Glutathione is essential for maintaining the intracellular redox environment and is synthesized from γ-glutamylcysteine, glycine, and ATP by glutathione synthetase (GS). To examine the reaction mechanism of a eukaryotic GS, 24 Arabidopsis thaliana GS (AtGS) mutants were kinetically characterized. Within the γ-glutamylcysteine/glutathione-binding site, the S153A and S155A mutants displayed less than 4-fold changes in kinetic parameters with mutations of Glu-220 (E220A/E220Q), Gln-226 (Q226A/Q226N), and Arg-274 (R274A/R274K) at the distal end of the binding site resulting in 24–180-fold increases in the $K_v$ values for γ-glutamylcysteine. Substitution of multiple residues interacting with ATP (K313M, K367M, and E429A/E429Q) or coordinating magnesium ions to ATP (E148A/E148Q, N150A/N150D, and E371A) yielded inactive protein because of compromised nucleotide binding, as determined by fluorescence titration. Other mutations in the ATP-binding site (E371Q, N376A, and K456M) resulted in greater than 30-fold decreases in affinity for ATP and up to 80-fold reductions in turnover rate. Mutation of Arg-132 and Arg-454, which are positioned at the interface of the two substrate-binding sites, affected the enzymatic activity differently. The R132A mutant was inactive, and the R132K mutant decreased $k_{cat}$ by 200-fold; however, both mutants bound ATP with $K_a$ values similar to wild-type enzyme. Minimal changes in kinetic parameters were observed with the R454K mutant, but the R454A mutant displayed a 160-fold decrease in $k_{cat}$. In addition, the R132K, R454A, and R454K mutations elevated the $K_a$ value for glycine up to 11-fold. Comparison of the pH profiles and the solvent deuteration effects of A. thaliana GS and the Arg-132 and Arg-454 mutants also suggest distinct mechanistic roles for these residues. Based on these results, a catalytic mechanism for the eukaryotic GS is proposed.
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![Overall reaction catalyzed by GS.](image)

Cluster of acidic amino acids (Glu-148, Asn-150, and Glu-371) coordinates binding of two magnesium ions to ATP. Analysis of the kinetic properties of human GS variants containing mutations found in patients with glutathione deficiency showed that certain residues in the active site, including those corresponding to Arg-132 and Arg-274, are important for enzyme function (20), but their possible catalytic roles have not been examined. Additional mutagenesis studies of human GS focused on four residues, corresponding to Glu-148, Asn-150, Lys-313, and Lys-367, in the ATP-binding site that are highly conserved in other ATP-grasp family members (21). Mutation of these amino acids showed that each was needed for efficient catalysis; however, the role of these residues to ATP binding was not demonstrated. Moreover, a chemical mechanism for the enzyme was not proposed.

To date, a complete analysis of the contribution of active site residues to the reaction mechanism of the eukaryotic GS has not been reported. In this study, we perform site-directed mutagenesis on the 15 amino acids forming the AtGS active site and examine the effect of these mutations on steady-state kinetics, ATP binding, pH dependence of catalysis, and solvent kinetic isotope effects. Overall, GS is sensitive to minor amino acid changes in the active site, which suggests that the enzyme is optimized for catalyzing the ATP-dependent peptide ligation reaction that results in the tripeptide glutathione. Our results, in combination with available structural information, suggest a chemical mechanism for glutathione synthesis in humans, plants, and yeast.

EXPERIMENTAL PROCEDURES

Materials—The QuikChange site-directed mutagenesis kit was purchased from Stratagene. All oligonucleotides were synthesized by Integrated DNA Technologies, Inc. E. coli C41(DE3) cells were purchased from Avidis, Inc. All other reagents were purchased from Sigma.

Mutagenesis, Expression, and Purification—Site-directed mutants of AtGS (R132A, R132K, E148A, E148Q, N150D, N150A, S153A, S155A, E220Q, E220A, Q226N, Q226A, R274K, R274A, K313M, K367M, N376A, E371Q, E371A, E429Q, E429A, R454A, R454K, and K456M) were generated using oligonucleotides containing the desired mutation and the QuikChange PCR method (Stratagene) with the pET28a-AtGS vector and the HEPES reaction buffer was adjusted to pH 7.9 using NaOD or DCl. Proteins were buffer-exchanged into 50 mM HEPES (pD 7.9), 50 mM NaCl, and 5 mM MgCl₂ in D₂O. Isotope effects on $k_{cat}$ and $K_m$ are expressed as $^{13}V = k_{cat}(\text{H}_2\text{O})/k_{cat}(\text{D}_2\text{O})$ and $^{13}V/K = (k_{cat}K_m)_{\text{H}_2\text{O}}/(k_{cat}K_m)_{\text{D}_2\text{O}}$.

Fluorescence Titration of ATP Binding—Binding of ATP to AtGS was determined by monitoring the change in protein fluorescence upon ligand addition. Measurements were made on a Varian Cary fluorimeter ($\lambda_{\text{excitation}} = 280$ nm and $\lambda_{\text{emission}} = 330$ nm; slit widths = 5 nm). Titrations were performed at 25 °C by addition of ATP (1 mM stock) to 0.5 ml of 100 mM HEPES (pH 7.5), 150 mM NaCl, and 20 mM MgCl₂, containing 100 µg of protein. To avoid dilution effects, volume change during the titration was limited to 3% of total volume. Control titrations with buffer alone did not produce any change in emission signal. The $K_d$ value was calculated using Kaleidagraph with the data fit to $\Delta F = (\Delta F_{\text{max}}[L])/(K_d + [L])$, where $\Delta F$ is the change in emission signal in the presence of ligand ([L]), and $\Delta F_{\text{max}}$ is the maximal change in signal. The data were also replotted as a linear transformation of the same equation ($R$ versus $R/[L]$), where $R$ is the fluorescence change. The pH dependence of ATP binding for wild-type AtGS was determined using the 50 mM sodium acetate, 50 mM MES, and 50 mM Tris buffer system in place of HEPES buffer. Data were fit to $\log Y = \log c/(1 + K_d/H)$, as described above (23).
RESULTS

Site-directed Mutagenesis, Expression, and Purification—Twenty four point mutants of AtGS were generated to evaluate the role of each active site residue in catalyzing the formation of glutathione. Wild-type and mutant AtGS was overexpressed in E. coli and purified using a two-step purification procedure (6). All of the mutant enzymes were isolated as soluble homodimeric proteins that displayed the same mobility on SDS-PAGE and elution profile by gel filtration chromatography as wild-type enzyme (not shown).

Effect of Mutations in the γ-Glutamylcysteine/Glutathione-binding Site—Based on comparison with the human and yeast GS, the AtGS γ-glutamylcysteine/glutathione-binding site includes Arg-132, Ser-153, Ser-155, Glu-220, Gln-226, and Arg-274 (Fig. 2). A series of point mutations (R132A, R132K, S153A, S155A, E220Q, E220A, Q226N, Q226A, R274K, and R274A) were used to probe the role of residues in the site. The effect of mutating these amino acids can be grouped into three outcomes as follows: minimal changes in the steady-state kinetic parameters, decreased turnover rate, and increased $K_m$ values for γ-glutamylcysteine (Table 1).

Minor changes in AtGS activity are observed with removal of the side-chain hydroxyl group from either Ser-153 or Ser-155 by mutation to alanine, which results in 2-fold lower $k_{cat}$ values and at most a 4-fold change in $K_m$ values for γ-glutamylcysteine. Mutation of Arg-132 drastically affects the turnover rate of AtGS. The rate of catalysis is decreased 200-fold by mutation of Arg-132 to lysine. The R132K mutant also shows a modest 5-fold increase in the $K_m$ value for glycine. The R132A mutant displayed no enzymatic activity above background using up to
100-fold more protein than used in wild-type assays and over a longer time. To evaluate if the loss of activity was because of compromised ATP binding to AtGS, a fluorescence titration assay was used. Addition of ATP quenches the fluorescence emission signal of AtGS (Fig. 3A). Titration of wild-type enzyme with increasing amounts of ATP yields a binding isotherm with a $K_d = 1.3$ mM (Fig. 3B and Table 2). Previous examination of the random sequential kinetic mechanism of AtGS yielded an estimated $K_m$ of 0.7 mM for ATP binding (6). The R132A and R132K mutants bind ATP with affinity similar to wild-type enzyme (Table 2). The effects of mutating Arg-132 imply a key role for this residue in catalysis.

Mutations of Glu-220, Gln-226, and Arg-274 at the distal end of the binding site alter $\gamma$-glutamylcysteine binding. Removal of the charged side chain of Glu-220 (E220A or E220Q) from the binding site results in a 24-fold higher $K_m$ value for $\gamma$-glutamylcysteine. Nearly 100-fold higher $K_m$ values are observed with substitution of Gln-226 by either an alanine or asparagine residue. Mutation of either Glu-220 or Gln-226 also slows $k_{cat}$ by 4–15-fold. The R274K mutant showed the largest (180-fold) change in the $K_m$ value for $\gamma$-glutamylcysteine with a 24-fold decrease in $k_{cat}$. Mutation of Arg-274 to alanine inactivates the enzyme, although the protein binds ATP with wild-type affinity (Table 2). These results suggest a primary role for Glu-220, Gln-226, and Arg-274 in the recognition of $\gamma$-glutamylcysteine.

**Effect of Mutations in the ATP-binding Site**—Substitution of residues interacting with the magnesium ions in the ATP-binding site (Fig. 2), i.e. Glu-148, Asn-150, and Glu-371, either abrogated enzymatic activity (E148A, E148Q, N150A, N150D, and E371A) or severely reduced the rate of catalysis (E371Q) (Table 3). The loss of activity in the E148A, E148Q, N150A, N150D, and E371A mutants results from the inability to bind ATP, as evaluated by fluorescence titration (not shown). Circular dichroism spectroscopy of these mutants indicates that each protein adopts a similar fold as wild-type enzyme (not shown). Addition of higher magnesium concentrations (up to 100 mM)
did not restore ATP binding or catalytic activity. Replacement of Glu-371 with a glutamine lowered the turnover rate by 80-fold with 4–7-fold increases in $K_m$ for ATP and γ-glutamylcysteine (Table 3). Fluorescence emission changes were observed for the E371Q mutant at 30 μM ATP, but insufficient quenching prevented accurate determination of $K_d$ values (Table 2).

Mutagenesis of residues contacting the bound nucleotide (Fig. 2) produced results similar to those observed for mutations of amino acids coordinating the magnesium ions to ATP (Tables 2 and 3). Binding of ATP was not observed by fluorescence titration for the K313M, K367M, E429A, and E429Q mutants, all of which showed no enzymatic activity, even though each mutant is properly folded as determined by circular dichroism spectroscopy (not shown). Mutation of Asn-376 to alanine and Lys-456 to methionine reduced $k_{cat}$ by 18- and 67-fold, respectively, and increased the $K_m$ values for ATP and γ-glutamylcysteine by 7–14-fold. $K_d$ values for ATP binding to the N376A and K456M mutants could not be accurately measured because of insufficient quenching of the fluorescence emission signal (Table 2). The R454K mutant displayed modest 3–4-fold differences in steady-state kinetic parameters and ATP binding affinity. Replacement of Arg-454 with an alanine resulted in an 80-fold decrease in $k_{cat}$, a 6-fold increase in the $K_m$ values for ATP, a 13-fold decrease in binding affinity for ATP, and an 11-fold increase in the $K_m$ values for glycine.

**pH Rate Profiles for Wild-type AtGS**—The effect of pH on the steady-state kinetic parameters of AtGS for γ-glutamylcysteine, ATP, and glycine was examined (Fig. 4 and Table 4). AtGS displayed the same breakpoint ($pK_a$ ~ 7.3) in the $V$ versus pH profile obtained for each substrate (Fig. 4A), which indicates that deprotonation of a charged species in the enzyme-substrate complex is required for maximal activity. Bell-shaped curves with $pK_a$ ~ 7.2 and $pK_a$ = 9.4–9.7 were observed in the pH profiles of $V/K$ for γ-glutamylcysteine and ATP (Fig. 4B). In contrast, the $V/K$ pH profile with glycine showed an increase with pH and a $pK_a$ = 8.6 (Fig. 4B). The pH dependence of ATP binding was analyzed and showed a decrease in binding affinity with increasing pH (Fig. 4C and Table 4), indicating that a protonated functional group increases binding affinity. The observed breakpoint for ATP binding ($pK_a$ = 9.6) was at a similar pH as the second inflection point observed in the $V/K$ pH profiles for ATP and γ-glutamylcysteine.

**pH Rate Profiles for the Arg-132 and Arg-454 Mutant AtGS**—Given the location of Arg-132 and Arg-454 at the interface between the two substrate-binding sites in the active site (Fig. 2B) and the effects of mutating these residues on catalysis, we examined the pH dependence of the steady-state kinetic parameters for the R132K, R454A, and R454K AtGS mutants (Fig. 5 and Table 5). The R132K mutant displayed curves that rose with increasing pH with $pK_a$ values ranging from 6.4 to 6.6 for the $V$ versus pH and the $V/K$ versus pH curves for each substrate (Fig. 5, A and B). This difference from wild-type enzyme suggests a change in the rate-limiting step of the chemical mechanism. Mutation of Arg-454 to an alanine abolished the pH dependence of $V/K$ for glycine, but the breakpoints for $V$ and $V/K$ for the other substrates were similar to those observed for wild-type AtGS (Fig. 5, A and C). The steady-state kinetic
parameters of the R454K mutant displayed wild-type behavior over the pH range examined (Fig. 5, A and D). The pKₐ values for ATP binding to the R132K, R454A, and R454K mutants were all similar to wild-type AtGS (Table 5), indicating that Arg-132 and Arg-454 are not responsible for the observed inflection point.

Solvant Kinetic Isotope Effects—The solvent deuterium isotope effects on wild-type, R132K, and R454A AtGS were determined (Table 6). With wild-type enzyme and the R454A mutant, similar normal solvent kinetic isotope effects on the turnover rate and V/Κ values were observed. In contrast, mutation of Arg-132 to a lysine, which reduces kₚcat by 200-fold, yielded a mutant enzyme with ΔV and ΔV/Κ values ranging from 1.0 to 1.2, which provides additional evidence that mutation of this arginine alters the rate-limiting step in glutathione formation and that Arg-132 plays an important catalytic function.

DISCUSSION

GS catalyzes the ATP-dependent formation of glutathione, the most abundant thiol peptide in eukaryotic cells (1). Although the three-dimensional structures of the human and yeast GS are known (15, 16), few functional studies of the eukaryotic GS have been reported (6, 19–21). To investigate the structure/function relationships in the eukaryotic GS, we used site-directed mutagenesis of AtGS to probe the role of active site residues. Our examination of AtGS identifies amino acids required for substrate binding and efficient catalysis.

The x-ray crystal structures of the human GS-glutathione:ADP complex and of the yeast enzyme bound with γ-glutamylcysteine and an ATP analog show extensive protein-ligand interactions in the γ-glutamylcysteine/glutathione-
binding site (Fig. 2B) (15, 16). In both structures, the carboxylate group of the \( \gamma \)-glutamyl moiety forms a charge-charge interaction with Arg-274 and hydrogen bonds with Ser-155, Glu-200, and Gln-226 (numbering for AtGS). In addition, a hydrogen bond between Gln-226 and Glu-220 orients the glutamate side chain toward the amide nitrogen of the substrate/product \( \gamma \)-glutamyl group. Ser-153 and Ser-155 also form hydrogen bonds with the amide group of the cysteinyl moiety.

The effect of mutating residues in the \( \gamma \)-glutamylcysteine/glutathione-binding site reveals varied contributions to substrate binding and catalysis (Table 1). For example, removal of the hydrogen bonds contributed by Ser-153 and Ser-55 only modestly reduced catalytic efficiency 10- and 3-fold, respectively, whereas mutations at the distal end of the binding site result in up to 2,600-fold changes in \( k_{\text{cat}}/K_m \). The charged nature of Glu-220 is important for maximal activity, as demonstrated by the effects of the E220A and E220Q mutants. Likewise, the respective 1,200- and 700-fold reductions in \( k_{\text{cat}}/K_m \) values for the Q226A and Q226N mutants are consistent with the dual role of Gln-226 in substrate interaction and orienting Glu-220. A key role for Arg-274 in binding \( \gamma \)-glutamylcysteine is revealed by the 2,600-fold decrease in \( k_{\text{cat}}/K_m \) values, which primarily results from \( K_m \) changes, in the R274K mutant. Likewise, the inactive R274A mutant, which binds ATP with wild-type affinity (Table 2), likely disrupts the bidentate interaction with the substrate carboxylate. The E220A, E220Q, Q226A, Q226N, and R274K mutants increase the \( K_m \) value for \( \gamma \)-glutamylcysteine, but the magnitude of changes in catalytic efficiency indicates that interactions between these residues and \( \gamma \)-glutamylcysteine also contribute to transition state stabilization.

In the human and yeast GS crystal structures, a common set of amino acids along the length of the nucleotide lock the molecule in the binding site (Fig. 2B). The adenine moiety binds in a largely apolar pocket with hydrogen bonds formed between the ribose and Glu-429 and Lys-456 (numbering for AtGS). A series of polar interactions occur along the phosphate tail of the nucleotide with Lys-313, Lys-367, and Asn-376. The \( \alpha \)- and \( \beta \)-phosphate groups of the nucleotide and Glu-148 coordinate one magnesium ion with a second magnesium ion bound by the \( \beta \)- and \( \gamma \)-phosphate groups, Glu-148, Asn-150, and Glu-371. Previously, mutagenesis studies of human GS focused on residues corresponding to Glu-148, Asn-150, Lys-313, and Lys-367, which are conserved in other ATP-grasp family members and showed that substitution of these residues decreased the specific activity of the enzyme (21); however, the effect of the mutations on ATP binding was not reported. Substitution of residues in the ATP-binding site of AtGS results in loss of measurable activity with the E148A, E148Q, N150A, N150D, K313M, K367M, E371A, E429A, and E429Q mutants. Analysis of these mutants by circular dichroism spectroscopy shows a similar overall fold as wild-type enzyme (not shown). Fluorescence titration assays indicate that these mutations compromise ATP binding, which ultimately affects catalysis. The E371Q, N376A, and K456M mutants also exhibit decreased affinity for ATP, as shown in the \( K_d \) and \( K_m \) values, and lowered catalytic efficiencies (Tables 2 and 3). Mutation of Glu-371 to a glutamine removes a negative charge from coordination of the second magnesium ion. Although the E371Q mutant retains the potential to coordinate the magnesium ion, the 80-fold reduction in \( k_{\text{cat}} \) emphasizes how sensitive the active site is to minor structural changes. The elevated \( K_m \) values for \( \gamma \)-glutamlycysteine observed in mutants of the ATP-binding site is consistent with earlier data suggesting that binding of ATP enhances binding of \( \gamma \)-glutamlycysteine, and vice versa (6). Likewise, the lower turnover rates of the N376A and K456M mutants also indicate that amino acids throughout the ATP-binding site stabilize the transition state of the GS reaction.

The eukaryotic GS and the prokaryotic GS are members of the ATP-grasp enzyme family, which includes other ATP-dependent enzymes such as biotin carboxylase and \( \beta \)-alanine-\( \alpha \)-alanine ligase (15–18, 24). Earlier work on human GS and biotin carboxylase examined the functional roles of a handful of conserved amino acids, which based on structural studies were proposed to function in coordinating the magnesium ions and interacting with the phosphate groups of ATP throughout the protein family (21, 24). By probing the role of each residue in the ATP-binding site of AtGS, our results imply that additional amino acids not conserved across the protein family are also critical for nucleotide binding. Because subtle changes in residues interacting with either ATP or the magnesium ions drastically alter binding affinity and turnover rates, it appears that the GS active site is highly evolved for its function.

To examine the AtGS reaction mechanism in more detail, we analyzed the effect of pH changes on the steady-state kinetic parameters for each substrate (Fig. 4 and Table 4). The overall GS reaction involves transfer of the \( \gamma \)-phosphate group of ATP to the C-terminal carboxylate of \( \gamma \)-glutamlycysteine, which yields an acylphosphate intermediate (1, 7). Next, nucelophilic attack of the glycine amine moiety on the intermediate leads to formation of glutathione with release of ADP and inorganic phosphate (Fig. 1) (1, 7). The pH dependence of AtGS shows that deprotonation of a charged species occurs for maximal activity with the same breakpoints (pK\(_a\) = 7.2–7.4) observed in the V and V/K profiles for \( \gamma \)-glutamlycysteine and ATP and that deprotonation of a second group (pK\(_a\) = 9.6) reduces activity (Fig. 4, A and B). Analysis of ATP binding versus pH (Fig. 4C) suggests that the second inflection results from titration of a group involved in nucleotide binding, potentially one or more of the lysine residues in the binding site. The V/K versus pH profile for glycine (pK\(_a\) = 8.6) differs from those of the other substrates (Fig. 4B and Table 4). Because glycine preferentially binds after addition of \( \gamma \)-glutamlycysteine and ATP in the kinetic mechanism of AtGS (6), the observed inflection represents titration of either glycine or a functional group on the AtGS-\( \gamma \)-glutamlycysteine-ATP complex involved in substrate binding (23).

Because acid-base catalysis should not be required for the GS mechanism, the reason for the pH dependence of reaction velocity is unclear. Examination of the AtGS active site does not suggest any amino acid accounting for the first inflection; however, the pH dependence may represent a change in the protonation state of a phosphate group (25, 26). Mechanistic studies of biotin carboxylase, another ATP-grasp family member using an acylphosphate intermediate, showed a similar pH dependence of catalysis that was attributed to protonation of a phos-
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Analysis of solvent kinetic isotope effects provides additional support for the catalytic role of Arg-132 (Table 6). Normal isotope effects on $k_{cat}$ and $k_{cat}/K_m$ values were observed with AtGS and the R454A mutant, indicating that the rate of the isotope-sensitive step is limiting in each protein and that Arg-454 is not involved in this step. In contrast, replacement of Arg-132 with a lysine abolishes the isotope effects on $V$ and $V/K$, suggesting that mutation of this residue destabilizes the transition state of the isotope-sensitive step of the reaction (27, 28).

Formation of the acylphosphate intermediate is a common feature in enzymes of the ATP-grasp family (1, 7, 26, 29, 30). The pH-rate profiles and solvent kinetic isotope effects demonstrate that the R132K mutation changes the rate-limiting step of the reaction catalyzed by AtGS. Although it is unclear which step of the overall reaction is limiting, the position of Arg-132 in the active site suggests that mutation of this residue destabilizes the transition state leading to formation of the acylphosphate intermediate and the transition state formed during nucleophilic attack of glycine on the intermediate. Local structural changes resulting from mutation of this residue may also allow bulk water access to the active site, which would affect the stability of the phosphorylated $\gamma$-glutamylcysteine intermediate and lead to an increased decomposition rate of the intermediate (8).

The functional analysis of AtGS mutants described here, together with available crystal structures of the human and yeast GS (15, 16), suggest a plausible catalytic mechanism for glutathione synthesis in eukaryotes (Fig. 6). In the first part of the reaction (Fig. 6A), formation of an electrophilic acylphosphate intermediate occurs by transfer of the $\gamma$-phosphate of ATP to $\gamma$-glutamylcysteine. During this step, the residues coordinating the magnesium ions to ATP are required for nucleotide binding and for orienting the $\gamma$-phosphate group in the active site (15–18, 21, 24). As noted above, Arg-132 plays a critical role in catalysis. The guanidyl group of the side chain likely stabilizes formation of the pentavalent transition state that yields the phosphorylated $\gamma$-glutamylcysteine intermediate and

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Proposed reaction mechanism for GS. \textit{A}, formation of the acylphosphate intermediate. \textit{B}, addition of glycine to the $\gamma$-glutamylcysteine phosphate intermediate.}
\end{figure}
ADP. In the second half of the reaction (Fig. 6B), the amino group of glycine acts as a nucleophile to attack the acylphosphate intermediate. Arg-132 and the magnesium ion bound by Glu-148 and Asn-150 are positioned to stabilize the tetrahedral transition state that decomposes to yield glutathione and inorganic phosphate. The side-chain guanidyl group of Arg-454 interacts with the carboxylate moiety of glycine to orient the substrate for attack on the acylphosphate intermediate.

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REFERENCES

1. Meister, A., and Anderson, M. E. (1983) Annu. Rev. Biochem. 52, 711–760
2. May, M. J., Vernoux, T., Leaver, C., Van Montagu, M., and Inze, D. (1998) J. Exp. Bot. 49, 649–667
3. Noctor, G., Arisi, A. C., Jouanin, L., Kunert, K. J., Rennenberg, H., and Foyer, C. H. (1998) J. Exp. Bot. 49, 423–447
4. Zenk, M. H. (1996) Gene (Amst.) 179, 21–30
5. Jez, J. M., Cahoon, R. E., and Chen, S. (2004) J. Biol. Chem. 279, 33463–33470
6. Jez, J. M., and Cahoon, R. E. (2004) J. Biol. Chem. 279, 42726–42731
7. Nishimura, J. S., Dodd, E. A., and Meister, A. (1964) J. Biol. Chem. 239, 2553–2558
8. Tanaka, T., Kato, H., Nishioka, T., and Oda, J. (1992) Biochemistry 31, 2259–2265
9. Tanaka, T., Yamaguchi, H., Kato, H., Nishioka, T., Katsube, Y., and Oda, J. (1993) Biochemistry 32, 12398–12404
10. Yamaguchi, H., Kato, H., Hata, Y., Nishioka, T., Kimura, A., Oda, J., and Katsube, Y. (1993) J. Mol. Biol. 229, 1083–1100
11. Kato, H., Tanaka, T., Yamaguchi, H., Hara, T., Nishioka, T., Katsube, Y., and Oda, J. (1994) Biochemistry 33, 4995–4999
12. Hara, T., Kato, H., Katsube, Y., and Oda, J. (1996) Biochemistry 35, 11967–11974
13. Dodd-Mooz, E., and Meister, A. (1967) Biochemistry 6, 1722–1734
14. Galil, R. R., and Board, P. G. (1997) Biochem. J. 321, 207–210
15. Polekhina, G., Board, P. G., Gali, R. R., Rossjohn, J., and Parker, M. W. (1999) EMBO J. 18, 3204–3213
16. Gogos, A., and Shapiro, L. (2002) Structure (Camb.) 10, 1669–1676
17. Farn, C., Moews, P. C., Shi, Y., Walsh, C. T., and Knox, J. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1172–1176
18. Galperin, M. Y., and Koonin, E. V. (1997) Protein Sci. 6, 2639–2643
19. Njalsson, R., Carlsson, K., Olin, B., Carlsson, B., Whitbread, L., Polekhina, G., Parker, M. W., Norgren, S., Mannervik, B., Board, P. G., and Larsson, A. (2000) Biochem. J. 349, 275–279
20. Njalsson, R., Norgren, S., Larsson, A., Huang, C. S., Anderson, M. E., and Luo, J. L. (2001) Biochem. Biophys. Res. Commun. 289, 80–84
21. Dinescu, A., Cundari, T. R., Bhansali, V. S., Luo, J. L., and Anderson, M. E. (2004) J. Biol. Chem. 279, 22412–22421
22. Ellis, K. J., and Morrison, J. F. (1982) Methods Enzymol. 87, 405–426
23. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
24. Sloane, V., Blanchard, C. Z., Guillot, F., and Waldrop, G. L. (2001) J. Biol. Chem. 276, 24991–24996
25. Swain, C. G., and Scott, S. B. (1953) J. Am. Chem. Soc. 75, 141–147
26. Tipton, P. A., and Cleland, W. W. (1988) Biochemistry 27, 4317–4325
27. Schowen, K. B., and Schowen, R. L. (1982) Methods Enzymol. 87, 551–606
28. Northrop, D. B. (1982) Methods Enzymol. 87, 607–625
29. Mullins, L. S., Zawadzhe, L. E., Walsh, C. T., and Rauschel, F. M. (1990) J. Biol. Chem. 265, 8993–8998
30. Tipton, P. A., and Cleland, W. W. (1988) Biochemistry 27, 4325–4331