



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 cyanobacteria, *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 C₁ 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 induced 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-

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Received July 16, 2012, Accepted October 30, 2012

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tion on the roles of functional proteins involved in CCM at different concentrations of CO₂.

A proteomic approach to this pertinent question has been undertaken in this study. Among algae in general, the only proteome to be studied is that of *Chlamydomonas reinhardtii*, with even with sub-cellular proteome investigations (Stauber and Hippler 2004, Atteia et al. 2009, Wienkoop et al. 2010). Recently, *C. reinhardtii* proteome, transcriptome and metabolome have been studied under varying CO₂ concentration. Cumulatively, these studies suggest the involvement of novel transport proteins involved in C_i transport and also reveal the effect of induction of CCM on whole genome (Yamano et al. 2008, Renberg et al. 2010, Baba et al. 2011, Fang et al. 2012). However, effect of CO₂ on other green alga and cyanobacteria has not been investigated so far. Hence, a look at the proteome of the other organisms at varying conditions may help corroborating the earlier efforts on *C. reinhardtii* as well as reveal any novel functional proteins involved in CO₂ metabolism. Moreover, this would serve as the first report on the effect of CO₂ on the proteome of the study organisms.

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).

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 PD-Quest imaging software (Bio-Rad). Those spots showing significant differential expression are then excised from the gel and processed for in-gel 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 μ L 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 μ L of 100 mM ammonium bicarbonate for 10 min. The solution was removed again and gel pieces were dehydrated with 100 μ L of 100% ACN for 20 min. For alkylation, 100 μ L of 50 mM iodoacetamide in 100 mM NH_4CO_3 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 μ L of 100 mM NH_4CO_3 for 10 min. Supernatant was removed again and gel pieces were washed with 100 μ L 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 μ L 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 CO_2 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 CO_2 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 CO_2 . Thus, the 2D PAGE obtained in the study has been compared with the *Arabidopsis thaliana* 2D database in the ExPASy (expert protein

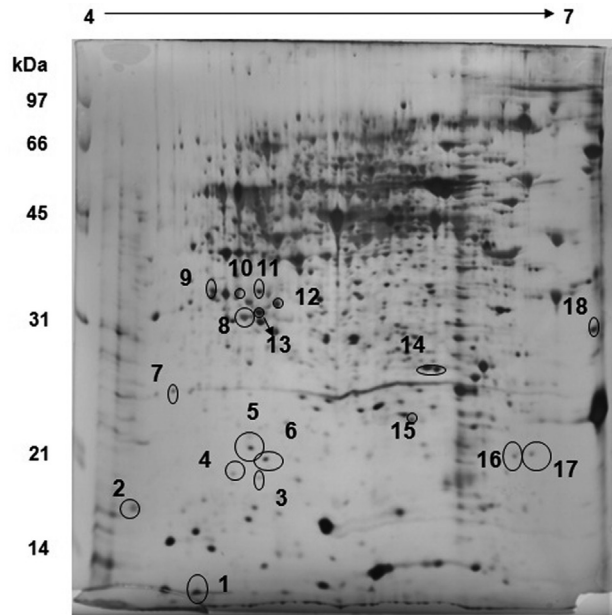


Fig. 1. The proteome profile of *Spirulina platensis* under atmospheric CO_2 concentration (350 ppm, control). The horizontal axis of 4-7 represents the first dimensional separation based on pI value of proteins. The vertical axis represents the second dimensional separation based on molecular weight. The proteins spots (1-18) which have been up regulated or only expressed in the control sample have been encircled.

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.

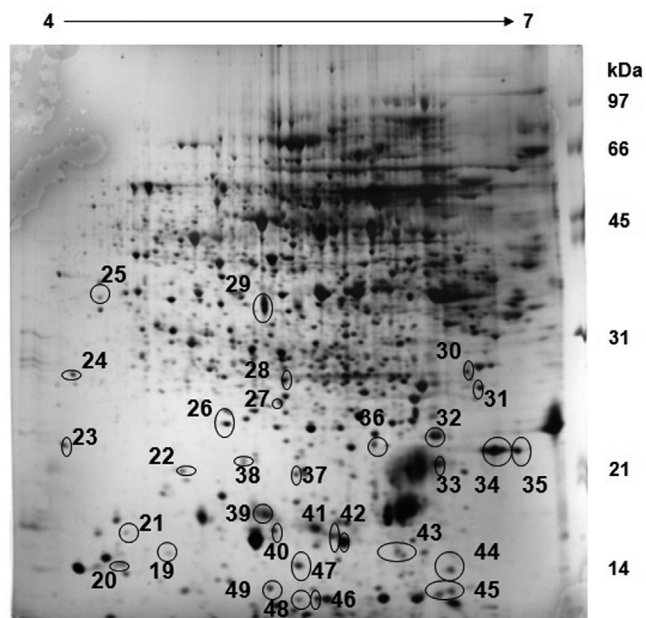


Fig. 2. The proteome profile of *Spirulina platensis* under elevated CO₂ concentration (100,000 ppm, exposed). The horizontal axis of 4-7 represents the first dimensional separation based on pI value of proteins. The vertical axis represents the second dimensional separation based on molecular weight. The proteins spots (19-49) which have been up regulated or only expressed in the exposed sample have been encircled.

Spirulina platensis: exposed sample

The exposed sample of *S. platensis* showed a completely different protein profile when compared to the control sample (Fig. 2). The regions of interest particularly that of the CCM related proteins and CA were analyzed for expression pattern. However, in the region of interest, the proteins were down regulated and lot of smaller molecular weight proteins were up regulated including the smaller subunit of Rubisco. This suggests that the induction of CCM is not required at high CO₂ concentrations.

Spirulina platensis: control vs. exposed sample

A comparative analysis of both sample gels reveals that 18 proteins are up regulated in the control sample whereas these 18 proteins are down regulated in the exposed sample (Fig. 2). Furthermore, about 31 proteins which were up regulated in the exposed sample were either down regulated or not expressed at all in the control sample. This reveals that some major metabolic activities, such as Calvin cycle, is altered under different CO₂ concentrations. The over-expression of Rubisco smaller sub-

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_i 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 *S. platensis* control sample (0.03% CO_2) were identified which are down regulated or not expressed at all in the exposed sample (10% CO_2). The LC-MS analysis of the proteins of interest in *Chlorella* sp. did not reveal

any results which may be because of the fact that the proteome database for *Chlorella* has not been established. The LC-MS analysis of the protein spot 17 from *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 *Pelotomaculum thermopropionicum* (Table 1).

The protein spot No. 17 pertaining ABC transport sys-

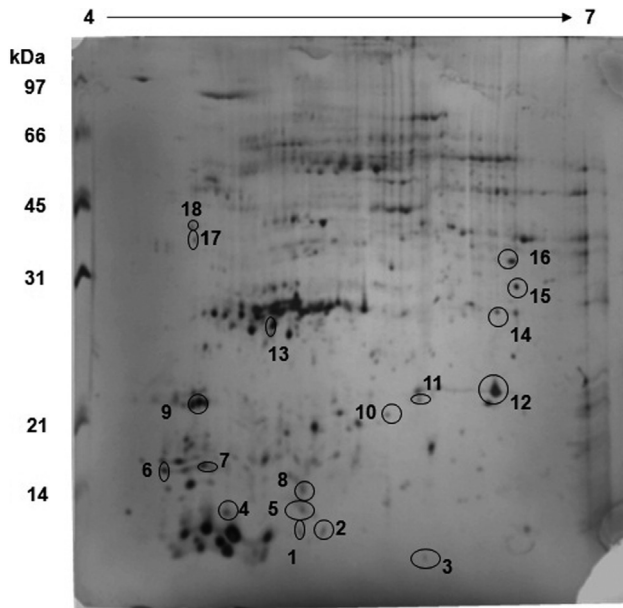


Fig. 3. The proteome profile of *Chlorella* sp. under atmospheric CO_2 concentration (350 ppm, control). The horizontal axis of 4-7 represents the first dimensional separation based on pI value of proteins. The vertical axis represents the second dimensional separation based on molecular weight. The proteins spots (1-18) which have been up regulated or only expressed in the control sample have been encircled.

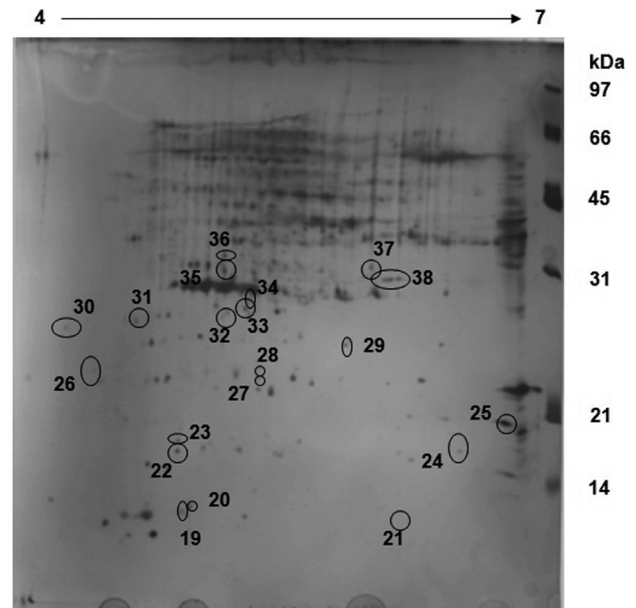


Fig. 4. The proteome profile of *Chlorella* sp. under elevated CO_2 concentration (100,000 ppm, exposed). The horizontal axis of 4-7 represents the first dimensional separation based on pI value of proteins. The vertical axis represents the second dimensional separation based on molecular weight. The proteins spots (19-38) which have been up regulated or only expressed in the exposed sample have been encircled.

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

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

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 C_i from the outside environment into the cell membrane for utilization. There are five reported transporters of C_i of which BCT1 is an inducible high affinity HCO_3^- transporter encoded by the *cmpABCD* 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 CO_2 concentration. This transporter protein is present in the cell membrane, and allows C_i to enter into the cell for further conversion into CO_2 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 C_i that accumulates in the cytosol is HCO_3^- , irrespective of the form of C_i entering the cells. An energized conversion of CO_2 to HCO_3^- , 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 CO_2 to HCO_3^- . 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 HCO_3^- from the external environment for assimilation of CO_2 inside the carboxysomes through the conversion of these HCO_3^- ions by CA (Guedeny 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- CO_2 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 CO_2 . On the contrary, the same pathways have not been activated under conditions of elevated CO_2 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 CO_2 , the CCM pathway is activated,

in the study organisms. At high concentrations of CO_2 , the CCM pathway need not be activated as the CO_2 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 CO_2 concentration with active C_i transport, whereas this mechanism is not activated at elevated concentration of CO_2 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 CO_2 on carbon concentrating mechanisms in *Chlorella* sp. due to the non-availability of a database.

ACKNOWLEDGEMENTS

Rishiram Ramanan is grateful to the University Grants Commission, New Delhi for the award of a Senior Research Fellowship and for providing financial support. The authors thank the Department of Science and Technology (DST), Government of India for the financial support provided for this project.

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