Plant Science 351 (2025) 112327

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Loss of PII-dependent control of arginine biosynthesis in *Dunaliella salina*

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ARTICLE INFO

Keywords:
Arginine biosynthesis
Dunaliella salina
N-acetyl-L-glutamate kinase
PII-signal proteins
Salinity tolerance

ABSTRACT

In cyanobacteria and most Archaeplastida, Arg regulates its formation via allosteric inhibition of the controlling enzyme, N-acetyl-L-glutamate kinase (NAGK) that requires PII protein to properly sense the feedback inhibitor. Although PII expression has been shown to be reduced in *Dunaliella salina* compared to other green algae, the potential impact of this protein on DsNAGK activity remains unclear. We here performed coupled enzyme assay and surface plasmon resonance analysis and show that DsNAGK is activated by NAG and inhibited by Arg but is not controlled by DsPII. Moreover, DsPII has likely lost its function as an effective glutamine sensor. Replacement of the C-terminus from DsPII with the C-terminus from *Chlamydomonas* PII restored sensitivity to glutamine in a recombinant DsPII protein, demonstrating the importance of C-terminal residues close to the Q-loop for PII functions. The findings are discussed in the context of the relationship between NAGK control and the acquisition of salinity tolerance during evolution.

1. Introduction

Arginine (Arg) biosynthesis has been one of key metabolic processes through the evolution of the adaptive responses of plants (Llácer et al., 2008; Slocum, 2005; Winter et al., 2015). Among the 21 proteinogenic amino acids, arginine has the highest nitrogen to carbon ratio, which makes it especially suitable as a storage form of organic nitrogen.

In plants, Arg regulates its formation via allosteric inhibition of the synthesis of N-Acetyl-L-glutamyl-phosphate (Llácer et al., 2008). This rate-limiting step in the ornithine/arginine (Arg) biosynthesis pathway is catalyzed by N-acetyl-L-glutamate kinase (NAGK), which phosphorylates N-acetyl-L-glutamate to N-acetyl-L-glutamyl-phosphate (Selim et al., 2020a). In accord with an endosymbiotic origin of the PII gene, the plant PII proteins are close relatives the proteins from cyanobacteria (Sant'Anna et al., 2009; Uhrig et al., 2009). In cyanobacteria and most Archaeplastida, NAGK is the target of the signaling protein PII, which orchestrates metabolic adaptations to nitrogen/carbon abundance (Forchhammer and Selim, 2020; Selim et al., 2020a). By binding to NAGK, PII relieves NAGK from Arg inhibition and allows nitrogen to accumulate as Arg (Beez et al., 2009; Chen et al., 2006; Lapina et al., 2018; Sugiyama et al., 2004).

PII proteins are highly conserved and widely distributed signal transduction proteins known in all domains of life (Fokina et al., 2010; Forchhammer and Selim, 2020; Huergo et al., 2013; Selim et al., 2020a). However, in the eukaryotes, PII homologs inherited from a cyanobacterial endosymbiont are restricted to Archaeplastida (Chellamuthu et al., 2013). In green algae and higher plants, NAGK activity is controlled by the glutamine (Gln) levels via Gln-dependent PII-NAGK complex formation, which leads to increased enzyme activity (Chellamuthu et al., 2014; Li et al., 2017; Minaeva et al., 2015). Gln sensing as the primary product of nitrogen (N) assimilation indicates the specialization of PII from green algae and land plants to respond to the cellular N status. Interestingly, PII has conspicuously lost across Asteraceae and in some red and green algae (Chellamuthu et al., 2013; Selim et al., 2020a; Shen et al., 2023).

Among the green algae, *Dunaliella salina* (*Dunaliella* hereinafter) has become a good model system for revealing important facts about the regulation of salt tolerance in the halophytes. Progress has been made in characterizing genes encoding components involved in salt stress response, with an emphasis on transport mechanisms, glycerol and carotenoid metabolism and photosynthetic apparatus remodeling (Polle et al., 2020; Ramachandran et al., 2023). The *Dunaliella* genome, at least in the strain used to sequence it, retains the gene encoding DsPII (Polle et al., 2020). However, unlike other green algae, DsPII expression in cells is reduced (Ermilova et al., 2013; Lapina et al., 2019; Minaeva and Ermilova, 2015; Zalutskaya et al., 2018; Vlasova et al., 2024). It

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https://doi.org/10.1016/j.plantsci.2024.112327
Received 16 August 2024; Received in revised form 19 October 2024; Accepted 22 November 2024
Available online 23 November 2024

remained unclear whether only the regulation of DsPII expression was altered or also the properties of this protein. The apparent gap in information about the control mechanisms of DsNAGK prompted us to study the biochemical features of this enzyme and PII-DsNAGK complexes. Surprisingly, we found unique features not described for NAGK regulation so far. The present study is the first to address the control of NAGK activity in the halophilic alga, where the signal protein PII is not involved in the regulatory network.

2. Materials and methods

2.1. Strains and cultivation conditions

The whole cloning and AQUA (advanced quick assembly) cloning procedures were performed in *E. coli* NEB 10-beta and TOP10 strains respectively, while protein expression and purification were done using *E. coli* LEMO-21(DE3) and PII-deficient *E. coli* RB9060 (Beyer et al., 2015; Bueno et al., 1985) in LB medium.

2.2. Cloning of DsNAGK and DsPII proteins

The sequences for DsNAGK and DsPII were derived from the algal genomics resource PhycoCosm (https://phycocosm.jgi.doe.gov/Dunsal1_1/Dunsal1_1.home.html) with sequence ID: for DsNAGK (Dusal.0374s00005.1) and for DsPII (Dusal.0350s00002.1). Gene Blocks, with optimized codon usage for cloning and expression into *E. coli*, encoding for amino acid sequences of mature DsNAGK and DsPII genes without plastid signal peptides, were synthesized by IDT, USA. The first Gene Block fragment for the amino acid sequence of the DsNAGK was derived from a DNA sequence starting with the 67th amino acid (ASDA) and was flanked at the 5' and 3' ends by the sequences: AGGAGCGGCCTGGTGCCGCGCGGCAGC and TATGCTCGAGGATCCG GCTGCTAACAAGC, respectively. The second Gene Block for the DNA sequence of DsPII was derived from the amino acid sequence starting with the 58th amino acid (ASLK) and was flanked by *Bsa*I restriction sites. Using advanced quick assembly, the Gene Blocks were cloned directly into *Nde*I-digested pET15b vector (Novagen, Darmstadt, Germany) and *Bsa*I-digested pASK-IBA3 vector (IBA, Munich, Germany), respectively, as described previously (Beyer et al., 2015). The potential signal peptide of DsNAGK was determined using a TargetP - 2.0 Server (https://services.healthtech.dtu.dk/services/TargetP-2.0/). The generated plasmids were verified by sequencing. The DsPII/Cr_C chimera was generated by PCR amplification with Q5 Hot start DNA polymerase (New England Biolabs) in two stages (Fig. S1). In the first PCR, the DsPII gene block and primers were used: DsPIIF1: 5'-GAATAGTTCGA-CAAAAATCTAGATAACGAGGGCAAAAAATGGCATCATTAAAACGTGC CAGCTACGC-3' and DsPIIR1: 5'-CCATTCCGCCCTCCATACG-3' (Evrogen company).

The PCR product was purified in 0.9 % agarose electrophoresis and used for a second PCR with site-directed mutagenesis primers: forward DsPIIF1: 5'- GAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAA ATGGCATCATTAAAACGTGCCAGCTACGC-3' and DsPIIR2: 5'-GGTGGC TCCAAGCGCTCTTTTTTTTCTTCATCATATCTTCCATTCCGCCCTCCATA C-3'. After purification on the agarose gel the second PCR product mixed with the linear form of the pET-15b vector (Novagen) and was transformed into *E. coli* Top10 cells by AQUA cloning (Beyer et al., 2015). The generated plasmid was verified by sequencing.

2.3. Expression and purification of DsNAGK, CrNAGK, DsPII, CrPII, and DsPII/Cr_C proteins

The overexpression of the recombinant N-terminal fused His_6-tagged DsNAGK and CrNAGK was performed in *E. coli* LEMO-21(DE3) and the proteins were affinity purified on a Ni-NTA columns according to (Lapina et al., 2018; Maheswaran et al., 2004). Overexpression of the recombinant C-terminal fused strep-tagged PII proteins (DsPII, CrPII and DsPII/Cr_C) were performed in PII-deficient *E. coli* RB9060 (Bueno et al., 1985) and the proteins were affinity purified on a Strep-Tactin II column according to Heinrich et al., 2004 (Fig. S2).

2.4. Gel filtration analysis

Gel filtration experiments were performed on chromatography system NGC Discover 10 (BioRad, USA) operated at room temperature. A precision column Superdex 200, GL 10/300 (Amersham Biosciences) was used with a running buffer consisting of 10 mM Tris pH 7.8, 300 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.02 % NaN_3 and 2 % glycerol. To determine the oligomeric structure of protein, the sample of 0.1 ml DsPII at a concentration of 1 μg/μl was applied to the sample loop and chromatographed at a flow rate of 0.05 ml/min. The elution profile of the protein samples were recorded by UV-detection at 280 nm. The Superdex 200 10/300 column was calibrated from a standard plot of Kav versus molecular mass for blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrohenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa).

2.5. Coupled NAGK activity assay

The activity of NAGK was assessed using a coupled enzyme assay, in which the production of ADP after the consumption of ATP for phosphorylation of NAG was associated with the oxidation of NADH by pyruvate kinase and lactate dehydrogenase as described previously (Beez et al., 2009; Lapina et al., 2018).

The standard reaction mixture consisted of 50 mM imidazole pH 7.5, 50-mM KCl, 20-mM $MgCl_2$, 0.4 mM NADH, 1-mM phosphoenolpyruvate, 5-mM ATP, 0.5-mM DTT, 11 U lactate dehydrogenase, 15 U pyruvate kinase and 50 mM NAG and the reaction was started by the addition of 3 μg NAGK. When necessary, PII protein was added to the reaction mix in equimolar concentration. When needed, the effector molecules Gln and Arg were added to the reaction mixtures at concentrations as indicated. The oxidation of NADH was measured at 340 nm for 10 min with a SPECORD-spectrophotometer (model-210 PLUS, Analytik Jena AG). One molecule oxidation of NADH is proportional to one molecule phosphorylation of NAG. One unit of NAGK catalyzes the conversion of 1 μmol of NAG min^{-1}, calculated with the molar absorption coefficient of NADH of 6178 L mol^{-1} cm^{-1} at 340 nm. Means of triplicate experimental determinations are shown with a standard deviation of less than 5 %. The enzymatic constants Km, kcat and IC_{50} were calculated from the velocity slopes using the GRAPHPAD PRISM software program (Graph-Pad Software, San Diego, CA, USA).

2.6. Surface plasmon resonance analysis

The interaction of NAGKs and PII proteins was measured at room temperature on an iMSPR mini-instrument (Icluebio, Seongnam, South Korea) using 10 mM HEPES pH 7.4, 150 mM NaCl, 0,05 mM EDTA, and 0.005 % (v/v) Tween-20 as a running buffer. The recombinant His_6-DsNAGK were immobilized on the surface of the Ni^{2+}-loaded FC2 channel of NTA–Au chip (iCLUEBIO) as ligands up to 2000–3000 response units. Then, the strep-tagged PII proteins (CrPII, DsPII) in a running buffer with or without glutamine (5 mM, 15 mM) were injected at a flow rate of 18-μl min^{-1} into both FC1 (control for unspecific binding of PII to the sensor chip) and FC2 (immobilized NAGK) at room temperature. The specific binding of PIIs to NAGKs was recorded as the difference in the response signal of FC2-FC1 (ΔRU). For the regeneration of the sensor chips at each cycle, 10 mM glycine-HCl, with a pH of 2.5, was used to remove bound proteins from the surfaces. Curve fitting and data analysis were performed using the iMSPR analysis software. The equilibrium dissociation constant (K_D) is calculated from the sensorgrams.

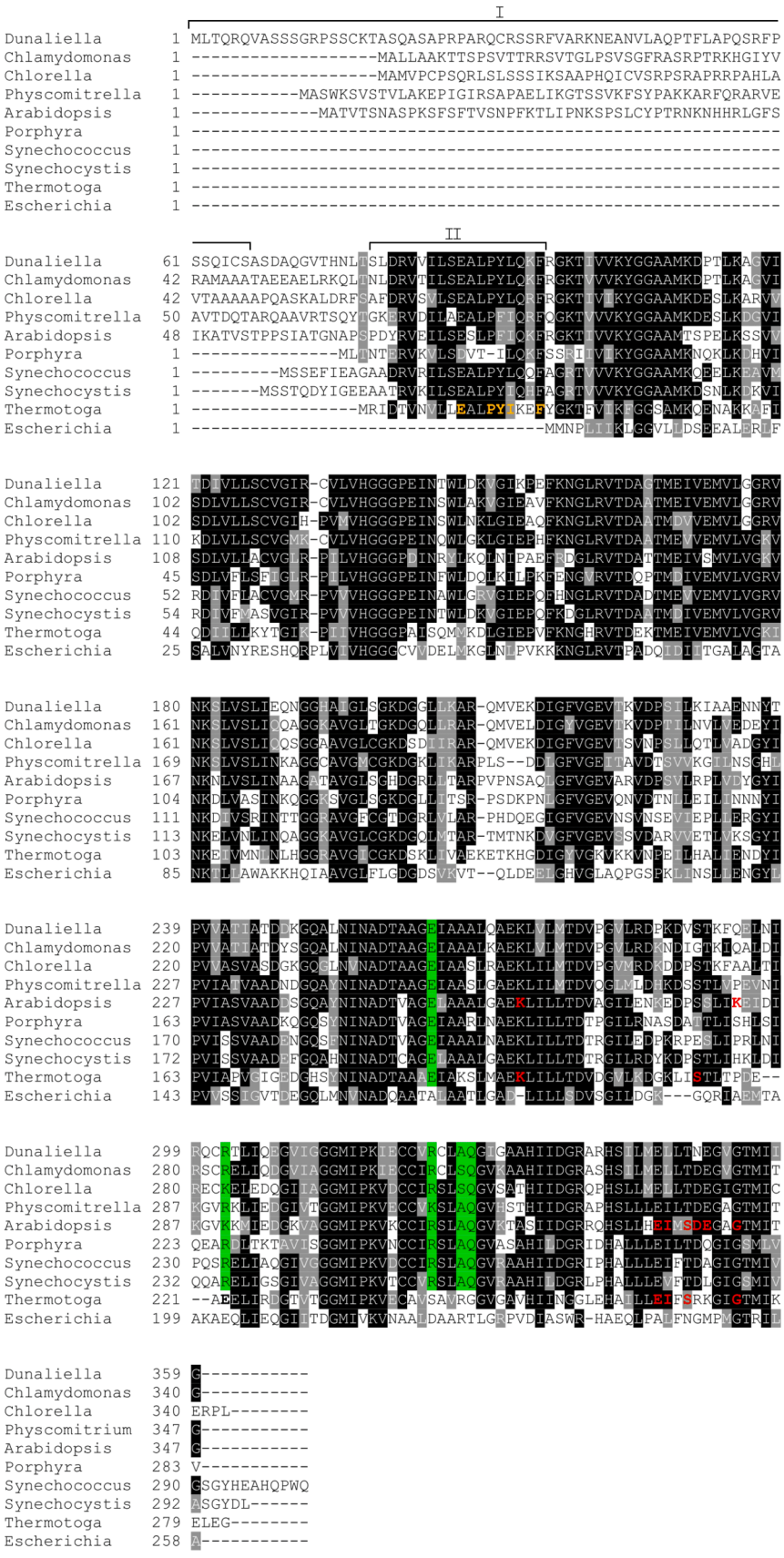

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Fig. 1. Multiple amino acid sequence alignment of NAGK proteins. The DsNAGK protein sequence was obtained from the PhycoCosm database, and the remaining sequences were obtained from the UniProt database. The sequences are derived from NAGK polypeptides of green algae *Dunaliella salina* (Dusal.0374s00005), *Chlamydomonas reinhardtii* (A8HPI1) and *Chlorella variabilis* (E1ZQ49), land plants *Physcomitrella patens* (A0JC02) and *Arabidopsis thaliana* (Q9SCL7), red alga *Porphyra purpurea* (P69365), cyanobacteria *Synechococcus elongatus* PCC 7942 (Q6V1L5) and *Synechocystis* sp. PCC 6803 (P73326), and bacteria *Thermotoga maritima* (Q9X2A4) and *Escherichia coli* (P0A6C8). Highlighted residues in black are invariant in at least 55 % of aligned NAGK proteins. Amino acids in grey represent similar residues. Box I refers to plastid-targeting signal peptides sequence (TargetP-2.0 server, https://services.healthtech.dtu.dk/services/TargetP-2.0/). Box II indicates an N-terminal signature extension of Arg-sensitive NAGK proteins, which is absent in Arg insensitive *E. coli* NAGK (Ramón-Maiques et al., 2006). In Box II, the previously identified signature sequence of Arg-sensitive NAGK from *Thermotoga maritima* is highlighted in yellow, which is involved in forming the allosteric Arg binding site (Ramón-Maiques et al., 2006). Amino acid residues directly involved in allosteric Arg binding are highlighted in red and are deduced from known structures of NAGK: Arg complexes from *Thermotoga maritima* NAGK (PDB: 2BTY) (Ramón-Maiques et al., 2006) and *Arabidopsis thaliana* (PDB: 2RD5) (Mizuno et al., 2007). Residues important for interaction with PII are highlighted in green. The alignment was done using the ClustalW program (https://www.genome.jp/tools-bin/clustalw) and manually refined.

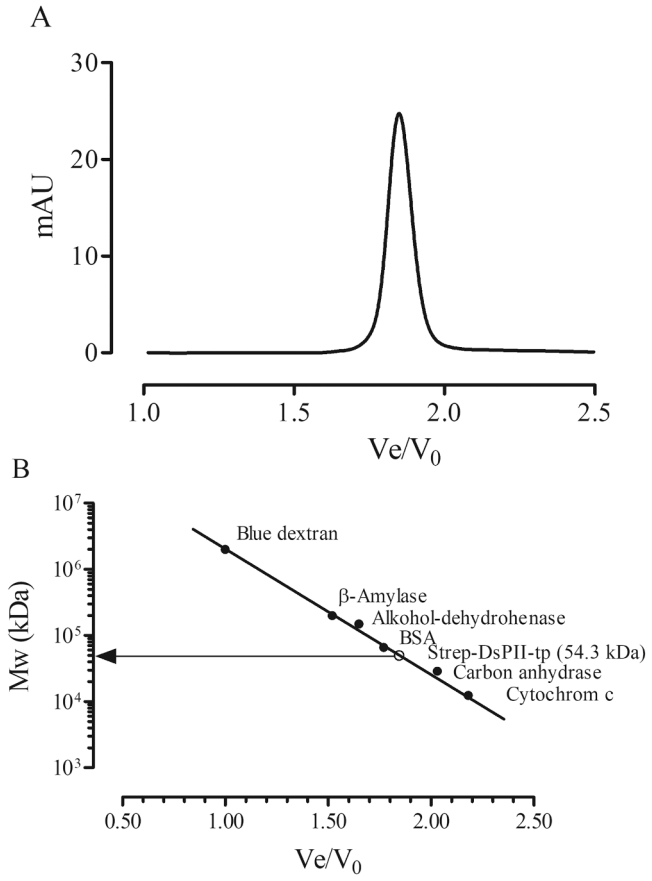

Fig. 2. Gel filtration chromatography of DsPII. A) Chromatographic plot elution volume (Ve/V0) versus absorbance (mAU) of the recombinant DsPII protein without the transit peptide sequence, showing trimeric peak, which corresponds to the following apparent molecular size (in parenthesis the size of the Strep-DsPII-tp trimer calculated from the amino acid sequences): Strep-DsPII-tp, 54.3 kDa (56.4 kDa). B) Standard calibration curve depicting elution volume and molecular size marker proteins: blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrohenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa).

3. Results

3.1. DsNAGK is a canonical plant NAGK protein

The predicted full-length NAGK polypeptide encoded by the *Dunaliella* Dusal.0374s00005.1 gene consists of 359 amino acids with a calculated molecular weight of 38,047.15 Da comprising a putative N-terminal plastid transit peptide (amino acid residues 1–66). The calculated molecular weight of the predicted mature DsNAGK polypeptide is 30,940.10 Da. The DsNAGK sequence exhibits the N-terminal signature pattern of arginine-sensitive NAGK enzymes and the allosteric arginine-binding site appears conserved (Fig. 1; Mizuno et al., 2007; Ramón--Maiques et al., 2006).

To gain further insights into biochemical properties of DsNAGK protein, a recombinant N-terminal His-tagged variant of the predicted mature DsNAGK protein without the plastid transit peptide (the theoretical molecular mass of monomeric recombinant DsNAGK protein is 32.96 kDa), was overexpressed in *E. coli* and affinity-purified (Fig. S2A).

3.2. Kinetic constants of DsNAGK determined by a coupled assay

To characterize the *Dunaliella* NAGK enzyme, the catalytic activity of DsNAGK was determined in the coupled NAGK enzyme assay as described (Beez et al., 2009; Lapina et al., 2018). The kinetic constants of the purified recombinant DsNAGK enzyme in the absence of the feedback inhibitor arginine exhibited an apparent Km value for NAG of 4.02 ± 0.70 mM and a Vmax of 10.93 ± 0.88 U mg^{-1} (corresponding to a k_{cat} of 11.25 ± 0.86 s^{-1}) (Fig. 2A). This Km value is 4.3-fold higher than that of AtNAGK (0.87 ± 0.11 mM) and is intermediate between that of *Chlorella variabilis* NAGK (CvNAGK) (1.5 ± 0.12 mM) and *C. reinhardtii* NAGK (7.8 ± 0.8 mM) (Chellamuthu et al., 2014; Minaeva et al., 2015). Notably, k_{cat} value is very similar to that of the cyanobacterial *Synechococcus elongatus* NAGK (SeNAGK) (13.1 ± 3.0 s^{-1}) (Beez et al., 2009).

Dunaliella gene Dusal.0350s00002.1 encodes PII protein, which consists of 220 amino acids with a calculated molecular weight of 23,690.82 Da. The alignment showed a high degree of conservation of the functional important regions of PII proteins, including the T-loop residues, which are involved in NAGK interactions (Fig. S3; Llácer et al., 2007, 2008; Mizuno et al., 2007). The mature recombinant DsPII protein without the transit peptide sequence, Strep-DsPII chromatographed as a 54.3 kDa protein during gel filtration implying that, like other PII proteins, DsPII is a homotrimer (Fig. 2).

In the presence of DsPII, the apparent Km for NAG and the specific activity were not changed significantly (Fig. 3A). This effect is similar to that reported for *C. reinhardtii* proteins, where PII has only minor effect on the catalytic constants of NAGK without Arg. Moreover, the overall catalytic efficiency (kcat/Km) was very similar for free (2.78 10^3 s^{-1} M^{-1}) or DsPII-complexed DsNAGK (2.42 10^3 s^{-1} M^{-1}). Strikingly, addition of Gln did not cause any increase in kcat/Km catalytic efficiency (2.70 10^3 s^{-1} M^{-1}), in stark contrast to the situation in *C. reinhardtii* (Chellamuthu et al., 2014), and therefore, the overall DsNAGK catalytic efficiency was not affected by DsPII, neither in the presence nor in the absence of Gln.

3.3. Arginine inhibition of DsNAGK and its modulation by PII proteins

As expected from sequence analysis (Fig. 1), Arg impairs *Dunaliella* NAGK activity (Fig. 3B). Feedback-inhibition of DsNAGK by Arg occurred with a half-maximal inhibitory concentration (IC_{50}) of 0.29 ± 0.05 mM, which is similar to the value determined for the CrNAGK (IC_{50}, 0.11 mM) and MiNAGK (IC_{50}, 0.14 mM) (Chellamuthu et al., 2014; Li et al., 2017). However, much higher concentrations of Arg were needed for inhibiting DsNAGK than cyanobacterial SeNAGK (IC_{50},

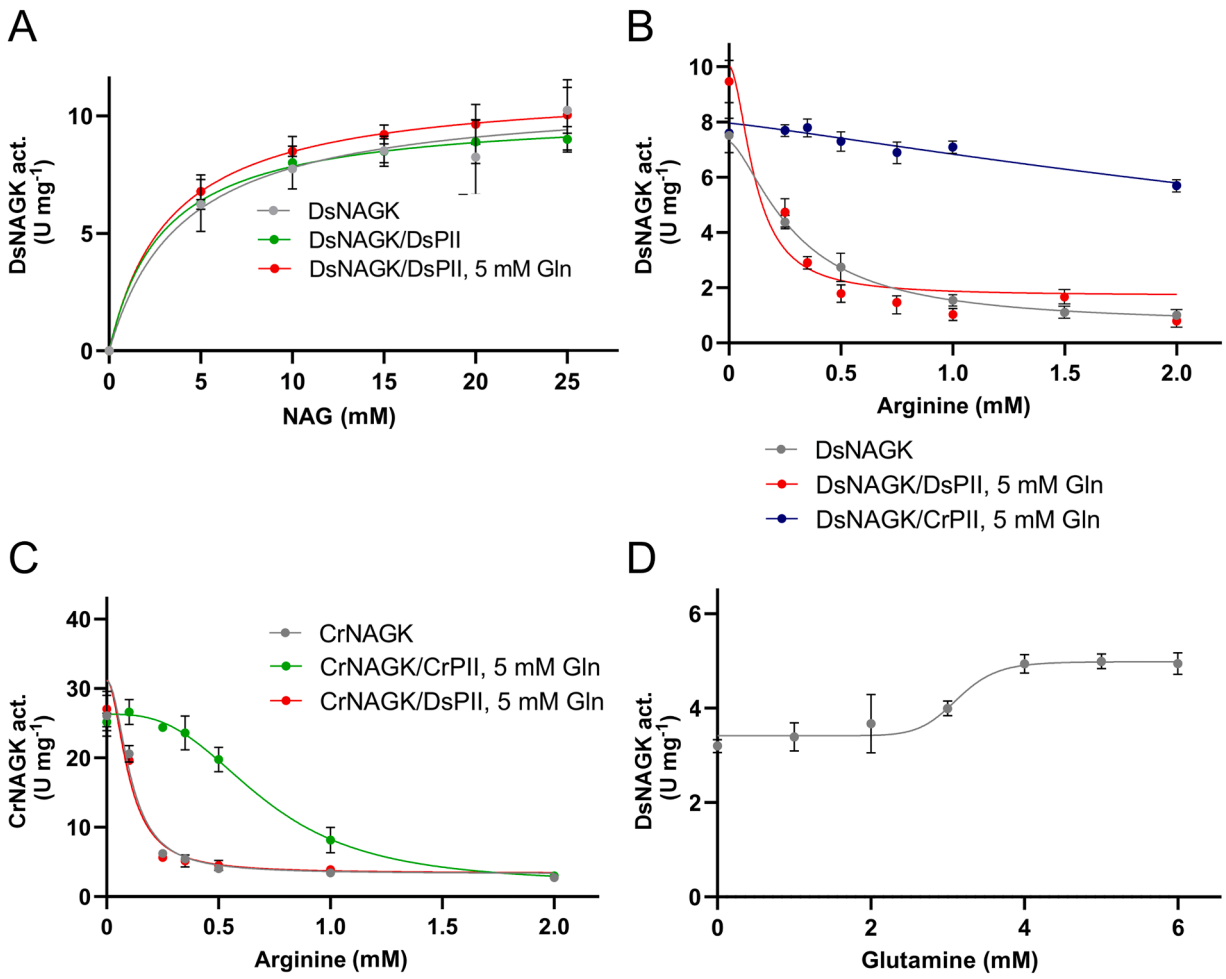

Fig. 3. Characterization of DsNAGK activity. A) Catalytic activity of DsNAGK in presence or absence of DsPII and of 5 mM Gln, as indicated. NAG was used as a variable substrate. B-C) Arg feedback inhibition of NAGK enzymes in presence or absence of PII proteins, with 5-mM Gln, as indicated. In B) DsNAGK with DsPII or CrPII; in C) CrNAGK with DsPII or CrPII. The Arg feedback inhibition data were fitted according to a sigmoidal dose response curve, yielding an IC_{50} for Arg. D) Activity of Arg-inhibited DsNAGK in the presence of DsPII and various concentrations of Gln. All data were fitted using a GraphPad prism program. SD as indicated by error bars, represents triplicate independent triplicate measurements.

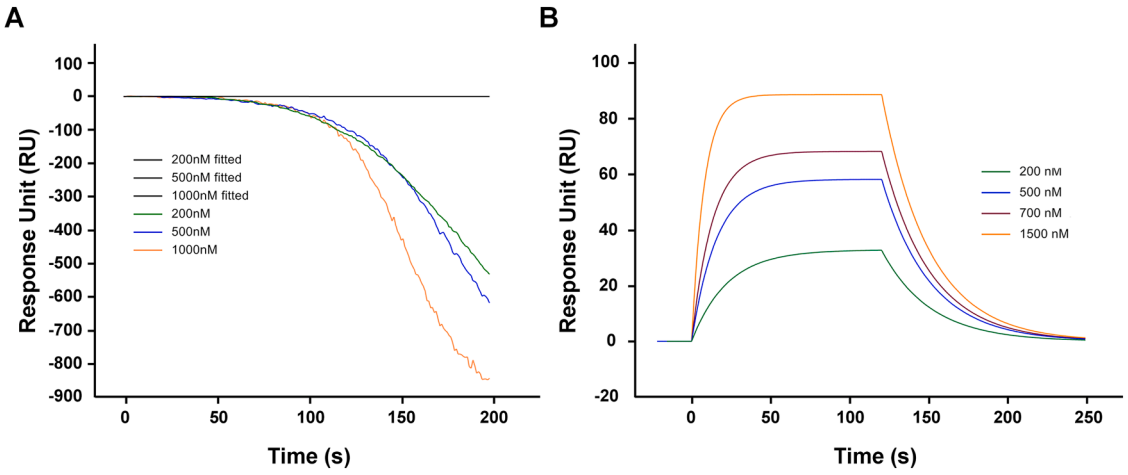

Fig. 4. Surface plasmon resonance spectroscopy analysis of PII-DsNAGK complex formation. Surface plasmon resonance spectroscopy analysis of PII-NAGK complex formation. DsPII (A) or CrPII (B) were injected to chip-immobilized with DsNAGK. In (A) the original (colored) and the fitted curves are shown. In (B) Each SPR sensorgram shows the interaction of the different concentrations (200-, 500-, 700 or 1500-nM) of CrPII.

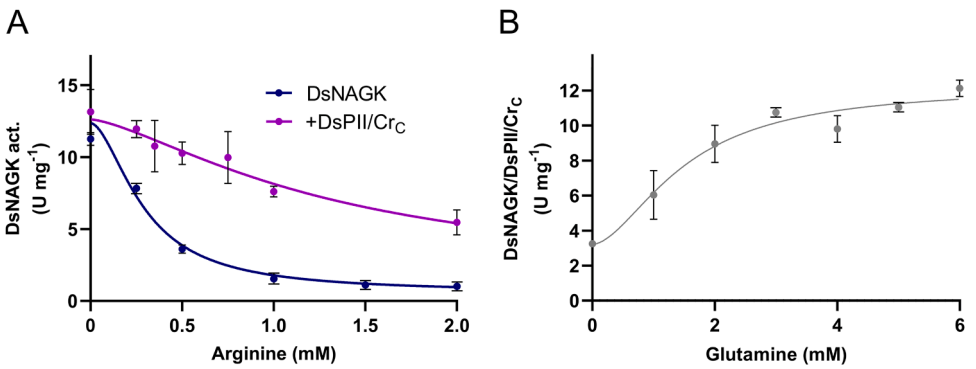

Fig. 5. Effects of a chimeric PII protein composed of DsPII with a C-terminus of *Chlamydomonas* (DsPII/Cr$_C$) on DsNAGK activity. A) DsNAGK with the DsPII/Cr$_C$ chimera was assayed under standard conditions containing Arg in the presence of 5 mM Gln. B) Glutamine-dependent activation of Arg-feedback-inhibited DsNAGK by the DsPII/Cr$_C$ chimera. Assays contained 0.4 mM Arg. All data were fitted using a GraphPad prism program. SD as indicated by error bars, represents triplicate independent triplicate measurements.

0.02 mM) (Beez et al., 2009).

Since the relief from Arg inhibition by PII-NAGK complex formation is crucial for metabolic control of Arg biosynthesis in cyanobacteria and Archaeplastida (Beez et al., 2009; Chellamuthu et al., 2014; Heinrich et al., 2004; Lapina et al., 2018), we asked whether the presence of DsPII could change the Arg inhibition profile of DsNAGK. When 5 mM Gln was added to the assay, DsPII did not relieve the Arg feedback inhibition ($IC_{50,}$ 0.19 ± 0.06 mM; Fig. 3B).

To test further whether the observed lack of Gln effect on the DsPII-DsNAGK complex is due to the properties of DsPII or DsNAGK, we performed a heterologous enzymatic assay using the *C. reinhardtii* proteins (CrPII and CrNAGK). In the presence of CrPII, Gln had a pronounced influence on DsNAGK activity (Fig. 3B). At the same time, unlike CrPII, DsPII did not raise the IC_{50} of Arg for CrNAGK in the presence of Gln (Fig. 3C).

To complement these observations, we titrated the effect of Gln with Arg (0.3 mM). Unlike other green algae (Chellamuthu et al., 2014; Minaeva et al., 2015; Li et al., 2017), no clear differences in DsNAGK activity are observed at different Gln concentrations (Fig. 3D).

Since in *Dunaliella* the resulting free Arg level depends on the flux of ornithine to putrescine and proline (Vlasova et al., 2024), these compounds were tested as potential effector molecules. Ornithine, proline, putrescine compounds, as well as glutamate and 2-OG did not rescue the activity of DsPII (data not shown).

3.4. Analysis of PII-DsNAGK complex formation

The enzyme tests described above suggested that DsPII-DsNAGK complex formation may be different from all previously tested cases (Chellamuthu et al., 2014; Minaeva et al., 2015; Selim et al., 2020b). We therefore analyzed complex formation using surface plasmon resonance (SPR) spectroscopy. The N-terminally His-tagged DsNAGK protein was immobilized on an NTA-Au sensor chip and probed with DsPII together with Mg^{2+}-ATP in combination with Gln (Fig. 4A). In no assays interaction between DsPII and DsNAGK was detected, neither in the presence nor absence of Gln. The variant without Mg^{2+}-ATP was used as a control. This result showed that the complex formation between DsPII and DsNAGK is disrupted. In agreement with the enzymatic assays, CrPII demonstrated formidable binding with DsNAGK with a K_D value of 0.53 µM (Fig. 4B)

3.5. Dependence of glutamine sensing on the C-terminus of PII

Sequence alignment of PII proteins (Fig. S3) reveals that the C-terminal residues of the Q loop in DsPII are part of a conserved motif present in all plant sequences (except the Brassicaceae family), but its C-terminus is the longest. To find out whether the missing Gln response of DsPII is a consequence of its too long C-terminus, we exchanged 18 amino acid amino acids of DsPII (FDQAASSSWIPANPANES) with the 8 amino acids from C-terminus of CrPII (EDMMKKKK) to obtain chimeric DsPII with the *Chlamydomonas* C-terminus (DsPII/Cr$_C$). The DsPII/Cr$_C$ chimera was functional and in the presence of 5 mM Gln, the inhibitory effect of Arg was strongly antagonized (Fig. 5A). In the presence of DsPII/Cr$_C$ and 5 mM glutamine, the IC50 of Arg for DsNAGK increased from 0.29 mM to 1.06 mM (Fig. 5A). The highest difference in activity between DsNAGK with or without DsPII/Cr$_C$ was with 0.4 mM Arg. Therefore, we titrated the effect of Gln with 0.4 mM Arg. Gln activated Arg-inhibited DsNAGK in the presence of chimeric DsPII/Cr$_C$ in a concentration-dependent manner (Fig. 5B). The half-maximal effective concentration (EC_{50}) of Gln for activation of DsNAGK by DsPII/Cr$_C$ was 2.6 mM. Therefore, the inability of *Dunaliella* PII to respond to Gln was indeed caused by the properties of its C-terminus.

4. Discussion

The halotolerant photosynthetic alga *Dunaliella* grows in extremely saline environments and exhibits efficient acclimation mechanisms (Polle et al., 2020; Ramachandran et al., 2023). Like non-halophilic green algae, the *Dunaliella* chloroplast carries out essential anabolic functions including amino acid biosynthesis. In general, the regulation of Arg formation appears easily to be adjusted to the respective metabolic situation in cyanobacteria and Archaeplastida due to the detailed properties of the PII proteins and PII-dependent control of NAGKs (Selim et al., 2020a, 2020b). Interestingly the expression/translocation of *Dunaliella* PII into chloroplast was significantly impaired (Vlasova et al., 2024). We aimed to understand whether the PII-mediated control of NAGK in *Dunaliella* is altered in this alga.

Like other Arg-sensitive NAGKs (Selim et al., 2020a; Slocum, 2005), the activity of the *Dunaliella* enzyme is activated by NAG and inhibited by Arg, consistent with its amino acid sequence (Fig. 1; Fig. 3A,B). The Arg sensitivity profile of free DsNAGK (Arg-IC_{50} of 0.29 mM) is intermediate between the more sensitive *Chlamydomonas* CrNAGK (Arg-IC_{50} of 0.11 mM; Chellamuthu et al., 2014), and the low-sensitive NAGKs from *Arabidopsis* AtNAGK (Arg-IC_{50} of 1.0 mM; Beez et al., 2009) or *Chlorella* CvNAGK (Arg-IC_{50} of 1.2 mM; Minaeva et al., 2015). Surprisingly, DsPII did not enhance the catalytic activity of Arg-feedback-inhibited DsNAGK in either the presence or absence of Gln (Fig. 3B). Moreover, *Dunaliella* PII did not mediate activation of NAGK from *Chlamydomonas* (Fig. 3C). By contrast, CrPII protein showed relieving feedback inhibition of DsNAGK (Fig. 3B). In addition, the DsPII

appears to have lost its function as an effective Gln sensor (Fig. 3D). This agrees with the fact that DsPII did not interact with NAGK (Fig. 4), which suggests that the flexible Q-loop not become structured for NAGK binding.

Grafting the C-terminus from *Chlamydomonas* PII onto the DsPII body restored Gln sensitivity and shows that *Dunaliella* PII can be converted back to a Gln-sensing protein (Fig. 5). In addition to loss of Gln sensing in DsPII and reduced expression of this protein, the resulting intracellular levels of free Arg are dependent on ornithine flux to putrescine and proline and are insufficient to inhibit DsNAGK in actively growing cells under high salinity conditions (Vlasova et al., 2024). It is believed that lowering the concentration of free Arg through promoting putrescine/-proline formation may bypass the requirement for PII-dependent control of NAGK in *Dunaliella*.

An intriguing question concerns the evolution of PII sensing in Archaeplastida. In contrast to cyanobacteria (Forchhammer and Selim, 2020), at the level of protein-protein interactions, there is no characterized target for Gln-regulated PII proteins other than NAGK. In this context, a key question is whether PII has become specialized to regulate NAGK during the evolution of oxygenic phototrophs? Loss of PII proteins in several red and green algae (Chellamuthu et al., 2013; Selim et al., 2020a) and all Asteraceae (Shen et al., 2023) supports the idea that NAGK may be the sole target in Archaeplastida. At the same time, we cannot completely rule out the presence of alternative, as yet unknown cellular targets for PII. A better understanding of the role of PII in plants requires an analysis of PII targets in a larger number of Archaeplastida representatives. Moreover, more studies to fully investigate the physiological and metabolic adaptation machinery in other Archaeplastida without functional PII are further needed.

Taken together, our results expand knowledge of NAGK regulation and control of Arg synthesis in plants. A deeper comprehension of NAGK properties in other marine Chlorophyta and Rhodophyta lacking PII will yield valuable insights into the molecular mechanisms involved in the acquisition of salinity tolerance during evolution.

CRediT authorship contribution statement

Vitalina Vlasova: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Tatiana Lapina:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Qi Cheng:** Writing – review & editing, Writing – original draft, Conceptualization. **Elena Ermilova:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest about the present work.

Acknowledgments

Sequencing was performed at the Research Resource Center for molecular and cell technologies of Saint-Petersburg State University. We thank Dr. Elena Dolgih and the Core Centrum 'Genomic Technologies, Proteomics and Cell Biology' in ARRIAM for assistance with SRP analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2024.112327.

Data availability

Data will be made available on request.

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