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EVIDENCE OF THE EFFECT OF *opcA* ON THE CATALYTIC ACTIVITY AND REDOX SENSITIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF *SYNECHOCYSTIS* SP. PCC6803

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Abstract

This study aimed at the analyses of G6PDH activity under presence of reducing agent DTT_{red} using cell-free extracts of *opcA*$^-$ mutant *Synechocystis* sp. PCC6803 cells. K_m values of enzyme for both G6P and NADP was higher in cell-free extracts of the *opcA*$^-$ mutant in comparison with those of the wild type showing that the OpcA polypeptide plays a role to increase the substrate affinity of G6PDH. The enzyme was insensitive to DTT_{red} in *opcA*$^-$ mutant cell-free extract at pH 7.5 while the activity reduced about 29.31% in wild type cell-free extracts indicating that presence of the OpcA was necessary to alter redox state of the enzyme by DTT_{red}. Results of this study support that the OpcA protein, as one of the components of a cellular level regulation, plays an important role in redox modulation of cyanobacterial G6PDH.

Key words: G6PDH, redox modulation, OpcA, *Synechocystis* sp. PCC6803

Introduction. The OpcA polypeptide has often been linked with cyanobacterial glucose-6-phosphate dehydrogenase (G6PDH) activity since the *opcA* gene was described first [1,2]. The effect of the OpcA was correlated with the synthesis, oligomerization or activation of cyanobacterial G6PDH [3–5]. In most cyanobacterial genomes, *opcA* and G6PDH genes (*zwf*) are located in same operon and

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an *opcA* mutation also negatively affects *zwf* gene [3, 4, 6]. In an *opcA* mutant of *Synechocystis* sp. PCC6803 (*S.* 6803), however, G6PDH activity in cell-free extracts was reported to be at a similar level with wild type activity in optimum substrate concentrations possible because *opcA* and *zwf* genes are located at a distance from each other on the chromosome [7].

Cyanobacterial G6PDH activity has known to be under control of redox regulation like in plastidic G6PDH, activated in the dark and deactivated in the light. In plastidic G6PDH, disulphide bonds are constructed between internal cysteine residues after the transition from light to dark while the bonds are broken after transition from dark to light [8–11]. In fact, critical cysteine positions of plastidic G6PDH are not conserved in *zwf* genes of cyanobacterial strains so far sequenced [12]. The question was raised as to whether disulphide bond formation/breaking mechanism was actually operating in the light/dark regulation of cyanobacterial G6PDH [13]. Instead, the OpcA polypeptide was reported to be under control of redox regulation and needed for reactivation/deactivation of cyanobacterial G6PDH [5, 14, 15]. These recent studies support the essential role of OpcA in light/dark regulation of G6PDH activity rather like a mechanism operating for plastidic G6PDH of plants.

Most of the studies on redox regulation of cyanobacterial G6PDH were performed by using purified G6PDH with traditional or recombinant DNA methods resulting in the loss of the OpcA polypeptide. Contrarily, cell-free extracts supply the reaction conditions more similar to in vivo condition containing more cellular actors which are likely to involve the regulation processes like OpcA protein. Therefore, this study aims to determine how G6PDH activity is affected by reducing agent reduced DTT (DTT_{red}) in absence of OpcA protein in an $opcA^-$ mutant *S.* PCC6803 SOI1 cell extracts. This mutant strain provides a suitable cellular condition for G6PDH activity analyses since the mutation in *opcA* gene has no polar effect on transcription of the *zwf* gene, both of the genes located far apart in the genome.

Materials and methods. Wild type and an $opcA^-$ mutant SOI1strains of the *Synechocystis* sp. PCC6803 [16] were used to prepare cell-free extracts for G6PDH assay. Both of the strains were grown in 1l BG11 medium [17] at 28 °C in a warm room by aerating and stirring continuously. These cultures were used for preparation of cell-free extracts.

The cell-free extracts were prepared by using the methods described previously [7,9]. Cell pellets were resuspended in 0.05 M Tris-Maleate buffer at pH 6.5 (Buffer A), and 400 µl aliquots of this cell suspension containing 400 µg glass beads (212–300 µm in size) were disrupted by agitation in a Fast Prep FP120 (BIO101) for 45 s at 5.5 speed level three times. The resulting extract was centrifuged at 10 000 × g and the supernatant was used as enzyme solution for G6PDH enzyme activity assays. Protein amount was determined by the method described by Bradford [18] using Micro Protein 1 kit (Sigma-Aldrich).

In a 1 ml spectrophotometer cuvette 200 μg protein containing cell-free extract, 2 mM G6P and 20 μM NADP were added and the volume was adjusted to 1 ml with nitrogen treated buffer D (0.05 M Tris-Maleate buffer at pH 7.5) [9]. NADP was finally added into reaction mixture to start reaction. Reduction of NADP to NADPH by G6PDH was followed in a spectrophotometer at 340 nm wavelength for 3 min. Activity unit was defined as the formation of 1 μmol NADPH in 1 min and specific activity as units per mg protein.

Reduction of G6PDH by using DTT_{red} was carried out according to the methods described previously [10,13,19]. Using a 500 mM solution, DTT_{red} was added in cell-free extract for a certain concentration in a range of 10 mM to 100 mM. Aliquots of this mixture were taken in after 10, 30 and 60 min after DTT_{red} treatment and used for G6PDH assay as described above. Buffer D stocks with pHs, 6.9, 7.5, 7.9, and 8.1 were used to find the effect of pH on G6PDH activity.

All the measurements of enzyme activity were repeated three times, and mean values were calculated with standard errors and plotted by using IBM Statistics package SPSS (IBM SPSS for Windows version 21).

Results. ***Effects of the opcA$^-$ mutation to G6PDH reaction velocity.*** To determine the effect of absence of the OpcA polypeptide to the G6PDH activity, specific activity was assayed against varying substrate concentrations in both wild type and *opcA*$^-$ mutant *S.* PCC6803 (SOI1) cell-free extracts until the velocity reached V_{max}. The K_m values of the enzyme for both substrates G6PDH and NADP were estimated as the substrate concentrations necessary for 0.5 V_{max}.

The K_m value for G6P was 8.5 mM in cell-free extracts of *opcA*$^-$ SOI1. For wild type G6PDH, the K_m for G6P was found as 6 mM in wild type cell-free extracts (Table 1). This result indicates that K_m value for G6P was 1.42 times higher in *opcA*$^-$ cell-free extracts and that supports possible effect of the OpcA polypeptide to keep K_m values lower for G6PDH of *Synechocystis* sp. PCC6803.

The K_m values for NADP were also determined in the cell-free extracts of *opcA*$^-$ mutant SOI1 and wild type. The K_m value for NADP was 0.025 mM in *opcA*$^-$ mutant SOI1 while the value was 0.0085 mM in the wild type. This result indicates that K_m value for NADP was 2.94 times higher in *opcA*$^-$ mutant SOI1 cell-free extracts and, therefore, OpcA polypeptide may also be needed to lower NADP K_m values for G6PDH activity.

Table 1

The K_m values for G6P and NADP determined by using cell-free extracts of the *opcA*$^-$ mutant SOI1 and wild type *S.* PCC6803

	WT	SOI1	Change (times)
K_m for G6PDH (mM)	6	8.5	1.42
K_m for NADP (mM)	0.0085	0.025	2.94

Effects of reducing agents to G6PDH activity in opcA$^-$ mutant SOI1 cell-free extracts. Initially, various concentrations of DTT_{red} (10, 25, 50, and 100 mM) were applied to the cell-free extracts for various time courses before assaying G6PDH activity. Maximum decreasing effect was reached at 50 mM DTT_{red} concentration in 10 min. Therefore 50 mM DTT_{red} concentration was used as the effective reducing agent concentration in further assays. DTT_{red} untreated cell-free extracts were also analysed for G6PDH activity (time = 0) and used as positive control. The G6PDH activity was decreased by 19.44% after 10 min DTT_{red} treatment while the decreases were 24.17% after 30 min and 29.31% after 60 min. However, specific activity did not exhibit meaningful decrease after DTT_{red} treatment in *opcA*$^-$ SOI1 cell-free extract (Fig. 1). Since no OpcA polypeptide is represented in *opcA*$^-$ mutant SOI1 cells, this result indicates that OpcA polypeptide is needed for reduction effect of reducing agent DTT_{red} on G6PDH activity.

Effects of reducing agents to G6PDH activity in various pH values. After DTT_{red} treatment at pH 6.9 for 30 and 60 min, G6PDH activity decreased both in cell-free extracts of *opcA*$^-$ SOI1 (Fig. 2) and wild type cells (Fig. 3). A lesser decrease in G6PDH activity about 17.65% was observed in *opcA*$^-$ SOI1 cell-free extracts while the activity decreased by about 44.65% in wild type cell-free extracts. At pH 7.5 no decrease was measured in *opcA*$^-$ mutant SOI1 cell-free extracts after 60 min treatment; however, the activity was still decreased by about

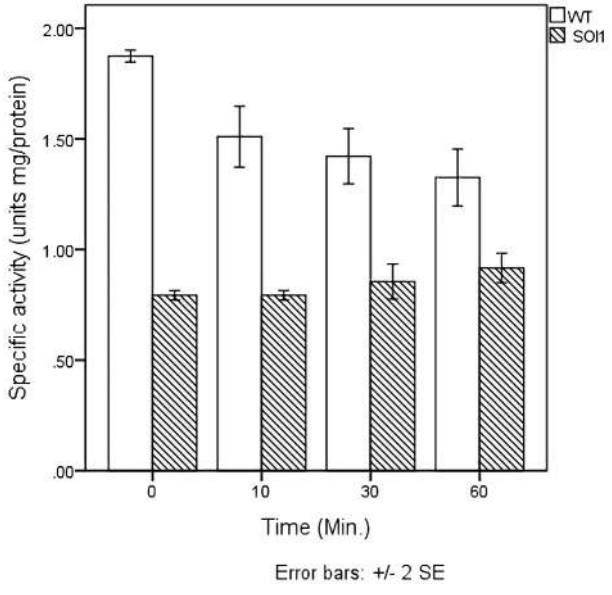


Fig. 1. Time-dependent effect of 50 mM DTT treatment on G6PDH activity in cell-free extracts of wild type and *opcA*$^-$mutant SOI1 cells. Time = 0 shows specific activities obtained form no DTT_{red} treated cell-free extracts

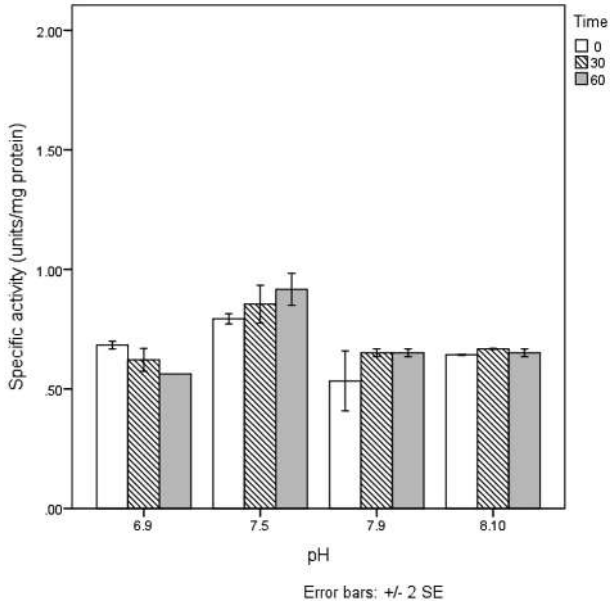


Fig. 2. Effect of DTT_{red} on G6PDH activity in cell-free extract of *opcA*$^{-}$ mutant SOI1 at various pH values. G6PDH activity at 0 min means activities in the cell-free extracts without DTT_{red} treatment at each pH value

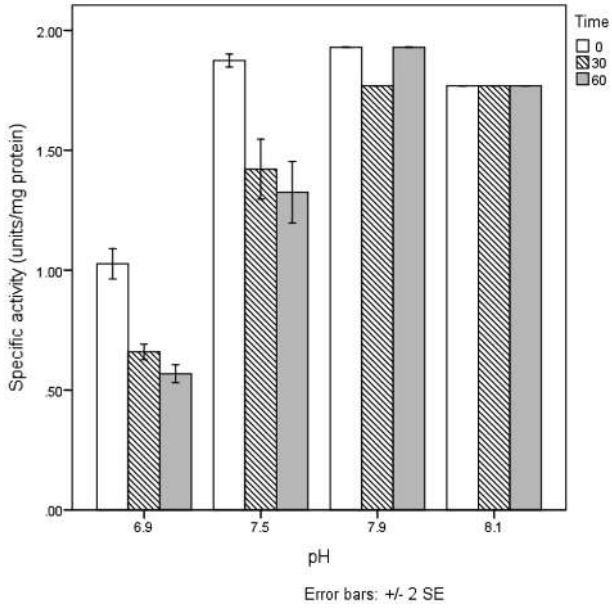


Fig. 3. Effect of 50 mM DTT_{red} on G6PDH in cell-free extract of wild-type *S.* PCC6803 at various pH values. G6PDH activity at 0 min means activities in the cell-free extracts without DTT_{red} treatment at each pH value

29.31% in cell-free extracts of wild type cells. In the cell-free extract of *opcA*$^-$ SOI1 cells, not containing OpcA polypeptide, G6PDH completely lost DTT_{red} sensitivity at the pH 7.5 while the wild type is still sensitive. Both in wild type and *opcA*$^-$ SOI1 cell-free extracts, G6PDH activity was, however, not affected by DTT_{red} treatment for 30 and 60 min neither at pH 7.9 nor at 8.1.

Discussion. A cyanobacterial polypeptide OpcA has been suggested to play an important role in regulation of G6PDH in cyanobacteria [3–5]. According to the results of this study K_m values of G6PDH for the substrates G6P and NADP in cell-free extracts of *opcA*$^-$ mutant SOI1 were 1.42 and 2.94 times higher than those of wild type, respectively. It indicates that the affinity of G6PDH to both the substrates G6P and NADPH were decreased significantly in the absence of OpcA polypeptide implying its role to increase the substrate affinity of G6PDH in *S.* PCC6803. Similar results were investigated for the substrate G6P in *Nostoc punctiforme* sp. strain ATCC29133 [5].

The observations of this study exhibited that DTT_{red} levels did not affect the activity state of G6PDH in cell-free extracts of *opcA*$^-$ mutant SOI1 while the activity clearly decreased in cell-free extracts of wild type in the presence of DTT_{red}. This result is consistent with the hypothesis that redox state of OpcA plays an essential role in light/dark modulation of G6PDH in *Anabaena* sp. PCC7120 [14] and that oxidised OpcA bound to G6PDH tetramer and induced conformational changes in active site of the enzyme [15]. The results of this study support that OpcA plays an essential role in the modulation of G6PDH activity during light/dark transitions.

Reducing agent DTT_{red} did not affect G6PDH activity at pH 7.5 in *opcA*$^-$ mutant SOI1 cell-free extract while the activity was still reduced by 29.31% in wild type. Also, the effect of the reducing agent to G6PDH activity in *opcA*$^-$ mutant SOI1 cell-free extract was lesser than the level in wild type at pH 6.9 indicating that the enzyme in *opcA*$^-$ mutant SOI1 cell-free extracts lost reduction sensitivity to some extent. These observations support that OpcA polypeptide in *S.* PCC6803 cells has a role in conversion of the G6PDH to higher active states during no photosynthesizing periods and to lesser active forms during photosynthetic periods since the enzyme G6PDH is expected to proceed into a lower activity state when the cells are photosynthesizing (at pH 7.4).

Since *opcA* is a circadian class 2 gene [20], light/dark regulation mechanism of G6PDH enzyme activity cannot be explained solely by disulphide bonds formation/reduction of Zwf or OpcA polypeptide alone. It may therefore be concluded that light/dark modulation of the G6PDH in cyanobacteria must be a total output of cellular level regulation of more than one mechanism like disulphide bond formation/reduction, the sensing substrate and product levels, the cell's photosynthetic status, and energy requirements at the cellular level.

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