

Subcellular distribution of glutathione and cysteine in cyanobacteria

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Abstract Glutathione plays numerous important functions in eukaryotic and prokaryotic cells. Whereas it can be found in virtually all eukaryotic cells, its production in prokaryotes is restricted to cyanobacteria and proteobacteria and a few strains of gram-positive bacteria. In bacteria, it is involved in the protection against reactive oxygen species (ROS), osmotic shock, acidic conditions, toxic chemicals, and heavy metals. Glutathione synthesis in bacteria takes place in two steps out of cysteine, glutamate, and glycine. Cysteine is the limiting factor for glutathione biosynthesis which can be especially crucial for cyanobacteria, which rely on both the sufficient sulfur supply from the growth media and on the protection of glutathione against ROS that are produced during photosynthesis. In this study, we report a method that allows detection and visualization of the subcellular distribution of glutathione in *Synechocystis* sp. This method is based on immunogold cytochemistry with glutathione and cysteine antisera and computer-supported transmission electron microscopy. Labeling of glutathione and cysteine was restricted to the cytosol and interthylakoidal spaces. Glutathione and cysteine could not be detected in carboxysomes, cyanophycin granules, cell walls, intrathylakoidal spaces, periplasm, and vacuoles. The accuracy of the glutathione and cysteine labeling is

supported by two observations. First, preadsorption of the antiglutathione and anticysteine antisera with glutathione and cysteine, respectively, reduced the density of the gold particles to background levels. Second, labeling of glutathione and cysteine was strongly decreased by 98.5% and 100%, respectively, in *Synechocystis* sp. cells grown on media without sulfur. This study indicates a strong similarity of the subcellular distribution of glutathione and cysteine in cyanobacteria and plastids of plants and provides a deeper insight into glutathione metabolism in bacteria.

Keywords Compartmentation · Electron microscopy · Glutathione · Immunogold labeling · Histochemistry · *Synechocystis*

Introduction

Glutathione (γ -L-glutamyl-L-cysteinyl-glycine) is the most important nonprotein thiol in plants, animals, and eukaryotes with the exception of those that do not contain mitochondria and chloroplasts (Fahey and Sundquist 1991; Rennenberg 2001; Masip et al. 2006; Forman et al. 2009; Foyer and Noctor 2009; Lafiti et al. 2009). Its production in prokaryotes is restricted to cyanobacteria and proteobacteria and a few strains of gram-positive bacteria. Glutathione has not been detected so far in any other subgroups of eubacteria or in archaeobacteria with the exception of green sulfur bacteria (Fahey et al. 1987; Fahey and Sundquist 1991; Newton et al. 1996; Masip et al. 2006). Nevertheless, some groups of bacteria that do not contain glutathione produce different low molecular thiols (cf. Fahey 2001) which fulfill similar functions as glutathione, thus demonstrating the importance of glutathione and low molecular

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thiols in bacteria. The roles of glutathione in metabolism of bacteria are similar to those in plants, animals, and eukaryotes. One of the most important functions of glutathione in bacteria is the protection against oxidative stress which occurs when cells are exposed to reactive oxygen species (ROS) such as H_2O_2 , alkyl hydroperoxides, hydroxyl radicals, and superoxides (Storz and Imlay 1999; Carmel-Harel and Storz 2000; Masip et al. 2006; Foyer and Noctor 2009; Lafiti et al. 2009). This is especially critical in cyanobacteria which perform aerobic photosynthesis and can produce large amounts of ROS that could destroy membranes, proteins, RNA, and DNA, leading to mutations and eventually cell death (Fahey 2001; Tausz 2001; Masip et al. 2006; van Breusegem and Dat 2006; Foyer and Noctor 2009; Lafiti et al. 2009). Other functions of glutathione in bacteria are the protection against osmotic shock (Csonka 1989; McLaggan et al. 1990; Smirnova et al. 2001), acidic conditions (Ferguson and Booth 1998; Riccillo et al. 2000), toxic chemicals such as methylglyoxal, chlorine compounds (HOCl , NH_2Cl), and heavy metals (Chesney et al. 1996; Ferguson and Booth 1998; Masip et al. 2006). Additionally, glutathione is important as a reducing agent for ribonucleotides and other substrates and protects proteins from being irreversibly oxidized by ROS and reactive nitrogen species through a process called glutathionylation (c.f. Hurd et al. 2005; Masip et al. 2006). Glutathionylation (the formation of mixed disulfides between proteins, cysteine, and glutathione) is mainly important during situations of oxidative stress. When cells are removed from the oxidative environment, glutathionylated proteins can be reduced to their native state, e.g., by glutaredoxins.

Glutathione synthesis in bacteria occurs in two ATP-depending steps. In the first step, cysteine and glutamate are linked together by γ -glutamyl-cysteine synthetase, and in the second step glycine is added to γ -glutamyl-cysteine to form the final product glutathione. This step is triggered by glutathione synthetase (c.f. Copley and Dhillon 2002; Masip et al. 2006). Glutathione synthesis is feedback-inhibited by the formation of γ -glutamyl-cysteine (glutathione itself can inhibit the activity of γ -glutamyl-cysteine synthetase) or by the availability of its amino acids. The concentration of cysteine seems to be the main limiting amino acid for glutathione synthesis which is especially essential in cyanobacteria that rely on sufficient sulfur supply from the growth medium and on the protection of glutathione, especially during oxidative stress situations (c.f. Copley and Dhillon 2002; Masip et al. 2006). Cysteine is the most important sulfur source for biosynthesis of most other thiols and essential for cell metabolism in bacteria (Fahey 2001).

Even though the roles of glutathione (and cysteine), its synthesis, and degradation are well understood, its subcel-

lular distribution within cyanobacteria still remains unclear. Nevertheless, information about the subcellular distribution of glutathione and cysteine is essential in order to gain a deeper insight into glutathione metabolism and into the subcellular importance of glutathione in bacteria. Therefore, the present study was aimed to develop a method that allows the visualization and quantification of the subcellular distribution of glutathione and cysteine in cyanobacteria. For this purpose, an immunohistochemical method was adapted to *Synechocystis* sp. which has been previously developed to detect the subcellular distribution of glutathione and cysteine in plant and animal tissue (Hjelle et al. 1994; Huster et al. 1998; Zechmann et al. 2006a, 2008). To verify the accuracy and specificity of this method, changes in glutathione and cysteine contents were quantified in cells grown on media with and without sulfur.

Materials and methods

Bacterial strains and culture conditions

Cyanobacteria (*Synechocystis* sp. PCC6803) were cultivated in BG11 medium at 30°C under constant light conditions ($20 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 7 days. One part of the culture was transferred into BG11 medium without sulfur. Fixation and embedding were performed with *Synechocystis* sp. grown on medium with sulfur for 7 days and without sulfur for 48 h as a stagnation of bacteria growth could be observed after that time (Fig. 1). Cell growth was monitored by spectrophotometric measurements of optical density at 730 nm. Before fixation cyanobacteria were centrifuged ($2,500\times g$ was used during centrifugation between each step throughout sample preparation) for 3 min, and the pellet was resuspended in 0.06 M Sørensen phosphate buffer (pH 7.2; Sørensen 1909). After another centrifugation step,

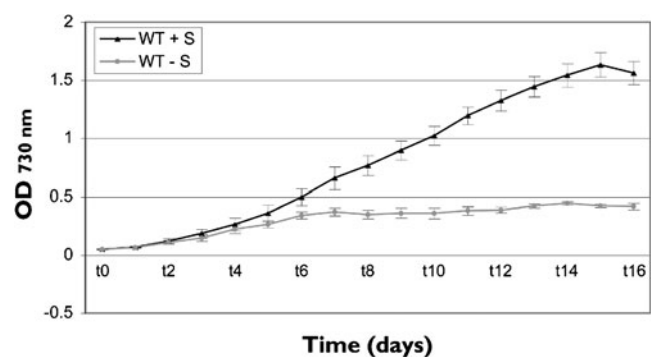


Fig. 1 Photoautotrophical growth curves of *Synechocystis* sp. PCC6803 wild-type cultivated in conditions with (WT + S) and without sulfur (WT - S) added to the BG-11 medium. Error bars indicate standard error of the mean

samples were resuspended and fixed for 90 min either in (a) 2.5% glutardialdehyde/2.5% paraformaldehyde in 0.06 M Sørensen phosphate buffer (pH 7.2; Sørensen 1909) for ultrastructural investigations or (b) in 2.5% paraformaldehyde/0.5% glutardialdehyde in 0.06 M Sørensen phosphate buffer (pH 7.2) for cytohistochemical analysis.

For ultrastructural analysis, samples were then rinsed in buffer (four times, 15 min each) and postfixed in 2% potassium permanganate in 0.06 M Sørensen phosphate buffer for 90 min at room temperature (RT). The samples were then dehydrated in increasing concentrations of acetone (50%, 70%, 90%, and 100%). Pure acetone was then exchanged by propylene oxide, and specimen were gradually infiltrated with increasing concentrations of Agar 100 epoxy resin (30%, 60%, and 100%) mixed with propylene oxide for a minimum of 3 h per step. Samples were finally embedded in pure, fresh Agar 100 epoxy resin (Agar Scientific Ltd., Stansted, UK) and polymerized at 60°C for 48 h.

For cytohistochemical investigations, samples were rinsed in buffer (four times, 15 min each) after fixation and then dehydrated in increasing concentrations of acetone (50%, 70%, and 90%) for two times for 10 min each. Subsequently, specimens were gradually infiltrated with increasing concentrations of LR-White resin (30%, 60%, and 100%; London Resin Company Ltd., Berkshire, UK) mixed with acetone (90%) for a minimum of 3 h per step. Samples were finally embedded in pure, fresh LR-White resin and polymerized at 50°C for 48 h in small plastic containers under anaerobic conditions. Ultrathin sections (80 nm) were cut with a Reichert Ultracut S ultramicrotome. For ultrastructural investigations, sections were poststained for 5 min with lead citrate and for 15 min with uranyl acetate at RT before they were observed with a Philips CM10 transmission electron microscope. For cytohistochemical investigations, sections remained either unstained or were stained for 15 s with 2% uranyl acetate dissolved in aqua bidest at RT.

Cytohistochemical investigations

Immunogold labeling of glutathione and cysteine was done with ultrathin sections on nickel grids as described in Zechmann et al. (2006b, 2008) for plant tissue. Briefly, samples were blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS, pH 7.2) for 20 min at RT. The samples were then treated with the primary antibodies (antiglutathione rabbit polyclonal IgG and anticysteine rabbit polyclonal IgG, Millipore Corp., Billerica, MA, USA) diluted 1:50 (glutathione antibody) and 1:300 (cysteine antibody) in PBS containing 1% goat serum for 2 h at RT. After a short rinse in PBS (three times, 5 min), the samples were incubated with a 10-nm

gold-conjugated secondary antibody (goat antirabbit IgG, British BioCell International, Cardiff, Great Britain) diluted 1:50 in PBS for 90 min at RT. After a short wash in PBS (three times, 5 min) and distilled water (two times, 5 min), labeled grids were either immediately observed in a Philips CM10 transmission electron microscope or poststained with uranyl acetate (15 s). Poststaining with uranyl acetate was applied to facilitate the distinction of different cell structures, enabling a clearer identification of the investigated organelles.

The selectivity and proper affinity of the primary antibody against glutathione have been tested with competition assays in tissue sections using bona fide GSH and GSSG as the target and a range of displacers. No measurable glutardialdehyde-fixed tissue cross-reactivity against L-alanine, γ -aminobutyrate, 1-amino-4-guanidobutane, D/L-arginine, D/L-aspartate, L-citrulline, L-cysteine, D/L-glutamate, D/L-glutamine, glycine, L-lysine, L-ornithine, L-serine, taurine, L-threonine, L-tryptophan, L-tyrosine was detected. Additionally, the antibody did not bind to glutathionylated proteins on Western blot experiments nor to glutathionylated proteins in sections fixed with formaldehyde (see technical note for glutathione antibody on the website of the manufacturer www.immunologics.com). Therefore, a reaction of the antibody with glutathionylated proteins bound by glutardialdehyde to the protein matrix of the tissue on ultrathin section seems very unlikely (Zechmann et al. 2008). The antibody does not discriminate between free reduced and oxidized glutathione (according to Signature Immunologics Inc.). The primary antibody against cysteine was tested against a spectrum of antigens to assure hapten selectivity and proper affinity. No measurable glutardialdehyde-fixed tissue cross-reactivity was found against methionine, glutathione, cysteic acid, and serine (according to Millipore Corp., Billerica, MA, USA). Several negative controls were made to confirm the specificity of the immunogold procedure. Negative controls were treated either with: (1) gold-conjugated secondary antibody (goat antirabbit IgG) without prior incubation of the section with the primary antibody, (2) nonspecific secondary antibody (goat antimouse IgG), (3) preimmune serum instead of the primary antibody, and (4) primary antibodies preadsorbed with an excess of glutathione or cysteine for 2 h at RT prior to labeling of the sections. For the latter, a solution containing 10 mM of glutathione or cysteine was incubated with 0.5% glutardialdehyde for 1 h. The excess of glutardialdehyde was then saturated by incubation for 30 min in a solution of 1% (w/v) BSA. The resulting solutions were used to saturate the glutathione antibody or the cysteine antibody for 2 h prior to its use in the immunogold labeling procedure described above.

Quantitative determination of glutathione levels using enzymatic method

For enzymatic method of glutathione determination, cyanobacterial cells were grown in BG-11 medium for 7 days as described above. For sulfur depletion, cells were harvested by 5-min centrifugation at $2,500\times g$ and resuspended in BG-11 without sulfur. This step was repeated for five times to ensure complete washing of sulfur-containing medium. Following that, cell cultivation was extended for additional 48 h in above-mentioned conditions. Cells were pelleted by 5-min centrifugation at $2,500\times g$ and washed with PBS (pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4). This step was repeated

three times. Then, cells were lysed for 30 min in lysis buffer (10 mM NaHPO_4 , 5 mM EDTA) and sonicated for 4 min (in 1-min intervals) at 40 W on ice. Cell debris was removed by $10,000\times g$ centrifugation step, at $+4^\circ\text{C}$ for 15 min. Using clear supernatant, the amount of GSH was measured enzymatically, based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by the glutathione reductase system (Tietze 1969).

Quantitative analysis of immunogold labeling

Micrographs of randomly photographed immunogold-labeled sections of cyanobacteria were digitized, and gold particles were counted automatically using the software

Fig. 2 Transmission electron micrographs showing the ultrastructure (**a, b**) and the distribution of gold particles bound to glutathione (**c, e**) and cysteine (**d, f**) within *Synechocystis* sp. cells grown in media with (+S) and without (−S) the addition of sulfur. Gold particles bound to glutathione and cysteine can be found in interthylakoidal spaces (arrows) but not in intrathylakoidal spaces. Note that cells cultivated on media without sulfur contained less gold particles bound to glutathione and cysteine than cells grown on media with sulfur. Bars = 0.5 μm . C carboxysomes, CG cyanophycin globules, V small vacuoles

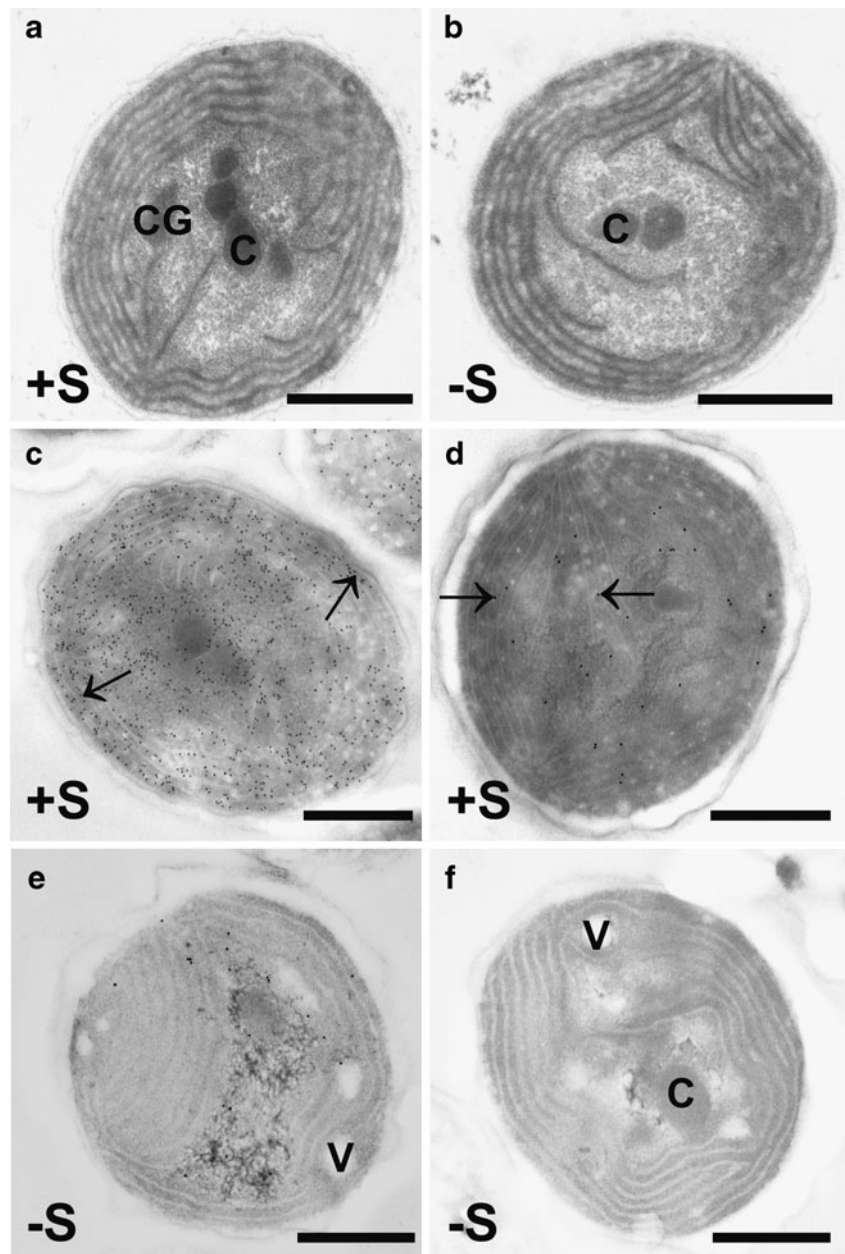


Table 1 Ratio of glutathione between cell compartments

	Ratio of glutathione in percent			
	Glutathione		Cysteine	
	Cytosol	Membranes	Cytosol	Membranes
<i>Synechocystis</i> sp. +S	49 ^a	51 ^a	58 ^a	42 ^b
<i>Synechocystis</i> sp. -S	48 ^a	52 ^a	nd	nd

Ratio (in percent) of the distribution of gold particles bound to glutathione between cell compartments where glutathione and cysteine could be detected. $P < 0.05$ was regarded significant, analyzed by the Kruskal–Wallis test, followed by post hoc comparison according to Conover. $n > 50$ nd not detected

package Optimas 6.5.1 (Media Cybernetics Inc., Bethesda, MD, USA) and Cell D with the particle analysis tool (Olympus, Life and Material Science Europa GmbH, Hamburg, Germany). For statistical evaluation, 20 to 60 sectioned cells from three different embeddings were analyzed for gold particle density. The obtained data were statistically evaluated using Statistica (Stat-Soft, USA, 2002) and presented as the number of gold particles per square micrometer. For statistical analyses, either the nonparametric Kruskal–Wallis test followed by a post hoc comparison according to Conover or the Mann–Whitney U test were used (Bortz et al. 2000).

Results

Ultrastructural investigations revealed no difference between intact cells grown on media with and without sulfur, 48 h after sulfur starvation, even though cell growth in bacteria cultivated in media without sulfur started to stagnate at this time (Fig. 1). Nevertheless, all cells showed a dense cytosol-containing thylakoidal membranes mainly organized in the form of concentric layers proximal to the plasma membrane. Membranes were well preserved and interthylakoidal and intrathylakoidal spaces could be distinguished clearly (Fig. 2a, b). Single thylakoid membranes could also be occasionally observed in the interior (Fig. 2a, b) of the cells. The cytosol contained carboxysomes which contain the enzyme RuBP-carboxylase in tightly packed crystalline arrays and cyanophycin granules (Fig. 2a, b). Additionally, small vacuoles could be occasionally observed within *Synechocystis* sp. Cells were surrounded by a well-preserved cell wall.

Immunohistochemical detection of glutathione and cysteine revealed that both substances occurred throughout the cytosol of *Synechocystis* sp. Gold particles were not found inside carboxysomes, cyanophycin granules, cell walls, intrathylakoidal spaces, periplasm, and vacuoles (Fig. 2c, d). Nevertheless, gold particles bound to glutathione and cysteine could be found in interthylakoidal spaces through-

out the cells (Fig. 2c, d). Glutathione was distributed evenly within cyanobacteria. No differences in the ratio of glutathione could be observed between the areas that contained membranes and the inner part of the cyanobacteria that did not contain membranes (Table 1). A higher ratio of gold particles bound to cysteine (16%) was found in areas that did not contain membranes than in those parts of the cyanobacteria that did contain membranes (Table 2).

The accuracy of the obtained immunogold labeling was supported by several observations. In *Synechocystis* sp. cells grown on media without sulfur, gold particles bound to glutathione and cysteine were strongly decreased by 98.5% and 100%, respectively, when compared to the control (Fig. 2e, f and Table 2). In addition, analysis of *Synechocystis* sp. with altered glutathione levels revealed a strong correlation between biochemically determined total glutathione levels. Cells grown on media without sulfur contained about 91% less glutathione than cells grown on media with sulfur (Fig. 3). No labeling was observed after sections were treated with (1) gold-conjugated secondary antibody without prior incubation with the primary antibody, (2) nonspecific secondary antibody, (3) with preimmune serum instead of the primary antibody and (4) primary antibodies against glutathione and cysteine which were preadsorbed with an excess of glutathione and cysteine (Fig. 4a, b).

Table 2 Data are means \pm standard errors and document the amount of gold particles bound to glutathione and cysteine per micrometer in *Synechocystis* sp. cells grown in media with (+S) and without (-S) the addition of sulfur

	Gold particles per micrometer	
	Glutathione	Cysteine
<i>Synechocystis</i> sp. +S	268 \pm 12.5*	39.38 \pm 3.7*
<i>Synechocystis</i> sp. -S	4.3 \pm 1.1	0 \pm 0

$n > 50$

* $P < 0.001$ (regarded as significant, analyzed with Mann–Whitney U test)

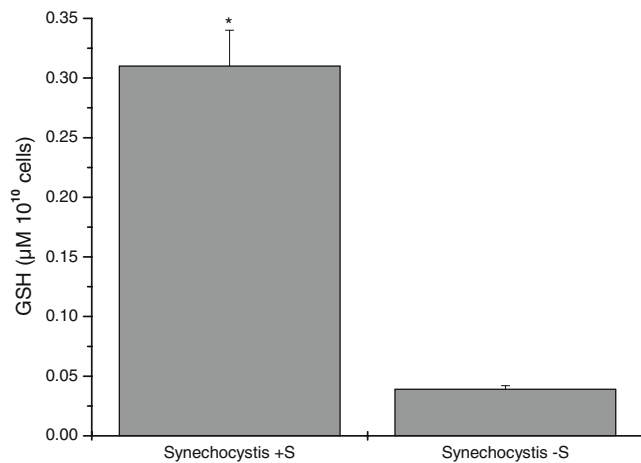


Fig. 3 Graph shows means with standard errors and documents the amount of glutathione in micromolar per cell number in *Synechocystis* sp. cells grown in media with (+S) and without (-S) the addition of sulfur. $P < 0.05$ (*) was regarded significant analyzed with Mann-Whitney U test

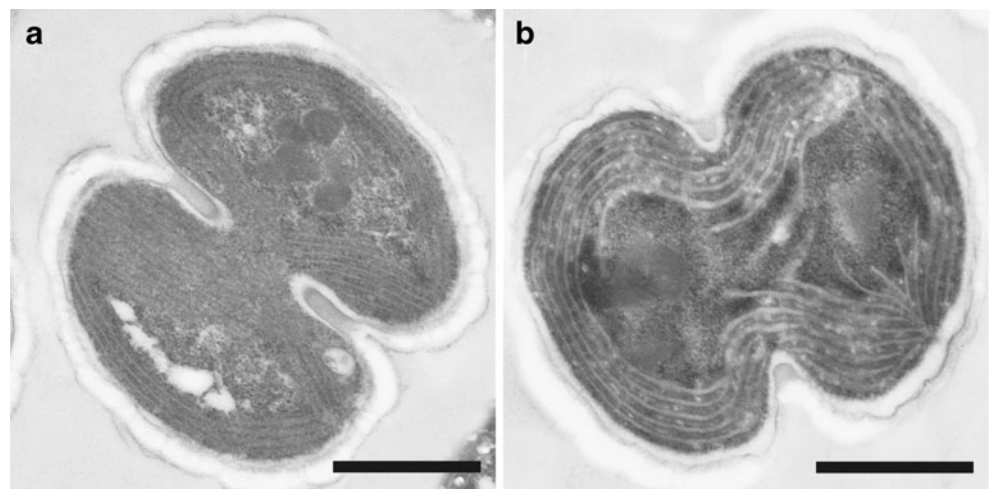
Discussion

Cells of *Synechocystis* sp. grown on media with sulfur showed typical ultrastructural features similar to those previously described for cyanobacteria (Lee et al. 2000; Liberton et al. 2006). No obvious ultrastructural differences were found between intact cells grown on media with and without sulfur, indicating that sulfur depletion for 48 h did not cause major ultrastructural changes in *Synechocystis* sp. cells, even though a stagnation of growth could be observed after that time when compared to bacteria grown in media with sulfur.

The present study revealed a deeper insight into the subcellular distribution of glutathione and cysteine in *Synechocystis* sp. The distribution of glutathione and cysteine was found to be mainly restricted to the cytosol and interthylakoidal spaces. Whereas glutathione was

evenly distributed in areas that contained membranes and areas that did not contain membranes, slightly more cysteine was detected within the latter one. No glutathione and cysteine was detected in carboxysomes, vacuoles, intrathylakoidal spaces, and the periplasm. These data indicate that glutathione and cysteine do not play an important role in these cell compartments in *Synechocystis* sp. Glutathione and cysteine were present in interthylakoidal spaces but not in intrathylakoidal spaces. Similar results were found for the subcellular distribution of glutathione in plastids of plant cells (Zechmann et al. 2006a, b, 2008) which are thought to be derived from endosymbiosis of cyanobacteria in a eukaryotic cells (Vesteg et al. 2009; Waters and Langdale 2009). In chloroplasts and plastids of plants, glutathione and cysteine were restricted to the stroma but did not occur in intrathylakoidal spaces (Zechmann et al. 2006a, b, 2008). Considering these data, there is a good correlation of the subcellular distribution of glutathione and cysteine between cyanobacteria and plastids in plant cells. These results indicate that the subcellular distribution of glutathione and cysteine in plastids remained conserved during evolution despite partly new and different subcellular functions and roles in plant cells when compared to cyanobacteria. Nevertheless, glutathione and cysteine syntheses in plants take place in plastids and primarily in the cytosol of plant cells. Additionally, cysteine, γ -glutamyl-cysteine, and glutathione can be transported across the chloroplast envelope and glutathione can also be imported into other cell compartments that are not able to synthesize glutathione such as mitochondria (Wachter et al. 2005; Zechmann et al. 2008; Foyer and Noctor 2009; Krueger et al. 2009; Maughan et al. 2010). Thus, it becomes evident that even though eukaryotes acquired glutathione metabolism through endosymbiotic events of cyanobacteria (Fahey et al. 1987, Fahey and Sundquist 1991) its metabolism and subcellular distribution in higher plants have been modified most probably due to

Fig. 4 Transmission electron micrographs showing sections of *Synechocystis* sp. treated as negative controls. Both images show lack of labeling as the primary antibody was saturated with an excess of glutathione (a) and cysteine (b) prior to its application. Bars = 0.5 μm



different and more effective defense strategies against environmental stress situations.

It is interesting that glutathione was not detected in the periplasmic spaces and cell walls. It has been shown in *E. coli* that γ -glutamyl-transpeptidase (responsible for glutathione degradation by cleaving the γ -peptide bond between glutamate and cysteine) is a periplasmic protein (Suzuki et al. 1986) whereas in *Neisseria meningitidis* it is associated with the inner membrane facing the cytoplasm (Takahashi and Watanabe 2004). The same has also been found for plant cells (Ohkama-Ohtsu et al. 2007). Additionally, it was postulated that bacteria excrete large amounts of glutathione into the extracellular space (Owens and Hartman 1986). The lack of glutathione labeling in the periplasm and cell walls in this study suggests that either glutathione degradation does not occur in the periplasm and cell walls of cyanobacteria or that it takes place very rapidly in these cell compartment so that free glutathione occurs in such low concentrations that it cannot be detected with this method. Additionally, these results indicate that the excretion of large amounts of glutathione through the periplasm in cyanobacteria is unlikely at least under physiological environmental conditions.

The accuracy of the observed labeling is supported by the results of the preadsorption experiments of the antibodies against glutathione and cysteine with glutathione and cysteine, respectively, and by the fact that a signal was not obtained after the preadsorption of the sections with preimmune serum. Additionally, sulfur starvation led to a complete loss of cysteine labeling and to a 98.5% decrease of glutathione labeling when compared to *Synechocystis* sp. cells grown on media with sulfur. These data also correlate well with results obtained from biochemical investigations which showed that cells grown on media without sulfur contained about 91% less glutathione when compared to cells grown on media with sulfur. From these investigations, it becomes obvious that in situations of sulfur starvation the rate of glutathione and cysteine degradation must exceed glutathione and cysteine synthesis due to the reduced ability of the cell to perform sulfur assimilation into cysteine.

Summing up the present study revealed a deeper insight into the subcellular distribution of glutathione and cysteine in cyanobacteria. Both components are restricted to the cytosol of *Synechocystis* sp. and do not occur in intrathylakoidal spaces, carboxysomes, cyanophycin granules, vacuoles, and the periplasm. Experiments under sulfur depletion revealed that both glutathione and cysteine were strongly decreased 48 h after the beginning of the sulfur starvation. This method can now be used to investigate the importance of subcellular glutathione and cysteine metabolism in cyanobacteria during different environmental (stress) situations such as oxidative and

osmotic stress, acidic conditions, and exposure to toxic components.

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