Journal of Plant Physiology 205 (2016) 105–112

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Metabolomic analysis of NAD kinase-deficient mutants of the cyanobacterium *Synechocystis* sp. PCC 6803

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ARTICLE INFO

Article history:
Received 9 February 2016
Received in revised form 30 August 2016
Accepted 9 September 2016
Available online 13 September 2016

Keyword:
CE-MS
Metabolomics
NAD kinase
Pyridine nucleotides *Synechocystis* sp. PCC 6803

ABSTRACT

NAD kinase (NADK) phosphorylates NAD(H) to NADP(H). The enzyme has a crucial role in the regulation of the NADP(H)/NAD(H) ratio in various organisms. The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 possesses two NADK-encoding genes, *sll1415* and *slr0400*. To elucidate the metabolic change in NADK-deficient mutants growing under photoautotrophic conditions, we conducted metabolomic analysis using capillary electrophoresis mass spectrometry (CE-MS). The growth curves of the wild-type parent (WT) and NADK-deficient mutants (Δ1415 and Δ0400) did not show any differences under photoautotrophic conditions. The NAD(P)(H) balance showed abnormality in both mutants. However, only the metabolite pattern of Δ0400 showed differences compared to WT. These results indicated that the two NADK isoforms have distinct functions in cyanobacterial metabolism.

1. Introduction

Pyridine nucleotides are cofactors involved in numerous redox reactions in all organisms. NAD^+ and NADH mainly function in catabolic reactions, while $NADP^+$ and NADPH participate in anabolic reactions and defenses against oxidative stress (Outten and Culotta, 2003; Pollak et al., 2007; Ziegler, 2000). Notably, $NADP^+$ is converted to NADPH by the donation of electrons from photosystem I through ferredoxin, and by the oxidative pentose phosphate pathway.

NAD kinase is the $NADP^+$ biosynthetic enzyme that regulates the balance between NAD(H) and NADP(H) (Ohashi et al., 2011). Genes encoding NAD kinase have been identified from many organisms, such as *Homo sapiens* (Lerner, 2001), *Escherichia coli* (Kawai et al., 2001a), *Saccharomyces cerevisiae* (Kawai et al., 2001b; Outten and Culotta, 2003), and *Arabidopsis thaliana* (Turner et al., 2004). Functional analyses have been performed in these organisms (Hashida et al., 2009; Kawai et al., 2000; Raffaelli et al., 2004; Sakuraba et al., 2005). In *A. thaliana*, three NAD kinase-encoding genes (*NADK1*, *NADK2*, and *NADK3*) have been identified (Berrin et al., 2005; Turner et al., 2005). NADK1 localizes to the cytosol (Chai et al., 2006). NADK2 is a chloroplast-localizing enzyme that can bind to calcium/calmodulin; this version is known to play a vital role in energy transduction through photosynthetic electron transport (Chai et al., 2005; Takahashi et al., 2006; Turner et al., 2004). NADK3 has been reported to localize to the peroxisomal matrix, where the enzyme serves as a source of NADPH (Waller et al., 2010). In our previous study, we demonstrated that alteration of the NAD/NADP balance in higher plants affected metabolism and reactive oxygen species (ROS)—specific responses (Takahara et al., 2010; Takahashi et al., 2009). Specifically, NADK2-overexpressing rice plants exhibited increased photosynthetic electron transport and CO_2 assimilation rates, as well as enhanced tolerance to oxidative stress (Takahara et al., 2010).

Cyanobacteria have flexible metabolic capabilities, permitting these microbes to adapt to various environments. Cyanobacteria have garnered interest as potential renewable sources of biomass for sustainable production of biofuels (Dismukes et al., 2008; Mussatto et al., 2010; Wijffels et al., 2013). Several species of cyanobacteria possess the ability to store large amount of glycogen and lipids that can be utilized for the synthesis of bioethanol (Aikawa et al., 2013, 2014). Furthermore, cyanobacteria have been

Abbreviation: AcCoA, acetyl CoA; ADH, alcohol dehydrogenase; CE-MS, capillary electrophoresis mass spectrometry; CIT, citrate; DHAP, dihydroxyacetone phosphate; DCPIP, 2,6-dichlorophenolindophenol; FBP, fructose-1,6-bisphosphate; FUM, fumarate; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; IsoCIT, isocitrate; LA, lactate; MAL, malate; NADK, NAD kinase; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PYR, pyruvate; RuBP, ribulose-1,5-bisphosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; SUC, succinate; 2OG, 2-oxoglutarate; 3PGA, 3-phosphoglycerate; 6PG, 6-phosphogluconate.

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http://dx.doi.org/10.1016/j.jplph.2016.09.002
0176-1617/

investigated in wide ranging metabolic flux and −omics analyses (You et al., 2015), including their potential use for the over-production of hydrogenases that might find application in industrial hydrogen synthesis (Belkin and Padan, 1978).

Synechocystis sp. PCC 6803 is a useful model species. The PCC 6803 genome includes two genes (*sll1415* and *slr0400*) that encode putative NAD kinases (Gao and Xu, 2012). It was found that both *sll1415* and *slr0400* included conserved amino acid sequence motifs for NAD binding (GGDG) (Mori et al., 2005; Raffaelli et al., 2004) and ATP binding (NE/D) (Liu et al., 2005). Based on BLAST searches of the cyanobacterial genomes available in the NCBL GenBank database, all cyanobacteria harbor two types of *NADK* genes, and each NAD kinase-encoding paralog belongs to either the *sll1415* or *slr0400* clade (Gao and Xu, 2012). Gao and Xu (2012) demonstrated that PCC 6803 strains mutated for either NAD kinase-encoding gene show distinct sensitivities to methyl viologen. As we show in the present work, metabolomic analysis of NADK-deficient mutants of PCC 6803 revealed that the metabolite patterns differ between two *NADK* mutants under photoautotrophic conditions.

2. Materials and methods

2.1. Strain and culture conditions

In this study, we used *Synechocystis* sp. PCC 6803 as the wild-type strain (WT). All strains were grown in BG11 culture medium (20 mL) on a shaker (100 rpm) at 30 °C under continuous light at 30 μE/m^2/s. To obtain NADK-deficient mutants (Δ1415 and Δ0400), antibiotic resistant cassettes (encoding spectinomycin resistance for Δ1415 or kanamycin resistance for Δ0400) were inserted by homologous recombination into the respective *NADK* gene. After selection of antibiotic-resistant colonies, segregation was checked by PCR. The following primers were used for *sll1415* (forward, 5′-GTGGAACTGAAACAGGTGATCATTGCCCAT-3′; reverse, 5′-TCAATTGACCTTGTTGTTACCATCGTACAA-3′), *slr0400* (forward, 5′-GTGCCAAAAGTCGGCATCATTTTCAACGAC-3′; reverse, 5′-TCATGGCAACTCCACCGATGTTGGTTTAGC-3′). To compare the growth of the *NADK* mutants, we measured using a spectrophotometer (Pharmacia Biotech, Ultrospec 3000) each culture's optical density at 730 nm (OD_{730}) at 24-h intervals.

2.2. Assays of NAD(H) kinase activity

To measure NAD(H)K activity, we used cells (40 mL) cultured for 4 days under photoautotrophic growth conditions. After centrifugation at 5,000 × *g* for 10 min at 4 °C, we collected the pellet and re-suspended by remaining cultured buffer and centrifuged at 15,000 × *g* for 1 min at 4 °C again. After removal of the supernatant, cells were sonicated in an extraction buffer consisting of 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, and protease inhibitor cocktail (Roche). After centrifugation at 15,000 × *g* for 10 min at 4 °C, the supernatant was desalted with a column (Zeba™ Spin Desalting Columns, Thermo, Japan) and protein contents were measured by the Bradford method. To measure $NADP^+$ (or NADPH) generation from NAD^+ (or NADH), an aliquot of desalted lysate supernatant corresponding to 10 μg protein was combined with reaction buffer formulated to provide final concentrations of 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 5 mM NAD^+(or NADH), and 5 mM ATP. The reaction was carried out for 30 min at 30 °C and quenched by incubation for 2 min at 95 °C. After centrifugation at 15,000 × *g* for 4 min at 4 °C, 10 μL of the resulting 10—fold diluted supernatant was combined with cycling assay buffer formulated to provide final concentrations of 57.1 mM HEPES-KOH (pH 7.5), 0.57 mM G6P, 0.14 mM DCPIP, and 1.1 mM phenazine methosulfate (Turner et al., 2004). The cycling assay reaction was initiated by adding 2.15 U of G6PDH (Sigma) to the mixture. The $NADP^+$ (or NADPH) generation rate over time was determined by measuring of the OD_{595} using a double-beam absorption spectrometer (PerkinElmer Lambda 25).

2.3. NAD(P)(H) contents

We determined the levels of NAD(P)(H) using the cycling assay, as described above in the section of assays of NAD kinase activity. We extracted NAD(P)(H) from *Synechocystis* cells that had been cultured under photoautotrophic condition for 4 days. After centrifugation at 5,000 × *g* for 10 min at 4 °C, we resuspended the pellet in 500 μL 0.2 N HCl (to extract NAD^+ and $NADP^+$) or 0.2 N NaOH (to extract NADH and NADPH). Cells were sonicated for three 10-s intervals. The lysate was centrifuged at 15,000 × *g* for 10 min at 4 °C, and the resulting supernatant was recovered and incubated for 1 min at 95 °C. For $NADP^+$ and $NADPH^+$ determinations, the heat-inactivated suspension was neutralized by the addition of 30 μL 0.2 M NaH_2PO_4 (pH 5.6) and 240 μL 0.2 N NaOH, and 35 μL of the neutralized supernatant was combined with the cycling assay buffer as described in the preceding section on measurement of NADK activity. For NAD^+ and NADH determinations, the heat-inactivated suspension was instead neutralized by the addition of 30 μL of 0.2 M HEPES-KOH (pH8.0) and 240 μL 0.2 N HCl. A volume of this neutralized supernatant was combined with a buffer formulated to provide final concentrations of 7.7% EtOH, 57.1 mM HEPES-KOH (pH 7.5), 0.14 mM DCPIP, and 1.1 mM phenazine methosulfate. Whether for $NADP^+$ and $NADPH^+$ or NAD^+ and NADH determinations, the cycling assay of the respective sample was initiated by adding ADH (Wako, Japan) at 180 U/mL to the assay mixture. Acetaldehyde generation rate over time was determined by measuring the OD_{595} using a double-beam absorption spectrometer (PerkinElmer Lambda 25).

2.4. Metabolite analysis

We extracted metabolites from *Synechocystis* cells that had been cultured under photoautotrophic condition for 4 days, using the extraction technique described in Takahashi et al. (2008) and Kaniya et al. (2013). After centrifugation at 5,000 × *g* for 10 min at 4 °C, the pellets were frozen with liquid nitrogen. Cells were resuspended/dissolved by vortexing each frozen pellet (approximately 30 mg each) in 100 μL methanol, which inactivated any enzymes. After complete dissolving of the pellets, internal standard solution containing 100 μM PIPES and 100 μM methionine sulfone was added to each suspension. The mixture was vortexed and then centrifuged at 15,000 × *g* for 5 min at 4 °C. The resulting supernatant was transferred to a 3-kD cutoff filter (Millipore, Japan) and centrifuged at 11,000 × *g* for 30 min at 4 °C. An aliquot of 13 μL of the resulting filtrate was used for metabolite analysis using capillary electrophoresis mass spectrometry (CE-MS) (CE:G1600AX, MS:G1965B, Agilent Technologies, Waldbronn, Germany) according to the methods of Miyagi et al. (2010), with minor modifications. Specifically, we used a polyethylene glycol-coated capillary (DB-WAX, Agilent Technology, 100 cm × 50 μm i.d.) with 20 mM ammonium acetate (pH 8.5) as the running buffer and applied a voltage of −25 kV for the electrophoresis step. For the stabilization of the mass spectrometer, 50% methanol containing 5 mM ammonium acetate as a sheath solution was applied to the capillary at 8 μL min^{-1} using an isocratic HPLC pump on an Agilent 1100-series HPLC equipped with a splitter (1:100). Glass capillary voltage of the quadrupole (−3500 V) and flow rate of the drying nitrogen gas (8 L min^{-1}) were maintained during the course of electrophoresis (20–30 min). Quantitative accuracy of the