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Protistology

ORIGINAL ARTICLE

Characterization of the unusual PII protein with elongated T-loop from *Micromonas pusilla*

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Summary

IThe PII superfamily consists of widespread signal transduction proteins found in all domains of life. The most conserved PII-interactor across oxygenic phototrophs from cyanobacteria to Archaeplastida is the key enzyme of the ornithine/arginine synthesis pathway, N-acetyl-L-glutamate kinase (NAGK). T-loops represent the major PII-receptor binding element and are involved in the interaction with NAGK. Within the class Mamiellophyceae, only the genus *Micromonas* contains species with the PII protein. Bioinformatic analysis revealed that the PII protein of *Micromonas pusilla* (MpPII) has an unusually prolonged T-loop. Here, we performed the coupled enzyme assay and showed that MpPII has no remarkable influence on NAGK activity. An engineered variant of MpPII with deletion of four additional amino acids (AATD) in the T-loop restored the ability of this protein to relieve NAGK from feedback inhibition by arginine in a glutamine-dependent manner. The findings are discussed in the context of unusual plasticity of the PII protein family during the evolution of Archaeplastida.

Key words: Mamiellophyceae, *Micromonas pusilla*, N-acetyl-L-glutamate kinase, PII proteins

Introduction

The PII proteins are highly conserved and widely distributed signal transduction proteins known in all domains of life (Fokina et al., 2010; Huergo et al., 2013; Forchhammer and Selim, 2020; Selim et al., 2020a). However, in the eukaryotes, the PII homologs inherited from a cyanobacterial endosymbiont are restricted to Archaeplastida (Chellamuthu et al., 2013).

The PII signalling proteins are characterized by highly conserved homo-trimeric structure. The three subunits assemble their orthogonally packed β-sheets into a triangular core surrounded by α-helices. Binding of the effector molecules occurs in the clefts formed at the contact sites of the subunits. Three loops (T, B and C) form important functional elements of the PII proteins (PDB: 2XUL) (Fokina et al., 2010; Truan et al., 2010) and are arranged in the order β1-α1-β2-(T-

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loop)-β3-α2-(B-loop)-β4-(C-loop). Three large and flexible T-loops protrude from the PII trimer body, which resembles a flat barrel, into the solvent. T-loops represent the major PII-receptor binding element. In general, the 2-oxoglutarate induced T-loop conformation abrogates most PII-protein interactions whereas different cell proteins may prefer the ATP- or the ADP-ligated state of PII (Zeth et al., 2014). Interestingly, in green algae and higher plants, C-terminal extension of the PII proteins forms a glutamine (Gln) binding site (Chellamuthu et al., 2014).

In bacteria, PII targets may be metabolic enzymes, transporters and transcription factors, which together regulate the metabolic status of cells (Forchhammer and Selim, 2020). In contrast to cyanobacteria, at the level of protein-protein interactions, there is no characterized target for the Gln-regulated PII proteins other than N-acetyl-L-glutamate kinase, NAGK (Vlasova et al., 2025). Notably, Gln, which is bound mainly through backbone interactions with two short helical segments of the extension that are connected by a tightly bent 4-amino-acids loop (Q-loop), induces interaction of plant PII with NAGK (Chellamuthu et al., 2014).

NAGK catalyzes the phosphorylation of N-acetyl-L-glutamate (NAG) to N-acetyl-L-glutamyl-phosphate, the rate-limiting step in the ornithine/arginine (Arg) biosynthesis pathway (Llácer et al., 2008; Selim et al., 2020b). Its activity is negatively regulated by Arg feedback inhibition and stimulated by PII interaction (Llácer et al., 2007; Forcada-Nadal et al., 2018; Selim et al., 2020b). In general, PII forms an activating complex with NAGK and thereby prevents feedback inhibition by Arg (Heinrich et al., 2004; Llácer et al., 2007; Mizuno et al., 2007; Beez et al., 2009; Chellamuthu et al., 2014; Lapina et al., 2018; Selim et al., 2020a).

NAGK is the most highly conserved target of PII in cyanobacteria and Archaeplastida (red algae and Chlorophyta) (Sugiyama et al., 2004; Chen et al., 2006; Beez et al., 2009; Chellamuthu et al., 2013, 2014). Surprisingly, among the class Mamiellophyceae, which gave rise to the core of Chlorophyta (Leliaert et al., 2011), only the genus *Micromonas* contains species with the PII proteins (*Micromonas pusilla* and *M. commoda*) (Selim et al., 2020b). Two other genera, *Bathycoccus* (Moreau et al., 2012) and *Ostreococcus* (Blanc-Mathieu et al., 2014), lost the PII proteins. In addition, compared with *Ostreococcus, Micromonas* has a richer set of nutrient-transporter gene families and appears to be the more flexible of the two in terms of environmental adaptability, which could explain its broader global distribution (Worden et al., 2009; Guo et al., 2024). Understanding the genus-specific differences in gene and genome structure and proteins content of the representatives of Mamiellophyceae will elucidate the differences in their biology and ecology. This prompted us to investigate the PII protein from *M. pusilla* (MpPII).

The present study is the first to address the control of NAGK activity by the PII protein with the unusual, prolonged T-loop.

Material and methods

Strains and cultivation conditions

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

Cloning of MpDsPII and MpPIIdel proteins

The sequence for MpPII was derived from the algal genomics resource PhycoCosm (https://phycocosm.jgi.doe.gov/pages/search-for-genes.jsf?organism=MicpuC3v2) with sequence ID for MpPII wlab.168725.1. Gene Block, with optimized codon usage for cloning and expression into *E. coli*, encoding for amino acid sequence of the mature MpPII gene without plastid signal peptide, was synthesized by IDT, USA. The Gene Block for the DNA sequence of MpPII was derived from the amino acid sequence starting with the 35th amino acid (AVQG) and was flanked by BsaI restriction sites. Using advanced quick assembly, the Gene Block was cloned directly into NdeI-digested pET15b vector (Novagen, Darmstadt, Germany), as described previously (Beyer et al., 2015). The MpPII with deletion of four amino acids in the T-loop (AATD) was generated by PCR amplification with Q5 Hot start DNA polymerase (New England Biolabs). In the first PCR, the MpPII gene block and primers were used: MpPIIF1: 5'-CTGGTGCC GCGCGGCAGCGCCT TCGCTGTAC -3' and MpPIIR1: 5'-TAAACTCGGTTCCTTTGTAAC G -3' (Evrogen company) (Fig. 1). The PCR product was purified in 0.9 % agarose electrophoresis and used for a second PCR with site-directed mutagene-

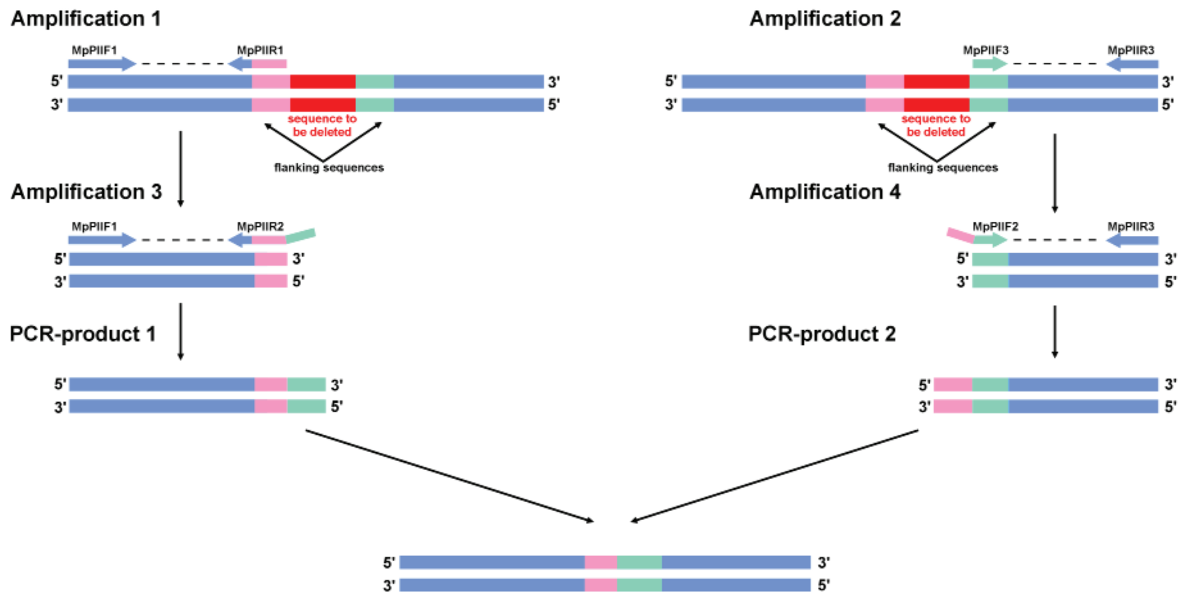

Fig. 1. A diagram showing the generation of the MpPIIdel. The primer sequences used are given in the text.

sis primers: forward MpPIIF1: 5'-CTGGTGCCG CGCGGCAGCGCCTTCGCTGTAC-3' and MpPIIR2: 5'-CTTTCTCGACCAGCGCGTCGG TTGTAAACTCGGTTCCTTTG-3'. The same procedure was performed to obtain the second fragment. In the first PCR, the MpPII gene block and primers were used: MpPIIF3: 5'-GACGCGCT GGTCGAGAAAG-3' and MpPIIR3: 5'-GCTTT GTTAGCAGCCGGATCCTCGAGCATACGC ACTGGCTTTCATGTC-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 MpPIIF2: 5'-CAAAGGAACCGAGTTTACAACCGA CGC GCTGGTCGAGAAAG-3' and MpPIIR3: 5'-GCT TTGTTAGCAGCCGGATCCTCG AGCA TACGCACTGGCTTTCATGTC-3'.

After purification on the agarose gel, the second PCR products were mixed with the linear form of the pET-15b (Novagen) and transformed into *E. coli* RB9060 cells by AQUA cloning (Beyer et al., 2015). The potential signal peptide was determined using a TargetP - 2.0 Server (https://services.healthtech.dtu.dk/services/TargetP-2.0/). The generated plasmid was verified by sequencing.

Expression and purification of the MpPII, MpPIIdel, CrPII and CrNAGK proteins

The overexpression of the recombinant N-terminal fused His_6-tagged MpPII, MpPII del and CrNAGK was performed in *E. coli* LEMO-21(DE3) and the proteins were affinity purified on a Ni-NTA columns according to the protocols described earlier (Maheswaran et al., 2004; Lapina et al., 2018). Overexpression of the recombinant C-terminal fused strep-tagged CrPII protein was performed in PII-deficient *E. coli* RB9060 (Bueno et al., 1985) and the protein was affinity purified on a Strep-Tactin II column according to Heinrich with co-authors (Heinrich et al., 2004).

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, the 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 meaured at 340 nm for 10 min with a SPECORD-spectrophotometer (model-210 PLUS, Analytik

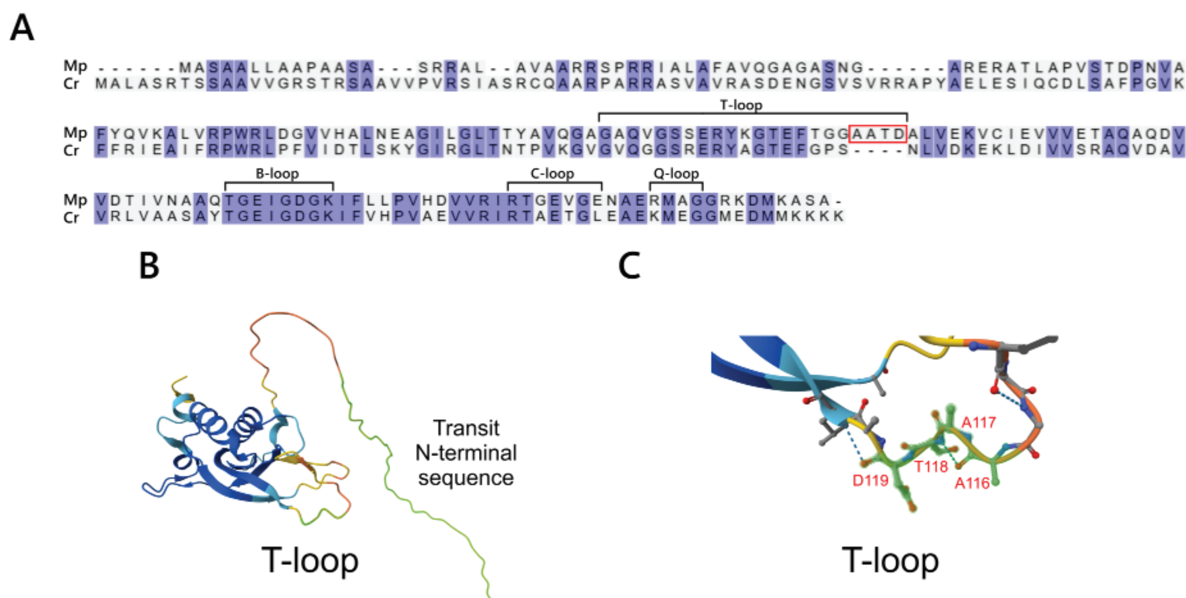

Fig. 2. Characterization of MpPII. A — Alignment of MpPII sequence with CrPII. The MpPII protein sequence was obtained from the PhycoCosm database. The other sequence was derived from PII polypeptide of *Chlamydomonas reinhardtii* (Cr; XP_001703658.1). The regions referring to T-, B-, C- and Q-loops are indicated (Chellamuthu et al., 2014). Highlighted residues in purple are invariant. The additional amino acids in the T-loop of MpPII C-terminal sequence of DsPII are shown in the red box. The alignment was done using the Clustal Omega program and manually refined; B — MpPII monomer structure. Additional amino acids in the T-loop are marked in green; C — a close-up of the T-loop reveals that a stretch of alanine, alanine, threonine and aspartate (A116, A117, T118 and D119) is an element of the T-loop. The structure model was done using the AlpfaFold program (https://alphafold.ebi.ac.uk/) and manually refined.

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).

Results

MpPII is a PII protein with unusual elongated T-loop

The predicted full-length MpPII polypeptide encoded by the *M. pusilla* wlab.168725.1 gene consists of 191 amino acids with a calculated molecular weight of 19659 Da and contains predicted plastid transit peptide using TargetP-2.0 Server (amino acid residues 1–34). As expected, the mature MpPII demonstrated the high degree of identity with *C. reinhardtii* PII (48.32 %). Similar to the PII homologues of Chloroplastida, the MpPII protein contains the unique C-terminal segment including the Q-loop, which is responsible for glutamine sensing (Fig. 2A; Chellamuthu et al., 2014). The alignment also showed a high degree of conservation of the functional important regions of the PII proteins, as B-, C- and T-loops (Llácer et al., 2007, 2008; Mizuno et al., 2007; Chellamuthu et al., 2014). However, MpPII contains an extended T-loop with four additional amino acids, AATD (Fig. 2A, B). Since the T-loop is involved in the interaction with NAGK, the presence of additional amino acids may indicate an unusual interaction of MpPII with this enzyme. To gain insights into the mode of interaction between MpPII and NAGK, we prepared a recombinant signaling protein.

Arg sensitivity of NAGK activity in the presence of MpPII

Since the relief from Arg inhibition by PII-NAGK complex formation is crucial for metabolic

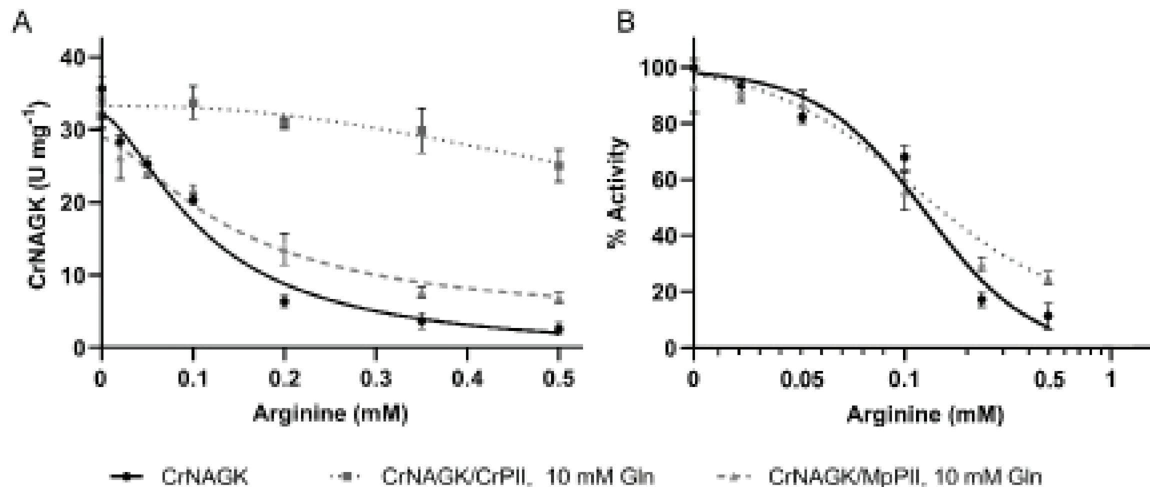

Fig. 3. Arg inhibition of CrNAGK activity in the presence of MpPII. A – Effects of CrPII and MpPII on CrNAGK activity in the presence of 10 mM Gln; B – Arg inhibition of CrNAGK in the presence of 10mM Gln or in the presence of MpPII and 10 mM glutamine. Data were fitted to a sigmoidal dose-response curve, yielding an EC50 (arginine) of 0.11 ± 0.04 mM and 0.14 ± 0.15 mM for free or MpPII-interacting CrNAGK, respectively. All data were fitted using a GraphPad prism program. SD as indicated by error bars represents independent triplicate measurements.

control of Arg biosynthesis in cyanobacteria and Archaeplastida (Heinrich et al., 2004; Beez et al., 2009; Chellamuthu et al., 2014; Lapina et al., 2018), we put a question whether the presence of MpPII could change the Arg inhibition profile of NAGK. We set up trials with heterologous CrNAGK, because the identity between MpNAGK and CrNAGK is 71.67 %. As shown previously, Arg impairs *C. reinhardtii* NAGK activity (Chellamuthu et al., 2014). Feedback inhibition of CrNAGK by Arg occurred with a half-maximal inhibitory concentration (IC50) of 0.11 mM (Fig. 3A; Chellamuthu et al., 2014; Vlasova et al., 2024). In the presence of CrPII and 10 mM glutamine, the IC50 of arginine for CrNAGK increased from 0.11 mM to 1 mM (Fig. 3). By contrast, in the presence of MpPII, glutamine had no remarkable influence on NAGK activity with IC50 0.14 mM (Fig. 3A, B).

MpPII variant with deletion in the T-loop relieved sensitivity of NAGK to Arg

To directly assess the role of elongation of the T-loop in MpPII in the loss of this protein's ability to recover NAGK from Arg feedback inhibition, we created a MpPIIdel variant, in which four additional amino acids, AATD, were deleted (Fig. 1). The MpPIIdel protein was functional and in the presence of 10 mM Gln, the inhibitory effect of Arg was strongly antagonized (Fig. 4A). Notably, this engineered MpPII was able to activate CrNAGK with IC50 1.56 ± 0.25 mM (Fig. 4B).

Discussion

During the evolution of Chlorophyta, the PII proteins that are ubiquitously present in archaea, bacteria and in the chloroplasts of Archaeplastida (Sant'Anna et al., 2009; Selim et al., 2020b) were lost in most of the analyzed Mamiellophyceae. The class Mamiellophyceae was defined in 2010 based on the nuclear 18S, the plastidial 16S and 23S rDNA (Marin and Melkonian, 2010) and is considered as the earliest divergent group within the Chlorophyta (Leliaert et al., 2011). Among Mamiellophyceae, genes encoding the putative PII proteins have so far been identified only in *M. pusilla* and *M. commoda*. In this work, PII from *M. pusilla* is characterized with respect to its structural similarities and differences compared to the PII proteins from cyanobacteria and Chloroplastida.

The sequence observed for the *M. pusilla* PII protein is very similar to the sequences identified for other canonical proteins in this family. Like its green algae and land plant PII homologs, the MpPII protein has the transit signal N- and the unique C-terminal sequences (Fig. 2). All PII proteins show

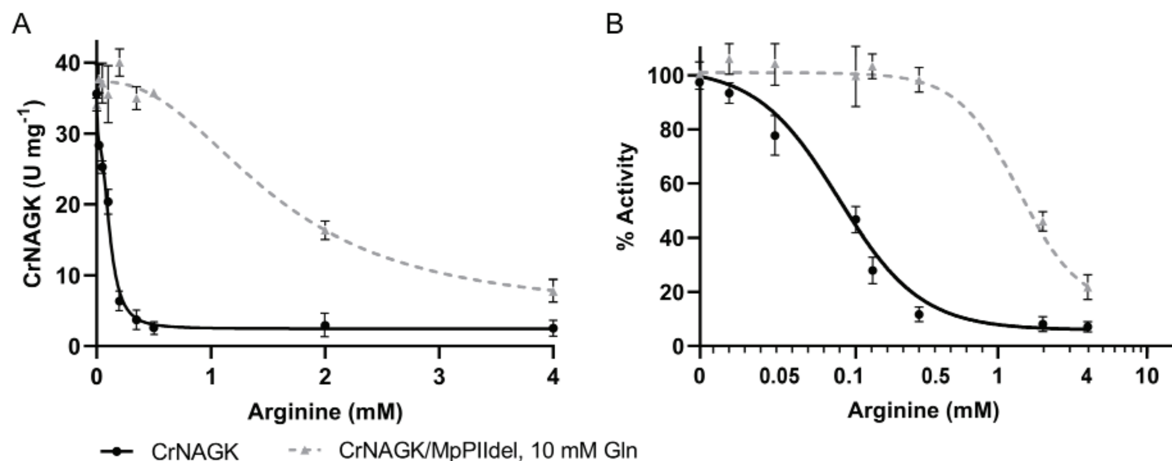

Fig. 4. Arg inhibition of CrNAGK activity in the presence of MpPIIdel. A – Effect of MpPII on CrNAGK activity in the presence of 10 mM Gln; B – Arg inhibition of CrNAGK in the presence of 10 mM Gln or in the presence of MpPIIdel and 10 mM glutamine. Data were fitted to a sigmoidal dose-response curve, yielding an EC50 (arginine) of 0.11 ± 0.04 mM and with IC50 1.56 ± 0.25 mM for free or MpPII-interacting CrNAGK, respectively. All data were fitted using a GraphPad prism program. SD as indicated by error bars represents independent triplicate measurements.

highly conserved T-loops, protruding from each subunit of PII trimers (Forchhammer, 2008). There is a feature of the MpPII protein, which appears unique – it is the unusually long T-loop (Fig. 2B). T-loops play key role in ligand binding and receptor interactions (Forchhammer, 2008; Huergo et al., 2013). Depending on the conformation of the T loop, the PII proteins can bind to various receptors, thereby exerting control at all levels of metabolic regulation (transport activity, metabolic reactions, gene expression) (Forchhammer and Selim, 2020).

We hypothesized that the elongated T-loop might influence the binding properties of this PII. To test this possibility, we analyzed the effect of MpPII on the catalytic activity of CrNAGK (Fig. 3). MpPII has lost its function in relieving Arg-mediated feedback inhibition by Arg, suggesting that the flexible T-loop is not structured to bind NAGK. Removal of additional amino acids from the T-loop of MpPII restores the effect of this protein on NAGK and shows that the *M. pusilla* PII can be converted back to a functional protein (Fig. 4). This confirms the role of amino acids (AATD) in the T-loop in altering the properties of MpPII. The PII protein of another member of this genus, *Micromonas commoda*, does not contain these additional amino acids in the T-loop. Thus, it is necessary to check whether McPII controls McNAGK.

Recently, the loss of PII-dependent control of arginine biosynthesis in halotolerant photosynthetic alga *Dunaliella salina* has been demonstrated (Vlasova et al., 2025). 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 *D. salina* (Vlasova et al., 2024). Interestingly, members of the class Mamiellophycea, *O. tauri, O. lucimarinus* and *Bathycoccus prasinos* that have lost the PII protein are marine picoeukaryotes. Thus, as in *D. salina*, Arg biosynthesis in them is independent of PII. In the case of *M. pusilla*, another scenario arises: this marine alga has lost PII-mediated control over Arg biosynthesis due to changes in the properties of this protein. Although further research is required on the molecular mechanisms underlying the control of Arg biosynthesis in other marine Chlorophyta and Rhodophyta lacking PII, this study shows clearly that different members of the class Mamiellophyceae do not use the PII signaling proteins to optimize NAGK activity.

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