Influence of CO₂ concentration on carbon concentrating mechanisms in cyanobacteria and green algae: a proteomic approach

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Carbon concentrating mechanisms play a vital role in photosynthesis in microalgae and cyanobacteria especially in the proper functioning of Rubisco and assimilation of carbon via the Calvin cycle. This study evaluates the role of carbon dioxide on carbon concentrating mechanism (CCM) in a cynaobacteria, Spirulina platensis and a microalga, Chlorella sp. 786. The study organisms were grown in both atmospheric (control sample, 0.035%) and high (exposed sample, 10%) CO₂ concentrations. Second dimension (2D) electrophoresis revealed a huge difference in the protein profiles of both organisms suggesting the induction of CCM related proteins in the sample maintained at atmospheric CO₂ concentration and the repression of CCM related proteins in the sample maintained at 10% CO₂. Liquid chromatography-mass spectroscopy analysis revealed the presence of two important Ci transporter proteins in the control sample of S. platensis, namely ferredoxin-NADP⁺ reductase and ATP binding cassette (ABC) transport system protein. These proteins were only expressed in the control sample and were downregulated or not expressed at all in the exposed sample. Consequently, this study conclusively proves that CCMs are only inducted at low CO₂ concentrations and are not functional at high CO₂ concentration.

Key Words: carbon concentrating mechanism (CCM); Chlorella sp.; cyanobacteria; proteomics; Rubisco; Spirulina platensis

INTRODUCTION

Natural photosynthesis in green plants achieves carbon dioxide (CO₂) fixation on a global scale. The incorporation of CO₂ into the biosphere by the photosynthetic action of plants and microorganisms has been estimated to amount to about 10¹¹ tons of CO₂ per year (Moroney and Somanchi 1999, Prentice 2001). However, the efficiency of solar energy conversion in plant production under optimal growth conditions is only 5-6%. The global average efficiency has been estimated as 0.15% (Price et al. 2008). Photosynthesis is much more efficient in single-celled organisms such as microalgae and cyanobacteria than in terrestrial C₃ and C₄ plants (Kaplan and Reinhold 1999). This high efficiency is primarily due to two factors: the action of carbonic anhydrase (CA), both extracellular and intracellular, and the CO₂ concentrating mechanisms (CCM) (Van et al. 2001, Spalding et al. 2002, Vance and Spalding 2005). CO₂ concentration plays a vital role in the induction or repression of CCM in microalgae and cyanobacteria. It has been proven that CCM is induced in low CO₂ concentrations, however, there is little informa-
exposed sample was maintained at a CO2 concentration of 10% (10,000 ppm). Both the cultures were grown for a period of 15-20 days in the reactor (Ramanan et al. 2010). The algal cells were centrifuged at 5,000 ×g for 5 min and to 0.5 g of algal pellet, 10 mL of algal culture medium with 2% Triton X-100 was added. The algal pellet was resuspended and pelleted. Supernatant which had greenish-yellow tint was poured off. The pellet was once again rinsed with culture medium and centrifuged again. The supernatant was poured off and 3.75 mL of extraction buffer containing 100 mM Tris, 100 mM EDTA and 100 mM NaCl, with one aliquot of Protein Inhibitor Cocktail was added to 10 mL of buffer. About 1-2 mL of acid washed glass beads (400-600 μm) were then added and the solution was vortexed for 30 s and then placed on ice for 30 s. Icing and vortexing were repeated 20 times. The suspension was then pipetted out and placed in microcentrifuge tubes. It was then centrifuged at 15,000 rpm for 5 min and the resultant supernatant with an orange tint was estimated for protein content by Lowry’s method (Lowry et al. 1951).

First dimension electrophoresis

For first-dimension electrophoresis, 300 μL of sample solution was applied to a pH 4-7 ReadyStrip IPG strips (Bio-Rad, Hercules, CA, USA). The strips were rehydrated for 18 h in 20°C. The strips were then placed on an isoelectric focussing (IEF) instrument, Bio-Rad, and were run according to the pre-set program. After the run was completed, strips were stored at -80°C until 2D electrophoresis was performed according to the pre-set program.

2D electrophoresis

For the 2D, the pH 4-7 IPG strips, were equilibrated for 10 min in equilibration buffer I and were then re-equilibrated for 10 min in equilibration buffer II. The equilibrated IPG strips were then kept in 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) shield with agarose. Precision protein standards (Bio-Rad) were run along with the sample at 50 mA for 4 h. The 2D gels were then fixed overnight, water washed and silver stained.

Image analysis

The 2D gels were stained with silver stain and images were captured and analysis was performed with the use of the Image Master software (GE Healthcare, USA).

MATERIALS AND METHODS

Selection of organisms

The organisms were selected as a result of parallel batch studies in the sequestration of CO2 which involved in the screening of high CO2 fixing organisms (Ramanan et al. 2010). Accordingly, *Spirulina platensis* and *Chlorella* sp. were selected for proteomic analysis at varied CO2 concentrations. Pure cultures of *S. platensis* (Cyanobacteria, Oscillatoriales) and *Chlorella* sp. (Chlorophyta, Chlorophyceae) were obtained from the algal culture collection centre, Indian Agriculture Research Institute, New Delhi, India and were cultured as mentioned in the previous study (Ramanan et al. 2010). 18S rRNA sequencing confirmed the identity of both organisms (Fulke et al. 2010).

Sample preparation for second dimension (2D) electrophoresis

Both organisms were grown under two different conditions of CO2 in the laboratory reactor maintained at 30°C under a 12 h light / dark illumination cycles of 30 μmol m−2 s−1 during light cycle supplied by artificial lighting. Three sets of control samples were maintained at an atmospheric CO2 concentration (~350 ppm) and three sets exposed sample was maintained at a CO2 concentration of 10% (10,000 ppm). Both the cultures were grown for a period of 15-20 days in the reactor (Ramanan et al. 2010).

The algal cells were centrifuged at 5,000 ×g for 5 min and to 0.5 g of algal pellet, 10 mL of algal culture medium with 2% Triton X-100 was added. The algal pellet was resuspended and pelleted. Supernatant which had greenish-yellow tint was poured off. The pellet was once again rinsed with culture medium and centrifuged again. The supernatant was poured off and 3.75 mL of extraction buffer containing 100 mM Tris, 100 mM EDTA and 100 mM NaCl, with one aliquot of Protein Inhibitor Cocktail was added to 10 mL of buffer. About 1-2 mL of acid washed glass beads (400-600 μm) were then added and the solution was vortexed for 30 s and then placed on ice for 30 s. Icing and vortexing were repeated 20 times. The suspension was then pipetted out and placed in microcentrifuge tubes. It was then centrifuged at 15,000 rpm for 5 min and the resultant supernatant with an orange tint was estimated for protein content by Lowry’s method (Lowry et al. 1951).

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The 2D gels were stained with silver stain and images were captured and analysis was performed with the use of the Image Master software (GE Healthcare, USA).
of PD-Quest imaging software (Bio-Rad). Those spots showing significant differential expression are then excised from the gel and processed for ingel digestion. The software analysis revealed the molecular weight and isoelectric point (pI) values as well as the levels of expression of all the proteins. Spot identification was made using liquid chromatography-mass spectroscopy (LC-MS) at The Centre for Genomic Application (TCGA), New Delhi, India.

Sample preparation for LC-MS analysis

The band of interest was cut from the gel and the gels were destained. The gel pieces were then reduced using 150 µL of 10 mM dithiothreitol in 100 mM ammonium bicarbonate with 5% acetonitrile (ACN) for 1 h at 55°C. The supernatant was removed and the gel pieces were dehydrated with 100 µL of 100 mM ammonium bicarbonate for 10 min. The solution was removed again and gel pieces were dehydrated with 100 µL of 100% ACN for 20 min. For alkylation, 100 µL of 50 mM iodoacetamide in 100 mM NH₄CO₃ was added to the gel pieces. Tubes were incubated in dark at room temperature for 30 min. Supernatant was removed and gel pieces were washed with 100 µL of 100 mM NH₄CO₃ for 10 min. Supernatant was removed again and gel pieces were washed with 100 µL of 100% ACN for 20 min. Supernatant was once again removed and gel pieces were dried in vacuum for 15 min.

Gel pieces were digested with trypsin and proteins were extracted from the digested solution. About 6 µL of the extracted protein sample was injected into nanoLC-MS (Agilent 1100 series LC/MSD Trap XCT; Agilent Technologies, Palo Alto, CA, USA).

RESULTS

The CO₂ environment plays a huge role in the metabolism of the algae (Beardall et al. 1998). This is revealed in the different protein profiles of samples treated with different CO₂ condition. Although the protein profiles are apparently distinct, comparison with an existing proteome would have given valuable insights on the differentially expressed proteins, however, algal proteome databases are not complete. There is no proteomic database for *S. platensis* and *Chlorella* sp. and this is the first study to elucidate the proteome profiles of these organisms under different conditions of CO₂. Thus, the 2D PAGE obtained in the study has been compared with the *Arabidopsis thaliana* 2D database in the ExPASy (expert protein analysis system) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/swiss-2dpage/viewer). Since *C. reinhardtii* proteome is not published, the leaf proteome of *A. thaliana* was selected, as it is the closest homolog to the organisms under study.

*Spirulina platensis*: control sample

The protein profile for the control sample reveals that a lot of proteins are expressed in the region of 30 kDa with an isoelectric point of about 5.0-5.5 (Fig. 1). This is the region where proteins involved in the CCM pathway, such as CA, are located and hence, all the proteins spots in the region were picked robotically for LC-MS analysis. A comparison between *A. thaliana* proteome and the *S. platensis* control sample reveals that CCM proteins such as CA, Rubisco larger subunit, and other proteins which are present in *A. thaliana* proteome, have been found to be expressed in the control *S. platensis* samples. The Rubisco smaller subunit, with a molecular weight of about 18 kDa and pI of about 6.0, was found to be expressed in the control sample, albeit to a lower degree.
unit in the exposed sample indicates that the CO₂ environment has a profound impact on photosynthesis and CCM as well.

**Chlorella sp.: control vs. exposed sample**

The protein profiles of both control and exposed samples of *Chlorella* sp. were completely different from one another (Figs 3 & 4). The control sample had 18 protein spots that were either expressed or up regulated in the control gel while the same proteins were down regulated in the exposed sample. In the exposed sample, 20 proteins were up regulated which were in turn down regulated in the control sample. This reveals that different metabolic pathways occur at atmospheric and 10% CO₂ concentrations.

**LC-MS analysis of proteins**

The LC-MS analysis of 10 selected protein spots in the control and exposed samples of *S. platensis* and *Chlorella* sp. did not yield results for any of the samples except for protein spots 9, 10, and 17 of the control sample of *S. platensis*. The LC-MS analysis and MASCOT search for the peptide sequence revealed that the protein expressed is ferredoxin-NADP⁺ reductase in the case of spots, 9 and 10. The individual ion score of 186 and 114 for the protein spots 9 and 10, respectively, confirms the identity of the protein. Individual ions scores >51 indicate identity or extensive homology (p < 0.05).

**DISCUSSION**

The 2D-PAGE data of both control and exposed samples of *S. platensis* and *Chlorella* sp. show that there is a significant difference in the protein profile of both organisms under these varying conditions of CO₂ as all the other factors, such as temperature, pH, light conditions and nutrients were optimal and identical for all the samples. A set of 18 proteins were found to be expressed only under low-CO₂ condition in both study organism. These proteins were not expressed at all in exposed samples. Similarly, Fang et al. (2012) observed that varying CO₂ concentrations had an effect on 25% of the transcriptome in *C. reinhardtii*. Proteomic studies on *C. reinhardtii* under varying concentrations of CO₂ reveal the role of 22 extracellular proteins which are only expressed under low-CO₂ concentration (Baba et al. 2011). Similarly, in a transcriptome study, a series of low-CO₂ inducible proteins have
been reported, which are membrane bound and possibly be C\textsubscript{3} transporter candidates playing a crucial role in CCM functioning (Yamano et al. 2008).

In the LC-MS analysis of proteins, a total of three proteins from \textit{S. platensis} control sample (0.03% CO\textsubscript{2}) were identified which are down regulated or not expressed at all in the exposed sample (10% CO\textsubscript{2}). The LC-MS analysis of the proteins of interest in \textit{Chlorella} sp. did not reveal any results which may be because of the fact that the proteome database for \textit{Chlorella} has not been established.

The LC-MS analysis of the protein spot 17 from \textit{S. platensis} control proteome revealed a similarity, with an individual ion score of 50, to the ATP binding cassette (ABC)-type multidrug / protein / lipid transport system from bacterium \textit{Pelotomaculum thermopropionicum} (Table 1).

The protein spot No. 17 pertaining ABC transport sys-

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**Table 1.** Details of the protein spots identified in \textit{Spirulina platensis} with accession number

<table>
<thead>
<tr>
<th>Protein spot No. / Organism</th>
<th>Accession No.</th>
<th>Matched protein</th>
<th>Molecular weight (Da)</th>
<th>Matched species</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 9 / \textit{S. platensis}</td>
<td>1BJK</td>
<td>Ferredoxin-NADP\textsuperscript{+} reductase</td>
<td>33,284</td>
<td>\textit{Anabaena} sp.</td>
<td>Active transportation of C\textsubscript{3} from the external environment</td>
</tr>
<tr>
<td>No. 10 / \textit{S. platensis}</td>
<td>RDSGXX</td>
<td>Ferredoxin-NADP\textsuperscript{+} reductase</td>
<td>33,618</td>
<td>\textit{Spirulina} sp.</td>
<td>Active transportation of C\textsubscript{3} from the external environment</td>
</tr>
<tr>
<td>No. 17 / \textit{S. platensis}</td>
<td>Q1WXW1_9FIRM</td>
<td>ABC-type multidrug / protein / lipid transport system</td>
<td>36,818</td>
<td>\textit{Pelotomaculum thermopropionicum} SI</td>
<td>Protein involved in transporting materials including C\textsubscript{3} for utilization inside the cell</td>
</tr>
</tbody>
</table>

The other protein spots which were differentially expressed could not be detected for the lack of proteome database for both organisms used in this study.
tem protein is an important protein involved in the transport of most materials including Cᵢ from the outside environment into the cell membrane for utilization. There are five reported transporters of Cᵢ, of which BCT1 is an inducible high affinity HCO₃⁻ transporter encoded by the cmpABC operon and belonging to the traffic ABC transporter family (Price et al. 2008). BCT1 has been found to be expressed in 10 other cyanobacteria and hence it is quite possible that BCT1 is the transporter which is expressed in *S. platensis* at the atmospheric CO₂ concentration. This transporter protein is present in the cell membrane, and allows Cᵢ to enter into the cell for further conversion into CO₂ in the cytosol, which further moves into the carboxysomes in cyanobacteria which is the site for carbon assimilation involving Rubisco (Giordano et al. 2005).

The form of Cᵢ that accumulates in the cytosol is HCO₃⁻, irrespective of the form of Cᵢ entering the cells. An energized conversion of CO₂ to HCO₃⁻ however, occurs on the cytosolic side of the thylakoid membrane. This energized conversion is aided by the electron transfer through NADH-1 complex which also hosts ferredoxin-NADP⁺ reductase enzyme, which catalyzes high energy conversion along with other proteins in the NADH-1 complex. According to Price et al. (2002), the reduced intermediate generated within the NADH-1 complex by the electrons donated by NAD(P)H or ferredoxin converts Zn-H₂O to Zn-OH at the active site of the complex. As in CA, the Zn-OH is then involved in hydrating CO₂ to HCO₃⁻. Thus, Ferredoxin-NADP⁺ reductase, which has been identified in proteins spots 9 and 10 from *S. platensis* proteome (Table 1), is a enzyme involved in the active transportation of HCO₃⁻ from the external environment for assimilation of CO₂ inside the carboxysomes by the conversion of these HCO₃⁻ ions by CA (Guedeney et al. 1996, Giordano et al. 2005). In *C. reinhardtii*, a variety of CA genes, especially mitochondrial CA, have been implicated for expression only under low-CO₂ condition (Yamano et al. 2008, Renberg et al. 2010, Baba et al. 2011, Fang et al. 2012). However, further studies are required to understand whether ferredoxin-NADP⁺ reductase implicated in this study is mitochondrial or membrane bound.

Thus, proteomic and subsequent LC-MS analyses reveal that normal CCM pathway has been actively pursued in cells maintained at atmospheric concentrations of CO₂. On the contrary, the same pathways have not been activated under conditions of elevated CO₂ as indicated by the down-regulation of the identified proteins along with 15 other proteins probably having functions in CCM and related mechanisms. This strongly proves that at lower concentrations of CO₂, the CCM pathway is activated, in the study organisms. At high concentrations of CO₂, the CCM pathway need not be activated as the CO₂ concentration is high and Rubisco naturally expresses carboxylase activity without the help of CCM (Fukuzawa et al. 2001, Moroney and Ynalvez 2007, Yamano and Fukuzawa 2009, Cannon et al. 2010). Thus, *S. platensis* seems to activate CCM at low CO₂ concentration with active Cᵢ transport, whereas this mechanism is not activated at elevated concentration of CO₂ in *S. platensis* (Figs 1 & 2).

In the case of *Chlorella* sp. the same trend of up-regulation of various proteins in exposed and control samples was observed, and it is quite possible that the same conclusion shall prevail (Figs 3 & 4). However, LC-MS analyses would not reveal the identity of the proteins which may be because of the absence of database on *Chlorella* proteome. Thus no definite conclusion could be drawn regarding the role of CO₂ on carbon concentrating mechanisms in *Chlorella* sp. due to the non-availability of a database.

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