Identification of Regions of the Tomato \(\gamma\)-Glutamyl Kinase That Are Involved in Allosteric Regulation by Proline*

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RUNNING TITLE: Allosteric regulation of tomato \(\gamma\)-glutamyl kinase
The first step of proline biosynthesis is catalyzed by γ-glutamyl kinase (GK). To better understand the feedback inhibition properties of GK, we randomly mutagenized a plasmid carrying tomato *tomPRO1* cDNA, which encodes proline-sensitive GK. A pool of mutagenized plasmids was transformed into an *E. coli* GK mutant, and proline overproducing derivatives were selected on minimal medium containing the toxic proline analog 3,4-dehydro-DL-proline (Dhp). Thirty-two mutations that conferred Dhp resistance were obtained. Thirteen different single amino acid substitutions were identified at 9 different residues. The residues were distributed throughout the amino-terminal two thirds of the polypeptide, but nine mutations affecting 6 residues were in a cluster of 16 residues. GK assays revealed that these amino acid substitutions caused varying degrees of diminished sensitivity to proline feedback inhibition and also resulted in a range of increased proline accumulation *in vivo*. GK belongs to a family of amino acid kinases, and a predicted three dimensional model of this enzyme was constructed on the basis of the crystal structures of three related kinases. In the model, residues that were identified as being important for allosteric control were located close to each other, suggesting that they may contribute to the structure of a proline binding site. The putative allosteric binding site partially overlaps the dimerization and substrate binding domains, suggesting that the allosteric regulation of GK may involve a direct structural interaction between the proline binding site and the dimerization and catalytic domains.

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Osmoregulation is of significance for agriculture, because water is a major limiting factor for crop productivity. Plants evolved a variety of mechanisms for adapting to environmental stresses, one of which is accumulation of proline under salinity, dehydration, or freezing environments (1,2). It has been proposed that proline plays an important role as a compatible solute, maintaining proper balance between the extracellular and intracellular osmolality. However, a number of other functions have been hypothesized for this imino acid, such as carbon or nitrogen reservoir, radical scavenger, or regulator of intracellular pH (3,4). Plants have two proline biosynthetic pathways: the glutamate pathway and the ornithine pathway, with the former appearing to play a predominant role under osmotic stress (5). In the glutamate pathway, glutamate is converted by GK to $\gamma$-glutamyl phosphate, which is reduced by GPR to $\gamma$-glutamyl semialdehyde. This product cyclizes spontaneously to P5C, which is reduced by NADPH to proline by P5CR. In bacteria and yeast, GK and GPR reactions are catalyzed by two separate enzymes. It has been shown in bacteria that the GK activity is sensitive to feedback inhibition by proline (6). In plants, the first two reactions of the pathway are mediated by a bifunctional P5CS, consisting of a GK domain at the N-terminus and a GPR domain at the C-terminus.

Genes specifying P5CS and P5CR have been cloned from plants, making it feasible to test the role of proline under osmotic stress and to aim to improve the salinity tolerance of agriculturally important crops by genetic engineering. It has been reported that the first step catalyzed by P5CS is the rate limiting step in proline biosynthesis in plants (7-9). Osmotic stress was shown to increase the P5CS mRNA levels along with the proline pool size in *Arabidopsis thaliana* and rice (*Oryza sativa*), suggesting that transcriptional regulation of P5CS could be important for a control of proline biosynthesis (10-12).

Tomato (*Lycopersicon esculentum*) has two distinct genes for P5CS, called *tomPRO1* and *tomPRO2* (13). Although this species accumulates over 15-fold higher levels of proline than *A. thaliana* or rice, the *tomPRO1* and *tomPRO2* mRNAs are expressed essentially at constitutive levels at low and high osmolality (13). These observations suggest that some post-transcriptional control mechanism must be taken into account for the regulation of proline synthesis in tomato.
The GKs encoded by the *tomPRO1* locus of tomato, the *P5CS* genes of *Vigna aconitifolia*, and grapevine (*Vitis vinifera*) have been demonstrated to be subject to feedback regulation by proline (14-16). Roosens *et al.* (17) described the isolation of a salt-tolerant mutant of *Nicotiana plumbaginifolia* that lost proline feedback inhibition of the P5CS. Also, Hong *et al.* (18) reported a direct role of feedback inhibition of P5CS for proline accumulation by constructing transgenic plants that expressed a mutated version of the *V. aconitifolia* P5CS, whose proline-feedback regulation was lost. These observations suggest that allosteric control of GK reaction also plays a key role in regulating proline synthesis in plants (16,18).

Studies with purified preparations revealed that the bacterial GKs and the plant P5CS enzymes are subject to feedback regulation by proline at very different sensitivities. For the bacterial GKs, the concentration of proline resulting in 50% inhibition of activity (apparent K_i) was in the range of 0.01 - 0.1 mM (19-21), whereas for the plant P5CS enzymes, the apparent K_i was estimated to be ≥5 mM (16,22). Single amino acid substitutions have been first isolated in the GK of *E. coli* and *S. marcescens* which lessened or eliminated the sensitivity of the enzyme to allosteric control. These mutations resulted in proline overproduction in whole cells (19,23-25), providing the most compelling proof that feedback inhibition is indeed important for the regulation of proline synthesis *in vivo*. In a site-directed mutagenesis of the *V. aconitifolia* P5CS, Zhang *et al.* (22) showed that amino acid substitutions at positions 126 and 129 in the *V. aconitifolia* P5CS abolished proline-feedback regulation. Other single amino acid mutations involving feedback regulation of GK have been recently reported in *Streptococcus thermophilus* and *Listeria monocytogenes* (26,27). In all, eight amino acid residues have been identified in six organisms as being important for proline feedback regulation of GK (19,22-24,26-28). However, because the three dimensional structure of GK has not been determined yet, there is no corroborating information about roles of specific amino acids in the allosteric binding site or other structure-function aspects of this protein.

On the basis of primary sequence similarity, GKs have been assigned to the so called amino acid kinase family of enzymes (PF00696 in Pfam database:...
http://www.sanger.ac.uk/Software/Pfam/), which also includes AK, CK, CK-CPS, NAGK, and UK. Initial sequence comparisons identified two sequence features that are common to GKS (Figure 1). A phosphate binding site and a leucine zipper have been suggested in the GKS, although neither seem to have a perfect match to the respective consensus sequences (11,14). The 3D structures for CK, CK-CPS, and NAGK are known (29-31), and the assignment of the phosphate binding site has been supported by the sequence information. Omori et al. (32) identified a motif common to GKS and AKs, which they suggested to be part of the catalytic site. This motif ("Region II" in Figure 1), which can be recognized in other members of the amino acid kinase family (see below), has been corroborated by the 3D sequence information to contain part of the nucleotide and amino acyl binding sites.

To identify residues in GK that contribute to allosteric regulation and to gain insights into the structure-function relationship of GK enzyme, we carried out random mutagenesis of tompRO1 cDNA to alter the allosteric properties of the enzyme. The tompRO1 clone, which has been isolated from a tomato cDNA library by complementation of a proB (GK) mutation in E. coli, specifies GK and GPR as two separate polypeptides in two non-overlapping open reading frames, similar to prokaryotic operons (15). Phylogenetic analysis revealed that the GK and GPR polypeptides encoded by the tompRO1 locus are more similar to prokaryotic enzymes than to eukaryotic counterparts, suggesting that GK may have been incorporated into the tomato nuclear genome from a prokaryote by horizontal gene transfer (13). Although unusual for eukaryotic transcripts, a similar bi-cistronic organization has been also observed in a locus specifying two subunits of another amino acid biosynthetic enzyme: carbamoyl phosphate synthetase in alfalfa (33). The tompRO1 GK seems to be suitable for the analysis of the determinants of allosteric control, because it is shorter by ~100 amino acid residues in its C-terminus than most other prokaryotic GKS. Based on sequence comparisons, this ~100 amino acid tail has been suggested to constitute an RNA binding domain (34), but the relevance of this element to the activity or regulation of GK is unknown. The tompRO1 GK is nevertheless contains the essentials for catalysis and is subject to proline feedback inhibition (15). In this
work, we describe the identification and analysis of 13 different amino acid substitutions at 9 different positions in \textit{tomPRO1} GK that eliminated feedback regulation by proline and we discuss the possible roles of these amino acids in a predicted model structure of GK.

EXPERIMENTAL PROCEDURES

\textit{Culture Media} – Minimal medium used was M63 (35) containing 10 mM glucose and 0.1 mM thiamine-HCl, unless otherwise stated. M63 was supplemented, as indicated, with a mixture of 19 amino acids (19 aa), consisting of 0.2 mM of each of the protein-amino acids, except proline. In media containing NaCl 0.6 M, we used an 18 aa mixture, which contained 0.2 mM of each amino acid, except proline and cysteine (because the latter amino acid has been shown to be inhibitory in media of high osmolality [36]). Complex medium was Luria-Bertani (LB) (37), supplemented with 100 \(\mu\)g/ml Ap, as indicated. When used, the proline analog Dhp (Sigma, St. Louis, MO) was present at 1.5 mM.

\textit{Isolation of Dhp-Resistant Mutants} – Basic techniques of DNA manipulation were carried out according to the procedures described by Sambrook \textit{et al.} (38). Plasmid pPRO1 is a derivative BluescriptKSII+ carrying the \textit{tomPRO1} locus (15). To introduce mutations into the \textit{tomPRO1} GK, this plasmid was transformed into the \textit{E. coli} mutator strain XL-1 Red (mutD mutS mutT) according to a supplier’s instructions (Stratagene, La Jolla, CA). Transformants were inoculated to LB plates containing Ap and grown at 37\(^\circ\) for 26 to 32 hours. Plates containing the transformant colonies were taken up and pooled in 2 ml LB, and plasmids were isolated from the pooled transformants with the QIAGEN plasmid purification procedure (QIAGEN, Valencia, CA). This plasmid preparation was transformed into the \textit{E. coli} proline auxotroph CSH26 (\textit{AproBA}) and transformants selected on LB Ap plates overnight. Over 2 \(\times\) 10\(^4\) transformant colonies were replicated to M63 + 19 aa plates containing Dhp and incubated at 30\(^\circ\) overnight.
Approximately 400 colonies that were able to grow on Dhp-containing plates were streaked again on the same medium. Dhp\textsuperscript{R} derivatives that grew faster than CSH26 carrying wild type pPRO1 were tested on 18 aa plates containing 0.6 M NaCl, as a further step in identifying proline over-producing derivatives by virtue of their increased salinity stress tolerance (36). Pilot experiments indicated that increasing the concentration of Dhp above 1.5 mM did not change the frequency of Dhp\textsuperscript{R}, suggesting that maximum Dhp uptake was accomplished at 1.5 mM. At this Dhp concentration, strain CSH26 carrying the wild type pPRO1 did not grow at all. Re-transformation of the mutagenized plasmids into an \textit{E. coli} proline auxotroph followed by subsequent screening for Dhp\textsuperscript{R} enabled us to identify mutations in the plasmid-borne gene, as opposed to chromosomal loci, such the \textit{putA} or \textit{proP} (proline transport) genes, where mutations could also result in Dhp\textsuperscript{R} (6).

\textit{GK/GPR Coupled Assays} – Derivatives of strain CSH26 carrying the wild type or mutated pPRO1 plasmids were grown overnight in LB + Ap, and 0.1 ml samples were inoculated to LB Ap plates and grown overnight at 30\textdegree. The lawn of cells was taken up in 5 ml of LB, sedimented by centrifugation, resuspended in 1 ml of 50 mM HEPES-KOH (pH 7.2) containing 10 mM dithiothreitol, 1 mM EDTA, 2 mM phenylmethysulfonyl fluoride, and 100 \textmu g/ml lysozyme. The crude extracts were centrifuged, and the supernatants were used to measure GK/GPR coupled activity in 50 mM MOPS-KOH (pH 6.5), 8 mM MgCl\textsubscript{2}, 75 mM Na glutamate, 4 mM ATP, 0.4 mM NADPH, and various concentrations of proline, as described by García-Ríos \textit{et al.} (15). The GK/GPR coupled activity was determined as the rate of NADPH consumption (decrease in OD\textsubscript{340}), and the enzyme specific activity was expressed as nmoles of NADPH consumed \cdot (min \cdot mg cell protein)\textsuperscript{-1}.

\textit{Determination of Total Proline Levels} – CSH26-derivatives carrying wild type or mutant versions of pPRO1 were inoculated into liquid M63 supplemented with 20 mM glucose, 0.2 mM thiamine and 18 aa and grown at 30\textdegree to OD\textsubscript{600} of 1.0. Lysozyme was added to 100 \textmu g/ml, and
cell debris removed by centrifugation. Culture media were passed through 0.22 \( \mu \text{m} \) membrane filters (Millipore, Bedford, MA), mixed vigorously with a drop of CHCl\(_3\) to kill surviving cells, and the CHCl\(_3\) separated by centrifugation. Proline content in the supernatant was measured by bioassay using *Salmonella typhimurium* strain TL131 (\( \Delta \text{proBA-47} \) putA842::Tn5). In this strain, in which proline biosynthesis is blocked by the \( \Delta \text{proBA-47} \) mutation and proline catabolism is blocked by the putA842::Tn5 mutation, the biomass yield is linearly dependent on the extracellular proline concentration in the range of 0 - 200 \( \mu \text{M} \) (39). Strain TL131 was cultured in M63 with 20 mM glucose, 18 aa and 0.2 mM proline at 37\( ^\circ \) for 12 h, collected by centrifugation, washed with the original volume of H\(_2\)O to minimize proline-carryover, and resuspended at the original cell density in M63 with 20 mM glucose. The suspension was inoculated at 1:200 dilution into 2 ml of cell lysates of the proline over-producing strains to be tested, glucose was added to 20 mM, the cells were grown at 37\( ^\circ \) for \~24 hours, and the final cell yield determined as the OD\(_{600}\). Because OD\(_{600}\) is not proportional to the cell density at OD\(_{600} > 1\), for those cultures which reached this high density, the OD\(_{600}\) was determined on appropriate dilutions. A standard curve of the cell yield as a function of proline concentration was generated with strain TL131, grown under identical conditions in M63 containing 20 mM glucose, 18 aa, and various input concentrations of proline.

**DNA and Amino Acid Sequence Analyses** – DNA sequences were determined using automated fluorescent sequence (ALFexpress; Pharmacia) or by the DNA sequencing service of Iowa State University. Nucleotide and predicted amino acid sequences were analyzed with programs in the Genetic Computer group (GCG) package of the University of Wisconsin. Amino acid sequence alignment was performed using PILEUP program and manually adjusted.

**Modeling of Tertiary Structure of tomPRO1 GK** – As there were no three-dimensional structures available for GKs from various organisms, we searched the Protein Data Bank (PDB) for homologous proteins with known three dimensional structures. The amino acid sequence of
tomPRO1 GK was submitted to 3D-PSSM servers (http://www.sbg.bio.ic.ac.uk/~3dpssm/), and CK, CK-CPS, NAGK (PDB entries 1B7B, 1E19 and 1GS5, respectively) were identified with the highest PSSM scores. Other modeling servers (GTOP, http://spock.genes.nig.ac.jp/~genome/gtop-j.html; UCLA-DOE fold server, http://fold.doe-mbi.ucla.edu/; PredictProtein server, http://www.embl-heidelberg.de/predictprotein/; 3D-Jigsaw, http://www.bmm.icnet.uk/servers/3djigsaw/) were used to search modeling templates to tomPRO1 GK and returned the same proteins as templates. A predicted tertiary model of tomPRO1 GK, based on crystal structures of these enzymes, was built and drawn using program Insight II, Homology and Discover (Accelrys, Tokyo, Japan).

RESULTS AND DISCUSSION

Isolation of Dhp-Resistant Mutants – Wild type E. coli is inhibited on minimal medium by the proline analog 3,4-dehydro-DL-proline (Dhp) (40). Because high level accumulation of proline antagonizes Dhp, proline over-producing mutants can be recovered by selecting Dhp-resistant (DhpR) derivatives on minimal media (36). Random mutations were introduced into the tomato GK by propagating pPRO1 in the mutator strain XL-1 Red (mutD mutS mutT). A pool of mutagenized pPRO1 plasmids was transformed into CSH26 (∆proBA), and derivatives carrying mutations in the tomPRO1 gene that confer DhpR were obtained, as described in Experimental Procedures. Plasmid DNA was isolated from 34 DhpR derivatives, transformed into strain CSH26, selecting ApR, and re-tested for DhpR. Of the 34 plasmid lines, 32 proved to be able to confer DhpR in the second round, confirming that this phenotype was caused by mutations on the plasmid.

The tomPRO1 gene contains a unique HpaI site between codons 186 and 187 of the GPR open reading frame, downstream of the GK sequence; it is possible to obtain the entire open reading frame specifying a functional GK without active GPR by deleting the sequences 3' to this
HpaI site (data not shown). GPR-coding sequences were removed in this manner from three of the 32 mutant pPRO1 plasmids, and the resultant plasmids were transformed into *E. coli* proB mutant G13, which is deficient in GK but has GPR (41). Each of these plasmids not only complemented the proB mutation of strain G13 strain but also conferred DhpR (data not shown), suggesting that the latter phenotype was due to mutation(s) within the GK sequence of *tomPRO1*. These data were consistent with the previous observations that single amino acid mutations in GK or the GK domain of bifunctional P5CS could result in proline analog-resistance and loss of allosteric control (19,22-28).

**Sites of the Mutations in GK** — The nature and location of mutations in the GK region of *tomPRO1* from each of the 32 plasmids were determined by DNA sequence analysis. Because previous mutations that conferred resistance to proline analogs have been reported to lie in the amino-terminal third of GK (19,23-25,27,28), initially, we concentrated on determining the nucleotide sequence of the N-terminal ~600 bases of the GK-coding region. Each plasmid had a single base-pair substitution in this region, resulting in an amino acid replacement (Table I), except for Dr214. The latter plasmid contained two substitutions: an (a → g) at nucleotide position 763, which caused an Asp → Gly substitution at amino acid position 162, and a (g → a) at nucleotide position 722, resulting in the change of a ggt (Val) codon to the synonymous gta codon at amino acid position 148. Initial sequence analysis identified 13 different amino acid substitutions at 9 different residues. The complete DNA sequence of a whole GK region for 15 clones (Dr246, 262, 161, 199, 220, 428, 122, 55, 319, 242, 385, 110, 63, 258 and 282), representing 13 different substitutions, was then determined, confirming that they had only a single amino acid substitution in the GK coding region that was caused by a single nucleotide change, except for Dr214. Of the 13 mutations, 11 were g/c ↔ a/t transitions and the other two were a → t/c transversions. Figure 1 shows the locations of the nine amino acid residues where replacements resulted in DhpR phenotype. The identified amino acid residues were distributed throughout two thirds of the amino terminal of GK, although a cluster was readily apparent.
between positions 147 to 162, where six of the target residues were concentrated. The facts, that some of the mutations were recovered multiple times and that some of the mutations resulted in the substitution of different amino acids at the same residue (Table I), suggest that we may have approached the saturation limit of mutations which could be generated in strain XL-1 Red resulting in a reduction in sensitivity to allosteric regulation while retaining sufficient catalytic activity for the synthesis of proline.

Enzymatic Confirmation That the Mutations Diminish Proline-Feedback Inhibition – To test whether DhpR resulted from a loss of proline-feedback regulation in the 13 mutants that were sequenced, the GK/GPR coupled activities of the wild type and mutant enzymes were determined in cell-free extracts. Each of the mutant enzymes had lower specific activities compared to the wild type in assays carried out in the absence of proline (Table II). The specific activities of the enzymes carrying mutations I79T, M94T, D147G, E153A, E153G, E153K, L154S, and S159P were 20 to 66% of the wild type activity. The most drastic reduction in specific activity was caused by the A62T, A62V, I149F, D162G, and D162N changes, suggesting that the residues at positions 62, 149, and 162 may have overlapping function in catalytic activity and allosteric regulation (see a section of 3D modeling below).

We also determined the effect of proline on the GK activity of the mutant enzymes. The proline-inhibition curve of the wild type enzyme was similar to the one observed previously with the purified enzyme (42). In contrast, all of 13 mutants exhibited decreased sensitivity to feedback regulation. Figure 2 shows the proline-inhibition curves in nine representative mutants. While the apparent K_i for proline of the wild type enzyme was about 0.09 mM, the apparent K_i of mutants ranged from 1.9 to 310 mM (Table II), representing a 20- to 3500-fold increase.

In order to verify that the DhpR phenotype indeed was due to increased intracellular proline levels and to check whether the proline levels were correlated with the residual feedback regulation of GK, the total amounts of proline (cellular + excreted into the medium) were measured in E. coli strains carrying the wild type or mutant versions of pPRO1. As can be seen

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in Table II, proline levels in mutants were 10 to 1000-fold greater than in the wild-type, indicating that in all the mutants, the DhpR phenotype was the consequence of proline overproduction. In general, there was a good correlation between the degree of deficiency in proline feedback regulation and the proline levels produced by the strains. The I79T, I149F, and S159P substitutions, which caused the least disruption of allosteric regulation (apparent Kᵢ = 1.9, 20, and 19 mM, respectively), resulted in the lowest proline production (10 to 25-fold increase over the wild type), and conversely, the L154S and E153K substitutions, which caused the most severe loss of allosteric control (apparent Kᵢ = 90 and 310 mM), conferred the highest production of proline (~900-fold increase). This generalization, however, does not hold for all of the mutants. The apparent Kᵢ of the GKS containing the A62V and M94T substitutions was comparable to that of mutants containing the I79T, I149F and S159P substitutions, but the former two mutations resulted in a ~4- to 19-fold higher production of proline compared to that caused by the latter three mutations. The D147G substitution increased the apparent Kᵢ to 180 mM, but caused lower production of proline than did the L154S substitution. Because it is possible that the mutations altered not only the allosteric properties of GK but also its in vivo stability or interaction with GPR, it will be necessary to carry out assays with purified GK in order to fully characterize the effects of the mutations on the enzyme.

Growth rates of mutants in the presence of Dhp were also determined. All mutants showed 2 to 3-fold faster growth rate than wild type in the presence of this anti-metabolite (Table II). The growth rate in the presence of Dhp was well correlated with the proline levels: strains expressing the GK with the I79T, I149F and S159P substitutions, which produced the lowest proline levels, also showed the lowest growth rate. However, E153K and L154S, which exhibited the most extreme loss of feedback inhibition and accumulated the highest levels of proline, nevertheless grew more slowly than most of other mutants. Inconsistency between proline levels and growth rates in the presence of Dhp might be due to physiological effects arising from limitation for some important intermediate or accumulation of a toxic metabolite arising from proline overproduction. In order to test whether loss of feedback sensitivity or
proline over-production might be accentuated by combining pairs of mutations, we introduced the A62T mutation in combination with the D147G, E153K, L154S and D162G mutations. However, none of the combinations of double mutations A62T/D147G, A62T/E153K, A62T/L154S, and A62T/D162G resulted in further enhancement of proline overproduction that would be consistent with an even greater loss of allosteric feedback regulation (data not shown).

A Cluster of Allosteric Mutations in the Region Between Residues 147-162 – We generated at least 13 different amino acid changes that diminished or eliminated the sensitivity of the tomPRO1 GK to feedback inhibition by proline. Figure 3 shows the alignment of 21 GKs from various species in the region corresponding to positions 147 to 162 of the tomPRO1 GK, where we obtained 6 substitutions giving rise to DhpR. The cluster of residues identified here might be a part of the allosteric regulatory domain. In all sequences, the residues at positions corresponding to 147 and 162 of tomPRO1 are invariably aspartates. The fact, that mutations at both of these positions (D147G and D162G; Table I) diminished sensitivity to feedback regulation but did not eliminate catalytic activity, suggests that these highly conserved aspartates might be important for allosteric regulation. Two other mutations were I149F and L154S. Positions corresponding to 149 and 154 show a characteristic preference for Ile, Leu, Met or Val in most GKs (except for the ProB enzyme of B. subtilis); mutation of these hydrophobic amino acids resulted in loss of allosteric control. Two other targets for our mutations were at residues 153, and 159, which are highly variable among species. Conceivably, these amino acids could make species-specific contributions to the feedback regulation that may be dependent on the context of other residues or might be required for the overall folding of the enzyme.

The fact that we obtained three different amino acid replacements at the highly variable position 153 (Table I), points out an intriguing aspect of the relationship among GK enzymes. One of these mutations changed the Glu to Ala. The identical substitution had been isolated at the corresponding site of the GK of E. coli (position 143 in that polypeptide) (25). This
replacement resulted in an 80- to 100-fold decreased sensitivity to proline feedback inhibition the
both in \textit{tomPRO1} GK and the \textit{E. coli} enzyme, as judged by an increase in their apparent $K_i$.

A second mutation at position 153 in \textit{tomPRO1} GK introduced Lys, resulting in a change
of charge from -1 to +1. \textit{GK} sequences of the plant P5CS enzymes invariably contain a
positively charged amino acid (Lys or Arg) at this position, whereas most of the bacterial GKS
have negatively charged residues (Asp or Glu) here (Figure 3). The presence of a Glu at this site
in the \textit{tomPRO1} GK is consistent with our classification of that protein as being more similar to
the prokaryotic type GKS than to the eukaryotic P5CS enzymes (13). Interestingly, the E153K
substitution caused the most marked loss of feedback inhibition in \textit{tomPRO1} GK, as indicated by
the highest value of apparent $K_i$ (Table II). The ~50-fold lower affinity of the plant P5CS
enzymes for proline as an inhibitor compared to that of the prokaryotic type GKS (please see
Introduction) might be due to the positively charged amino acid at the site corresponding to
position 153 in the \textit{tomPRO1} GK. It is possible that during evolution, the plant P5CS enzymes
acquired and fixed a residue at this position which diminished their sensitivity to allosteric
control, as compared to the primordial prokaryotic type GKS, resulting in a higher capacity for
the accumulation of proline in plants during osmotic stress.

\textit{B. subtilis} has two GKS: ProB, which is constitutive, and ProJ, which is induced by
osmotic stress (43). This organism elevates its proline levels upon osmotic stress by increased \textit{de
novo} synthesis. It has been hypothesized that the ProJ enzyme might be insensitive to allosteric
feedback inhibition by proline (43). With the exception of the \textit{B. subtilis} ProJ enzyme and the \textit{C.
glutamicum} enzyme, all other prokaryotic GKS have an acidic residue at position 153. The \textit{B.
subtilis} ProJ enzyme contains a basic amino acid (Arg) at this position, making it similar to the
plant enzymes. However, the possibility that the \textit{B. subtilis} ProJ enzyme has atypical allosteric
properties among prokaryotic GKS has not yet been investigated.

A third mutation at position 153 was Glu \textit{→} Gly (Table I). It is noteworthy that
\textit{C. glutamicum} already carries a Gly at the corresponding position (Figure 3), but there is no
information concerning the allosteric regulation of the enzyme from this organism.
**Mutations in N-Terminal Third of GK** – Four mutations, A62T, A62V, M94T, and I79T, which also decreased sensitivity to allosteric feedback, are outside the region shown in Fig. 3. Position 62 is occupied by an alanine in the GK/P5CS genes in 16 of 18 organisms. Position 94 is not highly conserved, but has a preference for a bulky, hydrophobic amino acid (sequence comparison not shown). Finally, the I79T mutation affected a residue that shows a high variability (sequence comparison not shown). Like the highly variable residues at positions 153 and 159, the Ile at position 79 might make a species-specific contribution to their allosteric regulation of the *tomPRO1* GK, or might be required for the proper conformation of the enzyme.

**3D Modeling and Putative Proline Binding Domain** – We were interested in seeing how the amino acid residues that were identified to be involved in the allosteric regulation of GK or P5CS might be positioned in three dimensional structure of the protein. Although crystallographic structure is not available yet for any GK and P5CS, it is possible to make predictions about the tertiary structure of GK by homology modeling programs based on the three related enzymes, CK, CK-like CPS, and NAGK, whose structures have been solved (29-31). CK catalyzes the phosphorylation of ADP by carbamoyl phosphate during arginine catabolism, CK-CPS makes carbamoyl phosphate by ATP-dependent phosphorylation of carbamate in the pyrimidine and arginine biosynthetic pathways, and NAGK carries out the phosphorylation of N-acetyl-L-glutamate by ATP in the arginine pathway. Because different secondary structure prediction algorithms or threading methods (see Experimental Procedures) recorded high scores of E-values using CK, CK-CPS, and NAGK as modeling template for *tomPRO1* GK (E values between e-14 and e-26 in 3D-PSSM program), the resultant hypothetical model could provide useful insights into the structure and regulation of this enzyme. However, it should be borne in mind that with the current status of protein structure calculation, this predicted model is highly speculative, and may be dependent on the modeling approaches used.
As might be expected from the similarities in the primary sequence of CK, CK-CPS, and NAGK and in the reactions they catalyze, these enzymes exhibit high degree of structural resemblance. All three are homodimers, made up of subunits that have open α/β structures composed of 16 β strands and 8 α helices, which are nucleated by a central β sheet of eight main strands sandwiched between two layers of α helices. Each subunit can be divided into N- and C-terminal domains, split by a large crevice. The N-terminal domain contains the amino acyl substrate binding site and entire dimer interface, the C-terminal portion has the nucleotide binding site, and the crevice constitutes the site for phosphate transfer from nucleotide to the carboxyl group. As shown in Fig. 4, the threading model predicted a structure made up of eight strands of the main β sheet surrounded by α helices in \textit{tomPRO1} GK, similar to those seen in CK-CPS. The conservation of eight strands of the main β sheet and the surrounding α helices is consistent with a proposal that the amino acid kinase family of enzymes share this basic characteristic (31). Therefore, it is likely that \textit{tomPRO1} GK catalyzes phosphoryl group transfer with similar amino acyl and ATP binding domains as CK-CPS. GK may form dimers with similar interfacial elements as the other three enzymes, although there are conflicting reports as to whether GK forms dimeric or hexameric structures in different organisms (20,44).

In addition to the 9 residues in \textit{tomPRO1} identified in this study, 7 other residues, corresponding to positions 115, 117, 118, 127, 150, 152 and 198 in \textit{tomPRO1} GK (Figure 4), were shown to be important for allosteric regulation of GK or P5CS from other organisms. With the exception of residue 198, all of the other residues are located within the region that corresponds to the N-terminal domain of the CK-CPS (residues 1-175 of \textit{tomPRO1} GK). The cluster of mutations between 147 and 162 was localized to a sequence corresponding to the region that encompasses the interval between β9-β10 and the interval between β11-αF of CK-CPS, which consists of a large loop containing hairpin β motif of β10 and β11. The three mutations that are between positions 115 to 118 in \textit{tomPRO1} GK can be mapped to the region that includes the β4-β5 interval and β5 of CK-CPS. This region, which exhibits different secondary structure between CK-CPS and NAGK, corresponds in CK-CPS to the junction

16
between a hairpin β motif of β4 and β8 and the peripheral domain and in NAGK to part of a hairpin β motif of β6-β7. Residues 115 to 118 and 147 to 162, where most of the mutations are concentrated, are folded very close to each other within our putative 3D structure of the GK (Figure 5), suggesting that they may constitute part of the proline binding site. This putative allosteric regulatory domain corresponds to a region of a small sheet consisting of the two sets of antiparallel strands (β11-β10 and β4-β8 in CK-CPS, β9-β10 and β7-β6 in NAGK) and a junction to a peripheral protruding domain in CK-CPS and a loop in NAGK that is emerging from the sheet. This region shows one of the major differences among the enzymes of the amino acid kinase family. The variation in the structures was interpreted to reflect differences of binding of the various substrates (31). We propose that the structural differences in this domain in GK, as compared to other family members, might also reflect the diversity in the regulatory properties of the enzymes. The E. coli NAGK is not an allosteric enzyme (although the orthologous enzyme from Pseudomonas aeruginosa and other bacteria is subject to feedback regulation) (45), and there are no reports on the feedback regulation of CK or CK-CPS. Thus, when the structure of GK is available, it will be interesting to compare the features that determine the allosteric properties of these related enzymes.

The predicted allosteric domain in tomPRO1 GK appears to be arranged to be able to make direct connection between the dimer interface and the substrate binding site. In fact, several residues within the regulatory domain can be assigned to residues of CK-CPS that play a role in dimer formation (residues 118, 149, 150, 152, 153, 154 in tomPRO1 GK) or in substrate binding (residue 162 in tomPRO1 GK). As the allosteric domain may be proximal to the dimer interface and substrate binding site, a conformational alteration of the domain upon proline binding could affect catalytic activity or inter-subunit interaction. Residue 62 in tomPRO1 GK corresponds to a residue that functions as part of the phosphate binding site in CK-CPS and the N-acetylglutamate binding site of NAGK, and residues 79, 94 and 127 were placed in α helices that are essential for dimerization in CK-CPS and NAGK. These results also support the possibility of reciprocal communication among substrate binding, subunit interaction, and
allosteric regulation. Residues 62 and 162, where mutations resulted in the greatest decrease in specific activity, are located at the borderline between the allosteric domain and the catalytic domain in our predicted 3D model, consistent with the possibility that these residues could have an overlapping role in feedback regulation and catalytic activity. The suggestion that there is an interaction between the catalytic and allosteric domains is consistent with previous biochemical analyses indicating that the binding of the proline could affect the substrate binding (22). The observation, that a mutation in the proline binding site was found to confer decreased stability of GK (21), is consistent with the suggestion that there is an interaction between the proline binding site and other parts of the molecule that determine subunit interaction, as suggested by our model.

The exceptional residue 198 is in the putative C-terminal domain, which has been suggested to be involved in nucleotide binding. Because it has been reported that ATP and proline bind independently (20,22), and because the predicted ATP binding region is distant from the proline binding motif in our proposed structure, the mutation at this position may influence effector binding in an indirect manner. It may be noted that the mutation at residue 198 actually caused a 4.5-fold increase in the specific activity of the enzyme, along with only a minor (~2-fold) increase in the apparent K_i for proline (26).

Two conserved regions, I and II (Fig. 1), have been suggested to be involved in phosphate and nucleotide binding in GKS, respectively (32). Region I corresponds to sequences located in αB of CK-CPS, and region II corresponds to sequences contained in structures αF to β12b in CK-CPS (Fig. 4). The latter contains the loop that forms most of the ATP binding site (β12-β13 loop in CK-CPS). These structural elements for Domains I and II are well conserved within the amino acid kinase family, suggesting that they constitute an important structure for ATP binding and phosphate transfer. The fact that these two conserved regions were arranged within a central core rather than two distant loops or unstructured domains corroborates the relevancy of our model.
**Conclusion** – Our data, obtained by genetic and biochemical approaches and tertiary structure prediction led to the identification of residues important for allosteric regulation of GK and provide an insight for structure-functional relationship of this enzyme. The effector binding domain may mutually interact with or partly overlap components of the substrate binding sites and subunit interaction domain. Further detailed studies, using a combination of various approaches including X-ray crystallography, will be necessary to dissect functional domains and residues essential for the catalytic and regulatory properties of the enzyme. Knowledge gained from such studies might allow us to control the flux of proline biosynthesis, which has potential applications for development of proline overproducing bacteria and engineering of improved adaptation to osmotic stress in important crops (2).

**Acknowledgments** – We thank E. Bremer for bringing to our attention the existence of the proline-insensitive GK in *B. subtilis*, D. Eisenberg for pointing out the similarities in the structure of GK and CK, H. Hatanaka for helpful insights on the 3D modeling of *tomPRO1* GK, and T. Smith for a critical reading of the manuscript. This work was supported by the U. S. Department of Agriculture, under grant No. 93-37100-8871.
REFERENCES

Abbreviations: ampicillin, Ap; aspartate kinase, AK; carbamate kinase, CK; carbamate kinase-like carbamoyl phosphate synthetase, CK-CPS; 3,4-dehydro-DL-proline, Dhp; $\gamma$-glutamyl kinase, GK; $\gamma$-glutamyl phosphate reductase, GPR; minimal medium 63, M63; optical density at 600 nm, OD$_{600}$; N-acetyl-L-glutamate kinase, NAGK; Protein Data Bank, PDB; $\Delta^1$-pyrroline-5-carboxylate, P5C; P5C reductase, P5CR; $\Delta^1$-pyrroline-5-carboxylate synthetase (bifunctional GK-GPR enzyme), P5CS; resistance to an antibiotic or antimetabolite is indicated by superscript R; uridylate kinase, UK. Nucleotides are denoted by lower case letters and amino acids by uppercase single letters or the three letter abbreviations.
FIG. 1. **Positions in the primary structure of *tomPRO1* GK where mutations resulted in decreased sensitivity to proline feedback inhibition.** The positions of nine amino acid where mutations resulted in DhpR are indicated by red arrowheads. Region I (blue) is the putative phosphate binding site and region II (green), which has been originally identified as the AK-homologous region (32), constitutes part of the nucleotide and acyl substrate binding sites.

FIG. 2. **Proline inhibition of wild type and mutant form of *tomPRO1* GK.** GK/GPR coupled assay was carried out in crude extracts of proline auxotroph *E. coli* strain harboring wild type or mutant versions of *tomPRO1*. Results are expressed as percent of activities in the absence of proline.

FIG. 3. **Sequence alignment of proteins in GK family from various organisms to the corresponding region of *tomPRO1* GK (positions 147 to 162).** The numbers are for the positions of the *tomPRO1* GK. Same or similar residues common to more than half of GKs are shaded, and conserved positively charged residues in plant P5CS enzymes at position 153 of *tomPRO1* are boxed (see text). Triangles indicate residues where amino acid substitutions were obtained in *tomPRO1* GK mutants lacking of feedback regulation; open triangles show positions that are occupied by an identical or similar amino acid in all species, and closed triangles point out divergent residues. Accession numbers for GK genes: *B. subtilis* ProJ, F69682; *B. subtilis* ProB, P39820; *C. glutamicum*, U31230; *E. coli*, P07005; *H. influenzae*, P43763; *L. esculentum* *tomPRO1* U27454; *S. marcescens*, P17856; *S. cerevisiae*, P32264; *S. thermophilus* GK, X92418; *Synechocystis* sp., D90903; *T. thermophilus*, D29973; *T. pallidum*, U61535; for P5CS genes: *A. thaliana* P5CSA, D32138; *A. thaliana* P5CSB, X86778; *Actinidia delicosa*, U92286; *C. elegans*, Z50797; *H. sapiens*, X94453; *L. esculentum* *tomPRO2*, U60267; *M. sativa*, X98421; *O. sativa*, D49714; *V. aconitifolia*, M92276.
**Fig. 4.** Comparison of the hypothetical secondary structure of *tomPRO1* GK with CK-CPS. Arrows and cylinders above and below the sequences depict β strands and α helices, respectively. Eight of main β strands are colored blue and a small β sheet in purple. All other strands and helix D included in a peripheral domain of CK-CPS are colored orange. Main helices that make up the inter-subunit interface are in yellow and the remaining helices in green. Red triangles and red circles show important residues for feedback regulation identified in this and other studies, respectively. Region I of *tomPRO1* GK, the putative phosphate binding site, and region II, which shows high conservation to amino acid kinase family, are highlighted by blue and green boxes, respectively.

**Fig. 5.** A proposed three-dimensional model of *tomPRO1* GK. (A) View from upper side of the molecule. (B) View from the dimerization surface. Nine amino acid residues that were identified in this study and seven residues that were identified by others are highlighted in red and purple, respectively. Regions I and II are shown in blue and green, respectively. Helices αD and αE in *tomPRO1* GK, which correspond to α helices that are important for inter-subunit interaction in CK-CPS, are colored yellow. The domain where mutations were closely positioned may constitute a structure involved in allosteric regulation by proline. The discontinuities in the ribbons correspond to regions of *tomPRO1* GK where we were not able to calculate significant conformational conservation against the template structure.
### Table I

*Location of Dhp-resistant mutations in tomPRO1 GK*

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Clones&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>g462 → a</td>
<td>A62 → T</td>
<td>Dr173, 246, 294, 433</td>
</tr>
<tr>
<td>c463 → t</td>
<td>A62 → V</td>
<td>Dr100, 262, 408</td>
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<tr>
<td>t514 → c</td>
<td>I79 → T</td>
<td>Dr161, 174, 404</td>
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<tr>
<td>t559 → c</td>
<td>M94 → T</td>
<td>Dr199, 395</td>
</tr>
<tr>
<td>a718 → g</td>
<td>D147 → G</td>
<td>Dr220, 428</td>
</tr>
<tr>
<td>a723 → t</td>
<td>I149 → F</td>
<td>Dr122</td>
</tr>
<tr>
<td>g735 → a</td>
<td>E153 → K</td>
<td>Dr55, 447</td>
</tr>
<tr>
<td>a736 → c</td>
<td>E153 → A</td>
<td>Dr319</td>
</tr>
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<td>a736 → g</td>
<td>E153 → G</td>
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<td>t739 → c</td>
<td>L154 → S</td>
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<tr>
<td>t753 → c</td>
<td>S159 → P</td>
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</tr>
<tr>
<td>g762 → a</td>
<td>D162 → N</td>
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<tr>
<td>a763 → g</td>
<td>D162 → G</td>
<td>Dr153, 214&lt;sup&gt;b&lt;/sup&gt;, 258, 282, 292, 311, 361, 383</td>
</tr>
</tbody>
</table>

<sup>a</sup>Clones whose complete sequence of GK region was determined are underlined.

<sup>b</sup>Dr214 also has a silent mutation (g to a) at nucleotide position 722.
# Table II

*Characterization of mutant alleles of *tomPRO1* GK*

<table>
<thead>
<tr>
<th>Mutation</th>
<th>γ-glutamyl kinase specific activity(^a) (nmol min \cdot mg protein(^{-1}))</th>
<th>Apparent K(_i) (mM)</th>
<th>Proline level (µmole/OD(_{600}))</th>
<th>Doubling time (min)</th>
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\(^a\)The enzyme specific activity was determined as described in Experimental procedures in crude extracts of *E. coli* strain CSH26 carrying the various mutant *tomPRO1* alleles on BluescriptKSII+. The number in this column represents 100% activity in Fig. 2.
Figure 2
<table>
<thead>
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Fig. 3
Figure 4
Figure 5
Identification of regions of the tomato gamma-glutamyl kinase that are involved in allosteric regulation by proline
Tomomichi Fujita, Albino Maggio, Mario García-Ríos, Cynthia Stauffacher, Ray A. Bressan and Laszlo N. Csonka

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