal packing. The solution experiment supports the decamer structure in solution in which the accessible surface area between the two pentamer is small (933 Å) but is thought to be sufficiently plausible.

Subunit Structure—The structure of one subunit consists of a smaller N-terminal domain (residues 1–103) and a larger C-terminal domain (residues 104–356), with the N-terminal domain of one subunit and the C-terminal domain of the neighboring subunit forming an active site (Fig. 2c). Structure-based sequence alignment with M. tuberculosis GS shows sequence identity of 22.2% (Fig. 3c) and secondary structure identity of 84.2% ([α-helix], [β-strand], or coil) for structurally corresponding 356 residues, whereas the root mean square deviation of the 356 C<sub>α</sub> atoms between aligned residues is moderate (3.92 Å), indicating the two structures have diverged from the common ancestor. The overall fold of the maize GS structure is similar to those of M. tuberculosis and S. typhimurium, but there are several large differences described as follows. Residues of M. tuberculosis GS lacking their counterparts in maize GS are mainly located at the molecular surface of M. tuberculosis GS. The C-terminal residues (residues 393–478) of M. tuberculosis lacking their counterparts in maize GS play a role as the adenylylation site and contribute to intimate interactions between two hexamer rings (helical thong). Residues 143–154 of M. tuberculosis GS lacking in maize GS are those of the [β-loop contributing to the hydrophobic interactions of the two hexamer rings. The conformations of maize GS and M. tuberculosis GS differ significantly for residues of 1–18 (1–17) and 145–152 (158–180) with residue ranges in parentheses for M. tuberculosis GS. Most of residues for binding substrate and divalent ions are conserved between maize and bacterial GS and hence the enzyme reaction mechanism of maize GS is likely to be essentially the same as that of bacterial GS. Each active site contains three Mn<sup>2+</sup> atoms in the middle of the catalytic cleft (Fig. 2c).

Structural Difference between Plant and Bacterial GSs—Remarkable differences between plant and
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bacterial GSs are notable in the mode of inter-ring subunit contact. Interface surface area between the two pentameric rings of plant GS is 933 Å², which is about 17 times smaller than that of the hexameric rings of bacterial GS (17,238 Å²) (Fig. 3, a and b). The inter-ring interaction is achieved by five pairs of two subunits, whose centroids are apart by 67.5 degrees with 5-fold axis (Fig. 2, a and b), and thus subunit interaction becomes very limited: only 4 hydrophobic and 2 hydrogen bonding interactions (supplementary Fig. S2). In contrast, the inter-ring interaction of the bacterial enzyme is much stronger: centroids of interacting subunits are totally overlapped with 6-fold axis and the two subunits are stabilized by 37 hydrophobic and 36 hydrogen bonding interactions contributed by 43 residues (supplementary Fig. S2). Plant GS has several internal deletions and a C-terminal truncation in comparison with the bacterial enzyme (Fig. 3c). Such structural differences occurring during molecular evolution have resulted in the appearance of unique quaternary structures, which have given rise to fundamental differences in the ways of regulation of activity of type I and type II GSs.

Substrate Binding Site—The GS decamer contains 10 active sites and each active site is located between two adjacent subunits in a pentamer. The active sites (20 Å deep) are formed between two neighboring monomers in a ring where its opening is roughly in parallel to the 5-fold axis (Fig. 2a). Well defined electron densities even without NCS averaging for each of ADP, AMPPNP, Mn²⁺, MetSox, phosphorylated MetSox, and phosphorylated PPT was found in all substrate binding sites in a decamer, respectively (Fig. 1). The ADP binding sites in the ADP/MetSox/P/Mn and ADP/PPT-P/Mn complex structures, and the AMPPNP binding sites in the AMPPNP/MetSox/Mn complex structure each are located near the openings in the 10 catalytic clefts. The phosphorylated MetSox (P-MetSox) binding sites were found at the bottoms of the 10 clefts. Three Mn²⁺ ions lie at the middle of the cleft. Three Mn²⁺ ions are called Mn-n1, Mn-n2, and Mn-n3, respectively, corresponding to the numbering of M. tuberculosis by Krajewski et al. (4) (Fig. 4). The positions of the three Mn²⁺ were confirmed by CuKα anomalous difference Fourier maps (Fig. 1). The ADP molecule in the ADP/MetSox-P/Mn complex are bound by Gly-127, Ser-187, Asn-251, Ser-253, Tyr-328, and Arg-316, through hydrophobic and hydrogen bonding interactions, and its β-phosphate is bound by Mn-n2 and Mn-n3 through coordination bond interactions (Mn-n2 and Mn-n3 coordinate phosphate oxygen atoms)(Fig. 4b). The P-MetSox molecule is bound mainly by the main chain of Gly-245 and the side chains of Glu-131, Glu-192, His-249, Arg-291, Arg-311, and Arg-332 through hydrogen bond interactions in addition to three Mn²⁺ ions (Fig. 4b). The phosphate group of the P-MetSox coordinates to the three Mn²⁺. The P-PPT is bound by the same protein residues and three Mn²⁺ ions as those of the P-MetSox (Fig. 4c). Interactions between AMPPNP and protein residues in the active site are approximately the same as those of GS1a in complex with ADP except for the γ-phosphate moiety of the AMPPNP (Fig. 4a). Similarly, interactions between P-MetSox and protein residues in the active site resemble those of GS1a in complex with MetSox except the phosphate part of the P-MetSox (Fig. 4b). Mn-n1 is coordinated by Glu-131, Glu-192, and Glu-199 in the three complex structures, and is further coordinated by γ-phosphate O of AMPPNP and δ-N of MetSox (AMPPNP/MetSox/Mn), phosphate-O and δ-N of P-MetSox (ADP/MetSox-P/Mn), and phosphate-O and δ-O of P-PPT (ADP/PPT-P/Mn). Mn-n2 is coordinated by Glu-129, His-249, Glu-330, and β-phosphate O of ADP or AMPPNP in the three complex structures, and further is coordinated by γ-phosphate O of AMPPNP (AMP- PNP/MetSox/Mn), phosphate O of P-MetSox (ADP/MetSox- P/Mn), and phosphate O of P-PPT (ADP/PPT-P/Mn). Mn-n3 is coordinated by Glu-129, Glu-199, and α-phosphate O of ADP or AMPPNP in the three complex structures, and in addition is coordinated by γ-phosphate O and Nγ of AMPPNP (AMP- PNP/MetSox/Mn), and β-phosphate O of ADP and phosphate O of MetSox-P (ADP/MetSox-P/Mn), and β-phosphate O of ADP and phosphate O of P-PPT (ADP/PPT-P/Mn). (Fig. 4).

In comparison with Type-I (M. tuberculosis), the GS active site structure in complex with ADP, Mg, and P-MetSox, residues interacting with ADP are significantly different from those of maize GS, although, residues interacting with metal ions and MetSox-P are conserved (4).

Structural Determinant of Stability of GS1a—Higher plants have isoenzymes for cytosolic GS1 with distinctive enzymatic properties. The presence of such isoenzymes is considered to be crucial for efficient ammonia assimilation in response to variable internal and external environments (7, 22, 23). We reported significant differences in heat stability and catalytic properties between maize GS1a and GS1d (7). Under low concentrations of divalent metal cations and high temperature, GS1d is easily inactivated as is the case for the bacterial GS from E. coli (3, 24). This inactivation is caused by dissociation of the oligomeric assembly of the enzyme (3, 7, 24). Even under such conditions, the activity and oligomeric structure of GS1a remains unchanged (7). As GS1a is very similar to GS1d in terms of primary structure (86% identical), we hypothesized that a small region or specific amino acid residue(s) would contribute to this physicochemical difference. To find such a region, we prepared GS1a–GS1d chimeric enzymes (Fig. 5a).

Of six chimeric proteins, in which the N-terminal region from GS1a and the C-terminal region from GS1d were fused at various points, a dramatic change of stability was observed when the GS1a region was longer than 146 amino acid residues. However, no further significant increase of stability occurred when the GS1a region was extended beyond 186 amino acid residues (Fig. 5, a and b). This result shows that the region from 146 to 186 is critical for stability. There are seven amino acid substitutions in this region between GS1a and GS1d. We changed these seven positions of GS1a separately to the corresponding residue of GS1d to determine that amino acid resi-
due(s) are critical for the stability difference. As shown in Fig. 5c, a single change of Ile-161 to Ala resulted in a drastic decrease in the stability of GS1a. Conversely, substitution of GS1d Ala-161 to Ile increased the stability to a level comparable with GS1a (Fig. 5, d and e). When these mutant GSs were subjected to gel filtration chromatography, unstable GS species were found to be easily dissociated into monomers (data not shown). These combined results strongly suggest that the residue at position 161 is a major determinant for differentiating the physicochemical property of maize GS1 isoenzymes.

Structural Basis for Differential Physicochemical Property of Plant Two GS1 Isoenzymes—In our GS1a structure, Ile-161, identified as a key residue for the differential stability (Fig. 5), of one subunit protrudes toward the neighboring subunit and forms extensive inter-subunit hydrophobic interactions with Leu-33', Tyr-219', Glu-222', and Arg-223' (Fig. 2d). Thus, the inter-subunit interaction within each ring is stabilized mainly by hydrophobic forces. It is notable that physiological differentiation of cytosolic GS isoenzymes is achieved by only a single amino acid substitution.

Reaction Mechanism of Phosphate Transfer—The catalytic reaction of GS proceeds in two steps. The first step is transfer of the terminal phosphoryl group of ATP to the side chain carboxyl moiety of the substrate glutamate, producing an activated intermediate of $\gamma$-glutamyl phosphate (25). In the second step, a bound ammonium ion is deprotonated, forming ammonia, which then attacks the carbonyl carbon of $\gamma$-glutamyl phosphate and subsequently releases a free phosphate to yield glutamine (25). In contrast to glutamate, MetSox, a noncovalent inhibitor of GS, is irreversibly phosphorylated and stops the catalytic reaction at the first step (18). Our crystal structures in complex with substrate or MetSox enable us to visualize the actual intermediate state structure of the
phosphoryl transfer (Fig. 4, a–c). Enzyme structures in complexes with AMPPNP/MetSox/Mn and ADP/MetSox-P/Mn correspond to the substrate-binding structures before and after the phosphoryl transfer, respectively. Between the two structures, there are no significant differences in the arrangements of the amino acid residues, Mn$^{2+}$, cofactor, or substrate analogues located in the active site, except for the 0.9-Å transposition of the phosphorus atom (Fig. 6). Considering that the distance between entering and leaving oxygens in this reaction is as short as 4.5 Å, we conclude that the phosphoryl transfer occurs without appearance of free orthophosphate by a partial associative mechanism (26, 27) through a transition state involving a trigonal bipyramidal transition structure, and therefore, the phosphorous atom undergoes a steric change (Fig. 6).

**Modulation of the Substrate Binding**—Many glutamate analogues are known to show inhibitory effects on GS activity with different $K_i$ values. The inhibitors 3-amino-3-carboxypropane sulfanamide and 2-amino-4-phosphonobutyric acid, which have $K_i$ values 50 times weaker than MetSox and PPT, have a carbonyl oxygen atom and a hydroxyl group in the positions corresponding to the $\delta$-methyl group of MetSox and PPT, respectively (28). 4-N-Hydroxy-L-2,4-diaminobutyric acid, which has no side group at the corresponding position, shows further stronger inhibition (26) than 3-amino-3-carboxypropane sulfanamide and 2-amino-4-phosphonobutyric acid. In our crystal structures of GS in complexes with MetSox-P and PPT-P, the $\delta$-methyl group is located close to Glu-297 and Asp-56’ with a distance of around 3.5 Å (Fig. 7a). Glu-297 and Asp-56’ are conserved among all GSs (Fig. 7b) and play a role as a flap to guard the substrate glutamate entrance to the active site and to bind ammonium substrate, respectively. The impor-
tance of Glu-297 and Asp-56 for plant GS activity was shown by site-directed mutagenesis (29, 30). Consideration of side chain structure corresponding to the δ-methyl group of Met-Sox and PPT in combination with GS polypeptide structure around Glu-297 and Asp-56 is important to obtain novel inhibitory drugs. Another important aspect for inhibitor design is consideration of the residues interacting with ADP. In comparison with the recent structure of *M. tuberculosis* GS (4), the maize GS1a structure shows a significant difference in this region. Because human GS belongs to type II GSs, this study is also applicable for understanding the reaction mechanism of human GS and in designing novel drugs specific for GS from pathogenic bacteria.

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