FEBS Letters 588 (2014) 466–471

journal homepage: www.FEBSLetters.org

Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803

Ancy Joseph [a,1], Shimpei Aikawa [a,1], Kengo Sasaki [b], Hiroshi Teramura [a], Tomohisa Hasunuma [b,c], Fumio Matsuda [d,e], Takashi Osanai [f], Masami Yokota Hirai [g], Akihiko Kondo [a,d,*]

[a] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan
[b] Organization of Advanced Science and Technology, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan
[c] Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, 3-5 Sanbancho, Chiyoda-ku, Tokyo 102-0075, Japan
[d] RIKEN Biomass Engineering Program, 1-7-22, Suehirocho, Tsurumi, Yokohama 230-0045, Japan
[e] Department of Bioinformatic Engineering, Graduate School of Information Science and Technology, Osaka University,1-5 Yamadaoka, Suita, Osaka 565-0871, Japan
[f] PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
[g] RIKEN Center for Sustainable Resource Science, 1-7-22, Suehirocho, Tsurumi, Yokohama 230-0045, Japan

ARTICLE INFO

Article history:
Received 8 October 2013
Revised 10 December 2013
Accepted 11 December 2013
Available online 24 December 2013

Edited by Stuart Ferguson

Keywords:
Cyanobacteria
Nitrogen starvation
Rre37
2-Oxoglutarate
Glycogen

ABSTRACT

Rre37 (sll1330) in a cyanobacterium *Synechocystis* sp. PCC 6803 acts as a regulatory protein for sugar catabolic genes during nitrogen starvation. Low glycogen accumulation in Δ*rre37* was due to low expression of glycogen anabolic genes. In addition to low 2-oxoglutarate accumulation, normal upregulated expression of genes encoding glutamate synthases (*gltD* and *gltB*) as well as accumulation of metabolites in glycolysis (fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate) and tricarboxylic acid (TCA) cycle (oxaloacetate, fumarate, succinate, and aconitate) were abolished by *rre37* knockout. Rre37 regulates 2-oxoglutarate accumulation, glycogen accumulation through expression of glycogen anabolic genes, and TCA cycle metabolites accumulation.

1. Introduction

Cyanobacteria, which perform oxygenic photosynthesis like eukaryotic algae and plants, inhabit almost all illuminated ecosystems and play key roles in the global carbon and nitrogen cycles [1,2]. In natural habitats, nitrogen, an essential macronutrient, is limiting factor for cellular growth, and unicellular cyanobacteria have developed mechanisms to respond to nitrogen depletion [3]. Most cyanobacteria, including non-nitrogen fixing, unicellular cyanobacteria, like *Synechocystis* sp., store glycogen under nitrogen or sulfur deficiency [4]. Metabolic changes after nitrogen depletion have been demonstrated [5,6], showing increases in levels of tricarboxylic acid (TCA) cycle metabolites (such as malate, fumarate, succinate) and decreases in purine and pyrimidine nucleotides. Several amino acids including glutamine and glutamate decrease following nitrogen depletion [6], and particularly reflect limitation of available nitrogen sources. Similarly to other bacteria, nitrogen compounds in cyanobacteria are reduced to ammonium and incorporated into 2-oxoglutarate (2-OG) via the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle, known as the GS–GOGAT pathway [7]. Levels of 2-OG increase during nitrogen depletion, which initiates nitrogen-starvation response in *Synechocystis* cells [8]. Thus, 2-OG is a key metabolite in *Synechocystis* for appropriate acclimation to nitrogen starvation.

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is one of the most widely studied cyanobacterial species due to its transformability and availability of its entire genome sequence [9,10]. Changes in gene expression under nitrogen depletion have been investigated by microarray analysis [11,12]. NtcA, which is a transcription factor belonging to the cAMP receptor protein family, regulates OmpR-type response regulator Rre37 and RNA polymerase sigma factor SigE, and binding of NtcA to the *rre37* promoter is

Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; HPLC, high performance liquid chromatography; LC/QqQ-MS, liquid chromatography/triple quadrupole mass spectrometry; OD, optical density; TCA, tricarboxylic acid; 2-OG, 2-oxoglutarate

* Corresponding author at: Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodaicho, Nada, Kobe, Hyogo 657-8501, Japan. Fax: +81 78 803 6196.
E-mail address: akondo@kobe-u.ac.jp (A. Kondo).
[1] These authors contributed equally to this work.

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.12.008

enhanced in the presence of 2-OG *in vitro* [13]. PII signaling protein (encoded by *glnB*) in *Synechococcus* sp. PCC 7942 also binds 2-OG in a cooperative manner with ATP and is phosphorylated during nitrogen starvation [2]. PII bound to 2-OG releases PipX, a small protein conserved among several cyanobacteria, and PipX associates with NtcA, possibly controlling the transcriptional activity of NtcA [14,15]. The expression of various sugar catabolic genes and glycogen content are both increased by nitrogen depletion [16]. Rre37 and SigE are involved in nitrogen-induced expression of sugar catabolic genes [13,16,17]. Rre37 activates the expression of two glycogen catabolic genes [*glgX* (slr1857, encoding glycogen isoamylase) and *glgP* (slr1367, encoding glycogen phosphorylase)] and two glycolytic genes [*gap1* (slr0084, glyceraldehyde 3-phosphate dehydrogenase) and *pfkA* (sll1196, phosphofructokinase)] under nitrogen starvation [13]. SigE activates the expression of glycolytic genes (*gap1* and *pfkA*) independently of Rre37 [12,13]. In contrast to studies of mechanism of transcriptional regulation after nitrogen depletion and regulatory factors controlling primary metabolism during nitrogen starvation, few studies of the metabolome have been performed with mutants of nitrogen regulators.

In this study, an Rre37 mutant of *Synechocystis* was constructed and analysis of gene expression and metabolites were carried out to clarify which pathways or genes are regulated by Rre37 in carbon or nitrogen flow.

2. Materials and Methods

2.1. Strains and growth conditions

The glucose-tolerant (GT) strain of cyanobacterium *Synechocystis* isolated by Williams [18] and the cognate *rre37* knockout mutant (Δ*rre37*) were precultured for 5 days in 1.4 L modified BG-11 medium, which contained (L^{-1}) $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4{\cdot}7H_2O$ 0.075 g, $CaCl_2{\cdot}2H_2O$ 0.036 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.001 g, Na_2CO_3 0.02 g, H_3BO_3 2.86 mg, $MnCl_2{\cdot}4H_2O$ 1.81 mg, $ZnSO_4{\cdot}7H_2O$ 0.222 mg, $NaMoO_4{\cdot}2H_2O$ 0.39 mg, $CuSO_4{\cdot}5H_2O$ 0.079 mg, $Co(NO_3)_2{\cdot}6H_2O$ 49.4 μg [19] under continuous illumination at 70 μmol photons m^{-2} s^{-1} at 25 °C with 2% (w/w) CO_2 bubbled at 80 mL min^{-1}. 10.0 g/L agar was added in a solid medium. For culturing Δ*rre37*, 50 μg L^{-1} kanamycin was added to the medium. After cells reached the mid-exponential phase, they were collected by filtration using a 1 μm pore size polytetrafluoroethylene filter (Millipore, Billerica, MA), washed 3 times with BG-11 medium without a nitrogen source (BG-11_0), and then inoculated into fresh BG-11_0 medium at 0.2 g dry-cell weight L^{-1}.

2.2. Glycogen analysis

Glycogen was extracted from dried cells as described previously [20]. Glycogen content was determined by high performance liquid chromatography (HPLC) (LC Prominence, Shimadzu, Kyoto, Japan) using a size exclusion HPLC column (OHpak SB-806M HQ; Shodex, Tokyo, Japan) and a reflective index detector (RID-10A; Shimadzu) as previously described [21].

2.3. Quantitative gene expression analysis

Cells were harvested at different time points by centrifugation. RNA was extracted using a Nucleospin RNAII kit (Macherey–Nagel GmbH & Co. KG Düren, Germany) and cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and loaded in an Agilent 2100 bioanalyzer (Agilent Technologies, La Jolla, CA) to check purity, concentration and integrity. To investigate expression of *rre37*, reverse transcription PCR was performed using cDNA and *rre37* primers (Supplementary Table S1). Real time quantitative PCR was performed with the gene-specific oligonucleotides (Supplementary Table S1) using Thunderbird SYBR qPCR mix (Toyobo). The transcript level of target genes was quantified using an Mx3000P qPCR system (Agilent Technologies). The *rnpB* gene, which encodes RNase P subunit B, was used as a housekeeping gene. The amplification efficiency (E) of each oligonucleotide was calculated using the equation $E = 10^{(-1/\text{slope})}$, and expression of target gene relative to reference gene was calculated using Pfaffl method [22].

2.4. Metabolic profile analysis using LC/QqQ-MS

Cell sampling was performed as described previously [5]. Dried extracts were dissolved in Milli-Q water and applied to an LC/QqQ-MS system (HPLC system: Agilent 1200 series, MS system: Agilent 6460 with Jet Stream Technology, Agilent Technologies) controlled with MassHunter Workstation Data Acquisition software v. B.04.01 (Agilent Technologies). LC/QqQ-MS was performed with multiple reaction monitoring as described previously [5].

3. Results

3.1. Delay of glycogen accumulation in Δrre37 under nitrogen starvation

During nitrogen starvation for 72 h, *rre37* was expressed in GT, whereas Δ*rre37* lacked expression of the *rre37* gene, confirming disruption of *rre37* in the mutant (data not shown). Disruption of *rre37* did not influence cell growth in Δ*rre37* during nitrogen depletion (data not shown).

After nitrogen depletion, *Synechocystis* started to accumulate glycogen and the glycogen content reached 44% of dry-cell weight after 72 h [5]. Glycogen accumulation was delayed in Δ*rre37* for 24 h, although the glycogen content in both GT and Δ*rre37* increased to 40% of dry-cell weight after 48 h (Fig. 1). Rre37 appears to have a functional role for glycogen accumulation up to 12 h after nitrogen depletion in GT.

3.2. Positive regulation of glycogen anabolic genes by rre37 under nitrogen starvation

To reveal the role of Rre37 during nitrogen depletion, changes in both metabolite content (related to glycogen catabolism and anabolism, glycolysis, pentose phosphate pathway, TCA cycle, and GS–GOGAT pathway) and gene expression, which are involved

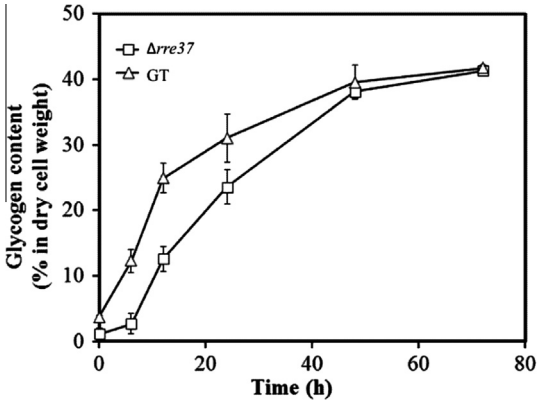

Fig. 1. Glycogen content in glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (GT) (Δ) and *rre37* mutant (□) after nitrogen depletion.