Membrane fluidity controls redox-regulated cold stress responses in cyanobacteria

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Abstract Membrane fluidity is the important regulator of cellular responses to changing ambient temperature. Bacteria perceive cold by the transmembrane histidine kinases that sense changes in thickness of the cytoplasmic membrane due to its rigidification. In the cyanobacterium Synechocystis, about a half of cold-responsive genes is controlled by the light-dependent transmembrane histidine kinase Hik33, which also partially controls the responses to osmotic, salt, and oxidative stress. This implies the existence of some universal, but yet unknown signal that triggers adaptive gene expression in response to various stressors. Here we selectively probed the components of photosynthetic machinery and functionally characterized the thermodynamics of cyanobacterial photosynthetic membranes with genetically altered fluidity. We show that the rate of oxidation of the quinone pool (PQ), which interacts with both photosynthetic and respiratory electron transport chains, depends on membrane fluidity. Inhibitor-induced stimulation of redox changes in PQ triggers cold-induced gene expression. Thus, the fluidity-dependent changes in the redox state of PQ may universally trigger cellular responses to stressors that affect membrane properties.

Keywords Cyanobacteria · Desaturase · Fatty acids · Fluidity · Fluorescence · Membrane · Lipids · Plastoquinone pool · Photosystem II · Photosystem I · Redox regulation

Abbreviations
ETC Electron transport chain
DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)
DBMIB Dibromothymoquinone
FA Fatty acid
FAD Fatty acid desaturase
FI Fluorescence induction
MR Modulated reflection
PQ Plastoquinone pool
UFA Unsaturated fatty acid

Introduction
Membrane fluidity is an important regulator of cellular responses to changing ambient temperature (Vigh et al. 1993; Los et al. 2013). Bacteria perceive cold by the transmembrane histidine kinases (Suzuki et al. 2000; Aguilar et al. 2001) (Hiks) that sense changes in thickness of the cytoplasmic membrane due to its rigidification (Saita et al. 2016). In photosynthesizing cyanobacteria, about a half of cold-responsive genes is controlled by the light-dependent
(Mironov et al. 2012b, 2014) transmembrane Hik33, which also partially controls the responses to osmotic, salt, and oxidative stress (Zorina et al. 2011; Sinetova and Los 2016a). This implies the existence of some universal, yet unknown signals that trigger the adaptive gene expression in response to various stressors (Sinetova and Los 2016b, c).

Similar to plants and eubacteria, cyanobacteria adjust the membrane fluidity in response to cold via fatty acid (FA) desaturation (Los and Murata 1998). Cold responses of the model cyanobacterium, *Synechocystis* sp. strain PCC 6803, are regulated, in part, by the red light-dependent Hik33 (Mironov et al. 2014), which similarly to bacillary thermosensor, DesK (Aguilar et al. 2001), senses cold-induced membrane rigidification (Saita et al. 2016). The photosynthetic apparatus of cyanobacteria is similar to that of higher plants in terms of structural and functional organization of photosystems I and II (PSI and PSII). However, in higher plants, respiratory and photosynthetic electron transport chains (ETCs) are allocated to different organelles. In cyanobacteria, photosynthetic ETC occurs solely in thylakoids, whereas respiratory electron flow takes place in both the thylakoid and cytoplasmic membrane systems (Mullineaux 2014). In cyanobacteria, water is not the only donor of electrons (via PS II water-oxidizing complex). Some substrates are oxidized by enzymes of the respiratory ETC that is co-localized and intersect with the photosynthetic ETC at plastoquinone pool (PQ), which, in turn, may be reduced via respiration (Cooley and Vermaas 2001; Kirilovsky 2015). The PQ redox state determines the activity of the photosynthetic ETC and redistribution of energy between PSI and PSII (Mao et al. 2002; Mullineaux and Emlyn-Jones 2005). Since the interaction of PQ with ETCs is a diffusion process, the critical factor for these interactions is the fluidity of thylakoid membranes, which depends on the activities of fatty acid desaturases (FADs) that dehydrogenate saturated FAs and produce unsaturated FAs (UFAs) with double bonds in carbon chains (Los and Murata 1998). X-ray crystals of cyanobacterial PSII and PSI clearly indicate the presence of significant amounts of lipids, which participate in the regulation of photosynthetic reactions (Mizusawa and Wada 2012), and provide the matrix for proper electron flow. Thus, among other factors, the changes in membrane physical properties (fluidity) may strongly affect interactions of PQ with ETCs. If so, a number of the vital processes, including stress-induced gene expression, may be controlled by the fluidity of the membranes.

Recently we analyzed stress transcriptomes of *Synechocystis* using bioinformatics approaches (Sinetova and Los 2016a, b). Systemic transcriptome analysis revealed that some universal chemical or electric stimuli may exist that trigger the expression of stress-dependent genes regardless of the physical nature of a stressor. The suggested candidates for such triggers are the reactive oxygen species (ROS) and changes in the redox status of plastoquinone pool (Sinetova and Los 2016b, c). The formation of ROS provoked by different stressors may affect the redox state of a cell, and thus may trigger the stress responses (Mittler et al. 2011; Schmitt et al. 2014; Dietz et al. 2016).

Here we selectively probed the components of photosynthetic machinery and functionally characterized the thermodynamics of cyanobacterial photosynthetic membranes with genetically altered fluidity. We show that the rate of oxidation of the PQ, which interacts with both photosynthetic and respiratory ETCs, depends on membrane fluidity. Thus, the fluidity-dependent changes in the redox state of PQ may universally trigger cellular responses to stressors that affect the membrane properties.

**Materials and methods**

**Cyanobacterial strain and culture conditions**

Cultures of the cyanobacterium wild-type (WT) *Synechocystis* sp. strain PCC 6803 (GT) were grown in BG-11 medium (Rippka 1988) at 26 or 33 °C under luminous lamps with continuous white light illumination at 70 µmol photons m−2 s−1, and constant bubbling by a sterile air-gas mixture enriched with 1.5% CO2 (Mironov et al. 2012a, b). The desAΔ/desDΔ mutant (AD) that lacks Δ12- and Δ6-FADs (Mironov et al. 2012a) was grown in the presence of kanamycin and spectinomycin at 25 and 30 µg ml−1, respectively. Prior to the experiment, cells were centrifuged at 4000g and resuspended in 200 µl of cultural media. Cold adaptation of WT and AD cells were carried out at 25 °C for 24 h. Then cells were harvested, aliquoted, and adapted to darkness at different temperatures (from 5 to 45 °C). For these cells, the kinetics of modulated reflection (MR) at 820 nm and the fluorescence induction curves were immediately measured. The duration of each measurement was not longer than 1 s. Phycobilisome purification was conducted according to the procedures described in (Sluchanko et al. 2017). Each experiment was conducted at least five times.

**Fluorescence induction transients and modulated reflection**

Time courses of fluorescence induction transients (FI) and MR were measured simultaneously from one sample by Multifunctional Plant Efficiency Analyser 2 (M-PEA2, Hansatech Instruments, United Kingdom). The M-PEA2 instrument was equipped with three types of LEDs: (1) 627±10 nm, for the actinic light, delivering up to
5000 µmol photons m⁻² s⁻¹ to the sample; (2) 820±25 nm, for the modulated light LED; and (3) 735±15 nm, for the far-red light LED; the latter uses a long-pass filter to remove any visible light component. Characteristic time courses of FI, MR, and amplitude of the DF are presented in Fig. S1. Since the actinic light is absorbed not only by Chl of photosystems, but also by phycobiliproteins (phyco- cyanin and allophycocyanin) of phycobilisomes, characterized by a high fluorescence quantum yield (up to 0.6 in solution), fluorescence signal detected by M-PEA2 (IF, or prompt fluorescence) is a superposition of PSI, PSII, and PBs fluorescence intensities (Goldsmith and Moerner 2010; Maksimov et al. 2011). It is evident that only fluorescence of PSII is characterized by non-linear time course of intensity due to gradual increase of reduced quinone acceptor concentration. So, one can easily distinguish characteristic time constants of characteristic OJIP steps, as it was previously proposed (Kolber et al. 1998). To control the temperature of the sample we used a custom-built cuvette holder with a Peltier element, providing temperature stabilization from −16 to +45 °C. Membranes were isolated and steady-state fluorescence anisotropy was measured as described before (Mironov et al. 2012b).

Raman spectra measurements

Raman spectra were obtained from Raman spectrometer BWTech (InnoRam, USA) with diode near IR laser 785 nm (max capacity 350 mW). Measuring range was from 64 to 3011 cm⁻¹ with 4 cm⁻¹ resolution. Laser beam was focused into the 100 µm spot via microscope objective (PL L 20/0.40). The samples for Raman spectroscopy were prepared by applying 3 µl aliquot of solutions on the aluminum foil. Time of spectra integration was 30 s; signals represent the average of ten measurements. Additional measurements with other excitation wavelengths were performed using a microscope-based system Ntegra Spectra (NTMDT, Russia) as described in (Maksimov et al. 2015).

Total membrane isolation and physical state measurement

WT and AD cells grown at 32°C or adapted to 25°C for 24 h were harvested for isolation of total membranes. 3–5 g of wet cell pellet was disrupted with glass beads (0.1 mm diameter) for 5 min (1 min of vortexing following 1 min on ice) in the PBS buffer supplemented with 300 mM sucrose and 5 mM EDTA. Broken cells were centrifuged at 12,000g for 10 min. Then supernatant was secondly centrifuged at 100,000g for 30 min. Membrane pellet was washed once, resuspended in Potter homogenizer, and re-centrifuged at 100,000g for 30 min. Membranes after second homogenization were stored in ice not longer than 2 days. The steady-state fluorescence anisotropy was measured as we described earlier (Mironov et al. 2012a, b).

RNA isolation and quantitative RT-PCR

Total RNA was isolated and qPCR was performed as described before (Mironov et al. 2014). Gene-specific primers (Table S1) were designed in Primer3Plus (Untergasser et al. 2007) and synthesized by Litech Ltd (Moscow, Russia). Relative expression level for genes of interest was calculated using calibration curbes (Pfaffl 2004) and triple reference was used for normalization (Vandesompele et al. 2002). Cells were exposed to high light (HL) of 2500 µE m⁻² s⁻¹ for 30 min or treated with photosynthetical inhibitors, 10 µM dibromothymoquinone (DBMIB), or 10 µM diuron (DCMU) also for 30 min. Then cells were fixed and total RNA was isolated as described. 1.5 µg of isolated RNA was treated with DNase I (Thermo Fisher Scientific) and 1 µg was separated in 1% TAE-based agarose gel and visualized under UV light with ethidium bromide. The rest 500 ng of RNA was reverse transcribed using Superscript III (Invitrogen) at 55°C in the presence of gene-specific primers. The qPCR was performed with SYBR® Green Master Mix (Bio-Rad) on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). qPCR included 95°C 5 min, and 40 two-step cycles 95°C 15 s and 63°C 45 s (with detection). Reaction contained 0.3 µM of each primer.

Results and discussion

FA composition of membrane lipids determines the rate of PQ oxidation at low temperature

Synechocystis cells grown at optimal (30–35 °C) temperature adapt to cold stress (20–22°C) by adjusting the membrane fluidity due to cold-induced FADs and increasing amounts of UFAs (Tasaka et al. 1996; Mironov et al. 2012a). We assumed that differences in membrane fluidity should affect plastoquinone diffusion rate and, accordingly, the rate of electron transport on the donor side of PSI. The reduction rate of P700⁺ was estimated from the modulated reflection (MR) data. The initial part of the MR time course (100 µs–10 ms) does not depend on temperature in 5–45 °C range (Fig. 1a), but strongly depends on photon flux density.
of the actinic light (Fig. S2), indicating that it is controlled by photoactivated formation of P700+. By contrast, in 10−2−10 s range, MR signal strongly depends on temperature (Fig. 1a) but not on photon flux density (Fig. S2). It means that P700+ reduction depends on the rates of PQ oxidation by cytochrome b_{6f} complex, which is a diffusion process. MR kinetics shows that P700+ reduction occurs much faster in WT cells grown at 25 °C (Fig. 1b). This is well explained by the cold-induced expression of FADs that produce UFAs to compensate the membrane rigidification. At 32 °C, WT and AD cells display similar P700+ reduction rates (Fig. 1b, c). At 25 °C, however, AD cells had much lower P700+ reduction rates than WT, showing the importance of FADs, mainly Δ12 (Tasaka et al. 1996), for the regulation of the ETC under cold stress. Arrhenius plots of P700+ reduction (Fig. 1b, c) indicate that activation energy of that process decreases above specific temperature that is close to the temperature of cultivation. Significant changes in the activation energy were observed in cold-acclimated cells. In WT cells grown at 25 °C, P700+ reduction occurred with a 20 kcal/mol barrier at temperatures below 25 °C, while above this specific temperature the activation energy decreased reaching 1 kcal/mol.

Placing cold-acclimated cells back to normal conditions shows that even short-term adaptation (300 s) causes significant changes of membrane structural and functional organization. At low temperature, (i) an adjustment of the redox state of the PQ pool depends on membrane fluidity regulated by the cold-induced Δ12- and Δ6-FADs, however (ii) the desaturation of membrane lipids is not the only mechanism, which controls PQ state otherwise AD cells should display similar P700+ reduction rates regardless of the actinic light (Fig. S2), indicating that it is controlled by photoactivated formation of P700+. By contrast, in 10−2−10 s range, MR signal strongly depends on temperature (Fig. 1a) but not on photon flux density (Fig. S2). It means that P700+ reduction depends on the rates of PQ oxidation by cytochrome b_{6f} complex, which is a diffusion process. MR kinetics shows that P700+ reduction occurs much faster in WT cells grown at 25 °C (Fig. 1b). This is well explained by the cold-induced expression of FADs that produce UFAs to compensate the membrane rigidification. At 32 °C, WT and AD cells display similar P700+ reduction rates (Fig. 1b, c). At 25 °C, however, AD cells had much lower P700+ reduction rates than WT, showing the importance of FADs, mainly Δ12 (Tasaka et al. 1996), for the regulation of the ETC under cold stress. Arrhenius plots of P700+ reduction (Fig. 1b, c) indicate that activation energy of that process decreases above specific temperature that is close to the temperature of cultivation. Significant changes in the activation energy were observed in cold-acclimated cells. In WT cells grown at 25 °C, P700+ reduction occurred with a 20 kcal/mol barrier at temperatures below 25 °C, while above this specific temperature the activation energy decreased reaching 1 kcal/mol.

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of the growth temperature. We assume that the significant decrease in the energy barrier for P700+ reduction (Fig. 1c) is related to the specific signaling state, which indicates that the diffusion of PQ is adjusted by FA desaturation and modulated membrane fluidity.

FA desaturation of membrane lipids at low temperatures determines the rate of Q_B reduction

Fluorescence induction (FI) curves were similar for WT and AD cells grown at optimal temperature (Fig. 1e, f). The rates of Q_A reduction (the initial part of the induction curve corresponding to OJ transition) were comparable in WT and AD cells at all temperatures examined (Fig. 1d; Fig. S1). Nevertheless, the rates of J–P transition, which correspond to Q_B reduction and the exchange of reduced secondary quinone (PSII fraction) with the PQ pool (membrane fraction), were affected by temperature in WT (Fig. 1b) but not in AD cells (Fig. 1c). Such a decrease in J–P rate probably reflects the specific regulation of PQ exchange between PSII and membrane pool, which decrease in J–P rate probably reflects the specific regulation and AD cells grown at optimal temperature (Fig. 1e, f).

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Correlation between FA unsaturation and membrane fluidity

Correlation between FA unsaturation and membrane fluidity was confirmed by the measurements of fluorescence anisotropy of DPH (Mironov et al. 2012b) in isolated crude membranes of WT and AD cells grown at 32 or 25°C (Fig. 3). Rigid membranes of AD mutant display higher values of fluorescence anisotropy than fluid membranes of WT cells.

Thus, PQ oxidation rate depends on the structural properties of the photosynthetic membrane and temperature. The diffusion coefficient of PQ depends on membrane fluidity, which is mainly regulated by FADs. The exchange rate of the PSII-reduced quinones with PQ pool also depends on membrane fluidity (and FA content of Q_B-binding pocket). Therefore, we hypothesize that fluidity-dependent changes in the redox rate of PQ may trigger cellular cold responses.
Fluidity-dependent changes in the redox state of PQ trigger cold-responsive gene expression

To validate the above-stated hypothesis, we simulated redox changes in PQ of WT and AD cells grown at 32°C with two well-known ETC inhibitors, oxidizing 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and reducing dibromothymoquinone (DBMIB), and followed stress-responsive gene expression (Fig. 4). DCMU blocks the electron transport at a site of PSII. This results in the oxidation of the following components of the ETC, including PQ. In its turn, DBMIB enters the Qo site of the cytochrome b6f complex from the membrane lipid phase, inhibits electron transfer at the Cytb6f complex, and reduces PQ (Yan et al. 2006).

In *Synechocystis*, cold-induced transcription of *ndhD2* for NADH hydrogenase and *desB* for the terminal ω3-FAD depends on light, membrane fluidity, and it is controlled by the cold sensor Hik33 (Mironov et al. 2012b, 2014). At 33°C, *ndhD2* was induced by strong light and DCMU in both WT and AD cells, however, the induction in AD was notably lower (Fig. 4). It seems that cold-treated WT cells compensate a decrease in PSII-dependent PQ reduction and support the H+ gradient via respiratory ETC. DBMIB induced *ndhD2* and *desB* to rather similar extent in WT and AD cells. It means that redox-dependent transcription of these two genes is less sensitive to PQ oxidation by strong light and DCMU in cells with rigidified membranes, whereas DBMIB-induced PQ reduction does not produce a significant difference between these two strains. Strong light and cold shock induce *hliB*, the product of which is associated with PSI trimers and protects it under stress conditions (He et al. 2001). Prg5 is involved in cyclic electron flow around PSI (Munekage et al. 2002). Unlike *ndhD2* and *desB*, *hliB* and *prg5* genes were highly induced in AD cells, and their transcription is regulated by PQ reduction.
Orange carotenoid protein (OCP) participates in non-photochemical quenching in cyanobacterial cells. The induction of ocpA by DBMIB was lower in AD than in WT cells; therefore the PQ reduction rate, also affects transcription of the genes related to stress regulation.

**Conclusion**

Temperature-dependent changes in the rates of PQ reduction/oxidation (Fig. 1) due to changes in FA composition and membrane fluidity (Figs. 2, 3) regulate stress-responsive gene expression (Fig. 4). Such regulation is important for the acclimation of phototrophic organisms that experience frequent (including daily) changes in the environment, e.g., fluctuations in ambient temperature, light intensity, etc. The cold-induced FADs adjust the membrane fluidity under cold stress by FA desaturation, which is probably triggered by changes in the redox state of PQ. Restoring the rate of PQ reduction in WT cells at 25 °C due to FA desaturation indicates that the acclimation process is completed. In rigidified membranes of the AD mutant, which is unable to adjust the fluidity, the redox signaling is interrupted. Thus, membrane fluidity regulates the energy flows in cyanobacteria. This is important for understanding the molecular mechanism that balances the use of light and regulates the genetic circuits involved in stress responses and acclimation.

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