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NOTE

Isolation and partial characterization of the *Chlamydomonas reinhardtii* γ-glutamyl kinase

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We isolated a *Chlamydomonas* gene encoding putative γ-glutamyl kinase (GK), an enzyme that catalyzes the first step of proline biosynthesis. Using an *Escherichia coli* auxotroph and a purified recombinant protein, we show that *Chlamydomonas* GK is a functional GK. The sensitivity of this kinase to feedback inhibition by proline was lower than in that of microbial GKs previously reported.

[**Key words:** γ-Glutamyl kinase; *Chlamydomonas reinhardtii*; Proline; Feedback regulation; Recombinant protein]

As a component of proteins, proline plays a crucial role in cellular metabolism and is also generally considered to act as an osmolyte to ameliorate environmental stress (1). Numerous studies have shown that proline accumulation occurs in photosynthetic organisms like plants and green algae when exposed to osmotic or salt stress (2). Recent studies have indicated that proline has multiple functions in addition to its action as an osmolyte. It has roles in maintaining redox balance, in developmental regulation and in metabolic signaling pathways linked to multiple physiological and molecular responses (3), highlighting the importance of the proline biosynthetic pathway and its key control enzyme.

In plants, the key enzyme in the biosynthesis of proline from glutamate is the well-conserved enzyme, 1-pyrroline 5-carboxylate synthase (P5CS). P5CS is a bifunctional enzyme that contains a γ-glutamyl kinase (GK) domain at its N-terminus, which catalyzes the conversion of glutamate to γ-glutamyl phosphate. Located at the C-terminus of P5CS is a γ-glutamyl phosphate reductase (GPR) domain, which catalyzes the reduction of γ-glutamyl phosphate (4). In green algae, such as *Chlamydomonas reinhardtii* (*Chlamydomonas* throughout), separate genes that putatively encode GK and GPR have been identified from gene models based on genome sequencing data (5). This is also true of bacteria and lower eukaryotes, such as *Escherichia coli* and *Saccharomyces cerevisiae*, in which two separate enzymes, GK and GPR, metabolize glutamate, as P5CS does in higher plants (6,7).

Enzymatic analysis has revealed that microbial GKs and plant P5CS enzymes are under metabolic regulation, such that GK activity is inhibited through feedback regulation by the end product proline (6,8–10). A number of mutations that weaken or eliminate enzyme sensitivity to proline have been described for GKs and P5CS enzymes. These mutations result in an increase in the intracellular proline content in a number of organisms and, occasionally, to an improved tolerance of environmental stress (2,9), demonstrating the importance of feedback regulation in *in vivo* proline synthesis and the stress response.

In plants, there are several lines of evidence suggesting a connection between photosynthesis and proline metabolism (3). For example, some genes involved in proline metabolism, including P5CS, have been suggested to be activated in a light-dependent manner (11) and to be located in chloroplasts, where photosynthesis occurs, under certain stress conditions (12,13). Studies of green algae, such as *Chlamydomonas*, is of interest in terms of offering further insight into the role and regulation of proline biosynthesis because these organisms appear to possess the microbial GK type but are classified together with higher plants, that is, in the green clade of eukaryotic photosynthetic organisms. However, the understanding of proline biosynthesis in green algae is still in its infancy when compared to the wealth of information about the biochemistry and function of GK in bacteria and yeast, and P5CS in plants. In this study, we isolated a *Chlamydomonas* gene putatively encoding GK from an expressed sequence tag (EST) library and partially analyzed its function and enzymatic properties.

Using the BLAST program, protein homology searches of amino acid sequences found in both bacterial GK and plant P5CS enabled us to identify a *Chlamydomonas* EST clone (AV641316) containing a sequence encoding at least part of a GK-like protein from the Kazusa *Chalmydomonas* EST library (http://est.kazusa.or.jp/en/plant/chlamy/EST/). Sequencing of the entire cDNA insert in AV641316 revealed that it was 3517 bp in length and consisted of a 2325 bp putative ORF encoding a polypeptide of 774 amino acids, a 140 bp 5′ UTR and a 1052 bp 3′ UTR.

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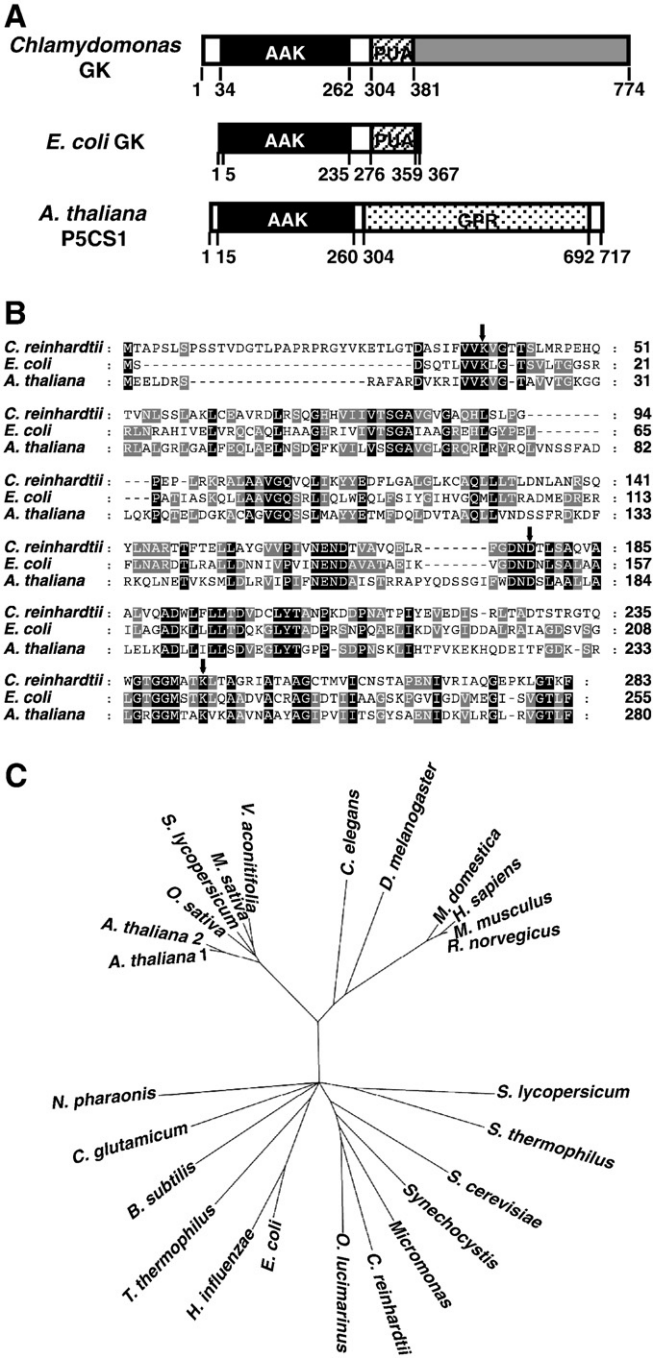
Having compared the sequence with data from the latest version of the *Chlamydomonas* genome (http://genomeportal.jgi-psf.org/Chlre4/Chlre4.home.html), the cDNA was found to be derived from the *PROB1* gene, one of two gene copies, *PROB1* and *PROB2*, which were predicted to encode γ-GK. However, the cDNA sequence was different from the annotated gene/transcript model of *PROB1* gene in its splicing pattern.

The putative GK encoded by the cDNA insert was longer than the GK sequence found in other species (Fig. 1A). GK and P5CS enzymes contain an N-terminal amino acid kinase (AAK) domain and, in most cases, microbial GKs contain a C-terminal RNA-binding domain known as PUA (14) (Fig. 1A). The function of the PUA domain in GK enzymes is not known; however, in *E. coli*, this domain was proposed to have a modulatory role in GK function, although it was not essential for GK enzyme activity (14). Analysis of the primary structural properties of the predicted *Chlamydomonas* GK enzyme using the Pfam (15) and SMART programs (16) suggested that GK harbored an AAK domain (amino acids 34–262 according to the both programs) and a PUA domain (amino acids 304–370 according to Pfam, and 304–381 according to SMART) (Fig. 1A). In addition, *Chlamydomonas* GK contained an extra N- and C-terminal region of around 30 and 400 amino acids, respectively. The C-terminal region exhibited no homology with any known domain or protein in the databases (Fig. 1A). Computer predictions suggested that a chloroplast transit peptide exists at the N-terminal region (iPSORT, http://ipsort.hgc.jp/), and that the GK protein is mainly located in chloroplasts (WoLF PSORT, http://wolfpsort.org/). The alignment of a highly conserved region of *Chlamydomonas* GK (amino acids 1–283) with *E. coli* GK and *Arabidopsis thaliana* P5CS is shown in Fig. 1B. The residues known to be involved in catalysis (4) were invariant in *Chlamydomonas* GK (Fig. 1B). A phylogenetic tree, constructed from the highly conserved region of GK and P5CS, revealed that *Chlamydomonas* GK appeared to belong to the same clade as bacterial and lower eukaryotic GKs and is distinct from the clade in which P5CS from higher eukaryotes belongs (Fig. 1C).



To investigate whether the *Chlamydomonas* GK possesses γ-GK activity, we examined its ability to complement the *E. coli* auxotroph. The host strain for functional complementation tests was JW0232, an *E. coli* strain in which the *proB* gene has been knocked-out (17). To construct expression vectors for *Chlamydomonas* GK, the entire ORF in AV641316 was amplified by PCR, using the primers 5′-GTGATGACCGCGCCATCGCTATCTC-3′ and 5′-ACGAGCTCACGCCGCCGGCAGTGTTGG-3′. The underlined sequence represents a *Sac*I site. The PCR fragment was ligated into pLEAD5 (Nippongene, Tokyo, Japan) to construct GK-pLEAD5, according to the manufacturer's instructions. The sequence of PCR products was confirmed by DNA sequencing. JW0232 was transformed with the GK-pLEAD5 and empty vector and grown on agar plates containing M9 medium with 1 mM IPTG at 37°C. The JW0232 strain transformed with the empty pLEAD5 vector grew only on medium containing 1 mM proline (Fig. 2A), and not in its absence (Fig. 2B). In contrast, expression of *Chlamydomonas* GK allowed the JW0232 strain to grow on the medium without proline (Fig. 2B), suggesting that *Chlamydomonas* GK is a functional GK enzyme.

To further evaluate the enzymatic properties of *Chlamydomonas* GK, recombinant His-tagged GK was expressed in *E. coli* and purified by affinity chromatography. First, to construct the His-tagged GK expression plasmid, the entire ORF in AV641316 was amplified by PCR with the primers 5′-TATATATCATATGACCGCGCCATCGCTATCTCCGAGTTC-3′ and

FIG. 1. Characterization of the *Chlamydomonas* GK protein. (A) Domain organization of *E. coli* GK, *Chlamydomonas* GK and *A. thaliana* P5CS. (B) Multiple sequence alignment of amino acids 1–283 of *Chlamydomonas* GK. Residues that are identical or similar are shown on black and gray backgrounds, respectively. The residues involved in the catalysis are indicated by arrows. (C) Phylogenetic relationship among GK homologs. An unrooted phylogenetic tree was constructed from a highly conserved region of deduced GK and P5CS amino acid sequences with Geneious Pro software (ver.Pro.5.1; Biomatters, Auckland, New Zealand) using the neighbor-joining method with a Jukes–Cantor genetic distance model. The bootstrapping value for each node was calculated from 100 replicates, with values of >50% shown at each divergence point. Sequence data can be found in the GenBank/EMBL databases under the following accession numbers: BAA06864.1 for *A. thaliana* P5CS1; NP_191120.2 for *A. thaliana* P5CS2; CAA90672.1 for *Caenorhabditis elegans* P5CS; AF51799.1 for *Drosophila melanogaster* P5CS; CAA64224.1 for *Homo sapiens* P5CS; XP_001374686.1 for *Monodelphis domestica* P5CS; NP_062672.2 for *Mus musculus* P5CS; CAA67069.1 for *Medicago sativa* P5CS; BAA19916.1 for *Oryza sativa* P5CS; NP_001101994.1 for *Rattus norvegicus* P5CS; AAB67875.1 for *Solanum lycopersicum* P5CS; P32296.1 for *Vigna aconitifolia* P5CS; CAB13740.1 for *Bacillus subtilis* GK; AAC44174.1 for *Corynebacterium glutamicum* GK; NP_414777.1 for *E. coli* GK; AAC22560.1 for *Haemophilus influenzae* GK; XP_002505450.1 for *Micromonas* GK; Q3IP73.1 for *Natronomonas pharaonis* GK; XP_001419513.1 for *Ostreococcus lucimarinus* GK; AAA34904.1 for *S. cerevisiae* GK; AAB67876.1 for *Solanum lycopersicum* GK; CAA63147.1 for *Streptococcus thermophilus* GK; BAA17096.1 for *Synechocystis* GK; and BAA06237.1 for *Thermus thermophilus* GK. Alignments of overall amino acid sequences of *Chlamydomonas* GK with GKs and P5CSs of various other organisms are shown in Fig. S1.