DISSERTATION

The central body of the cyanelles of *Cyanophora paradoxa* : A eukaryotic carboxysome?

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ABSTRACT

Aquatic microorganisms contribute approximately 50% to the total CO_2 fixation in the biosphere (Behrenfeld et al., 2001). Among them, first the cyanobacteria and then, after the endosymbiotic event, cyanobacteria and algae together have been responsible for creating the present atmosphere of 21% oxygen. Furthermore, all cyanobacteria investigated and most algae developed similar solutions to cope with the low CO_2 content (0.037%; Keeling and Whorf, 2004) of the atmosphere and their dependence on diffusive entry of CO_2 by evolving CO_2 concentration mechanisms (CCM) different from those emerging later in C_4 and CAM plants. However, the type of the CCM microcompartment harboring Rubisco is different in prokaryotic and eukaryotic oxygenic phototrophic microorganisms: carboxysomes and pyrenoids, respectively. Carboxysomes could have been transferred via endosymbiosis and then changed to pyrenoids in algae or, alternatively, carboxysomes and pyrenoids arose independently (Badger et al., 2006; Giordano et al., 2005). Each cyanelle is surrounded by two envelope membranes, their thylakoids are peripheral and concentrically arranged, between them intrathylakoidal phycobilisomes are present.The centre of the cyanelles of Cyanophora is occupied by a large electron dense roundish sometimes polyhedral body, which has been shown, by protein A-gold immuno electron microscopy, to contain the bulk of ribulose- l,5-bisphosphate carboxylase/oxygenase (Mangeney and Gibbs, 1987). Phosphoribulose kinase which catalyzes the regeneration of ribulose-l,5-bisphosphate from ribulose-5-phosphate is localized outside the carboxysome, in the thylakoid region (Mangeney et al., 1987). Carboxysomes of cyanelles and of cyanobacteria thus resemble pyrenoids in eukaryotic algae.

Cyanophora paradoxa, the best-investigated alga of the *Glaucocystophyta,* contains plastids (cyanelles) surrounded by a prokaryote-type peptidoglycan wall, which represent a very early stage in organelle evolution. The unusual plastids of C. *paradoxa* display several cyanobacterial features besides the peptidoglycan wall. Therefore, our assumption is that the glaucocystophyte cyanelles bear a cyanobacterial central body for acclimation to low [CO_2] by virtue of a cyanobacterial CCM.

The characterization of the central body of C. *paradoxa* and its CCM as well as the identification of homologues of cyanobacterial and algal proteins involved in carboxysome and/or pyrenoid biogenesis and functioning were the goals. An isolation procedure for central bodies was developed to be able to perform mass spectrometry on in-gel digested bands of SDS-PAGE gels. Two cDNA libraries, for conditions of low- and high [CO_2], were constructed. At the University of Illinois, high-throughput sequencing of 4992 clones generated a collection of expressed sequence tags (ESTs). Genes involved in the CCM were among the abundant transcripts (shown by S. Burey). Through a different collaboration, phylogenetic analyses using EST data from C. *paradoxa* provided further evidence for the monophyletic origin of the kingdom plantae. A subset of largely unique ESTs was PCR-amplified and PCR products spotted on coated slides for microarray analysis of transcript levels at various timepoints after shift from high (5%) to low- (ambient, 0.037%) CO_2. Among the genes showing differential regulation upon high- and low-CO_2 a number of CCM candidate genes were identified, such as Rubisco activase and a putative bicarbonate transporter - to date the only Ci transporter (Ci, inorganic carbon) candidate in C. *paradoxa.* Emphasis was placed on identifying various carbonic anhydrases (CA): a putatively cytosolic CA and two isoforms of putatively mitochondrial CA were described. The results presented support the existence of a CCM in C. *paradoxa,* though more data is required to support or refute our hypothesis of "eukaryotic carboxysomes".

The morphology and size of the central bodies were compared for high and low CO2 cells using a JEOL 1210 transmission electron microscope (collaboration with Dr. Siegfried Reipert from Department of Molecular Cell Biology). High pressure cryofixation (Empact HP-freezer, LEICA Microsystems) in Combination with freeze substitution (AFS, LEICA Microsystems) were employed to preserve both the fine structural details. Peptide pattern and sequence information for components of isolated central bodies smaller than 57 kDa were obtained after in-gel protease digestion bands via MALDI-TOF- and nanoelectrospray tandem mass spectrometry, respectively (collaboration with Günter Allmaier and Martina Marchetti, Technical University of Vienna).

Rubisco activase was identified as a *bona fide* componend of the central body. *In vitro* assembly of this chaperone together with Rubisco could be show after import of labeled precursor into isolated cyanelles and subsequent fractionation.

ZUSAMMENFASSUNG

Die Photosynthese stellt die Grundlage des Lebens auf unserem Planeten in seiner heutigen Form dar. Neben der Landpflanzen spielen die Algen und die Cyanobakterien eine wesentliche Rolle bei der globalen Kohlendioxid-Fixierung und tragen damit zum derzeit noch annähernd bestehenden Gleichgewicht zwischen der Produktion und dem Verbrauch des (Treibhaus-Gases) bei. Angesichts der relativ geringen Affinität des Schlüsselenzyms Ribulose-1,5-bisphosphatcarboxylase (Rubisco) für sein Substrat ist die Kohlendioxidkonzentration in der Atmosphäre (0.037%) tatsächlich als zu niedrig anzusehen. Im Verlauf der Evolution haben zunächst die Cyanobakterien, dann die durch Endosymbiose aus ihnen entstandenen Algen und schließlich die höheren Pflanzen Kohlendioxid- Konzentrierungsmechanismen (CCM) entwickelt, um dieses Manko auszugleichen.

Cyanophora paradoxa ist die am besten charakterisierte einzellige Alge aus der Gruppe der *Glaucocystophyta*. Sie enthält photosynthetisierende Plastiden (Cyanellen), welche von einer zwischen den Envelopemembranen liegenden Peptidoglykanschicht, ähnlich wie bei den Cyanobakterien, umgeben sind. Dieses Merkmal lässt erkennen, dass diese Plastiden eine Modell für eine frühe Stufe in der Evolution der Organellen darstellen. In jeder Cyanelle befindet sich ein elektronen-dichter Zentralkörper, der den Großteil der vorhandenen Ribulose-1,5-bisphosphat- Carboxylase/Oxygenase (Rubisco) beinhaltet. Vermutlich ist der Zentralkörper von *C. paradoxa* die wesentliche Struktur für den CO_2-Konzentrierungs-Mechanismus (CCM) und entspricht daher den cyanobakteriellen Carboxysomen oder den Pyrenoiden in Algen. Ein mögliches Szenario für die Evolution des CCM postuliert, dass Cyanobakterien schon vor dem primären endosymbiotischen Ereignis Carboxysomen entwickelt haben und nach der Endosymbiose der cyanobakterielle CCM umgestaltet wurde zu einem Algen-CCM. Die ungewöhnlichen Plastiden von *C. paradoxa* zeichnen sich neben ihrer Peptidoglykanschicht noch durch mehrere cyanobakterielle Eigenschaften aus. Daher unsere Annahme, dass die Cyanellen der *Glaucocystophyta* einen

cyanobakteriellen Zentralkörper besitzen, um sich mittels eines cyanobakteriellen CCM durch veränderte Genexpression an niedrig-CO_2 Zustände anpassen zu können („Acclimation“).

Im Rahmen dieser Arbeit sollten Zentralkörper und CCM von *C. paradoxa* erforscht werden und beteiligte Proteine (d.h. homologe Proteine zu Carboxysom- und/oder Pyrenoid- Komponenten) identifiziert werden. Eine Isolierungsmethode für Zentralkörper wurde entwickelt, um Massenspektrometrie an im Gel verdauten Banden aus SDS-PAGE zu ermöglichen. Zwei cDNA Libraries wurden gemacht, aus Zellen unter unterschiedlichen Wachstumsbedingungen, nämlich hoch- und niedrig-CO_2. An der University of Illinois wurde mittels High-Throughput Sequenzierung von 4992 Klonen eine Sammlung von Expressed Sequence Tags (ESTs) geschaffen (durch S. Burey). Unter den häufig vorkommenden Transkripten befanden sich auch Gene, die möglicherweise eine Funktion im CCM haben. Mittels Microarray-Technologie konnte das mengenmäßige Verhältnis von Transkripten zu unterschiedlichen Zeitpunkten nach dem Übergang von hoch- (5%) zu niedrig- (0.037%) CO_2 erfasst werden. Unter den Genen, die bei hoch- bzw. niedrig- CO_2 Bedingungen unterschiedlich reguliert sind, wurden auch einige für CCM-Kandidaten identifiziert, wie Rubisco-Activase (der Einbau des markierten Proteins in isolierte Zentralkörper konnte durch import in isolierte cyanellen und ausschließende Fraktionierung nachgewiesen werden) und ein putativer Bicarbonat-Transporter, bis dato der einzige in Frage kommende Ci-Transporter (Ci, anorganischer Kohlenstoff). Ein weiterer Forschungsschwerpunkt war die Charakterisierung der Gene für Carbo-Anhydrase (CA): eine putative cytosolische CA und zwei Isoformen einer putativen mitochondrialen CA wurden beschrieben. Die dargelegten Befunde unterstützen die Existenz eines CCM in *C. paradoxa*, jedoch bedarf es weiterer Ergebnisse um unsere Hypothese von „eukaryotischen Carboxysomen“ bestätigen oder verwerfen zu können.

Ein wichtiges Teilprojekt war die Etablierung der ELMI, auch in Hinblick auf künftige Immuno-EM. Leichte Unterschiede in Größe und Morphologie der Carboxysomen wurden beobachtet. Die Carboxysomenpräparation wurde ebenfalls untersucht. Hinweis auf quasikristalline Arrays aus Rubisco-Molekülen (L8S8), ähnlich wie in Cyanobakterien, wurden erhalten.

Die zahlreichen Aufnahmen erbrachten den eindeutiger Nachweis daß das Cyanellen-Mikrokompartment während der Teilung bestehen bleibt und durch das Septum in zwei Hälften getrennt wird. Weiters kann jetzt mit Sicherheit behauptet werden, daß nie Thylakoidmembranen durch den Zentralkörper gehen.

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1. INTRODUCTION

Cyanelles (muroplasts) are the peptidoglycan-surrounded plastids of glaucocystophyte algae. This prokaryotic wall, a clear indication of cyanelle origin from endosymbiotic cyanobacteria, is found in no other plastid type. Another cyanobacterial feature of cyanelles shared with some primitive plastids, such as those from red algae or cryptomonads, is the presence of light-harvesting phycobiliproteins encoded by clustered plastid genes. There is one large electron-dense central body in *C. paradoxa* cyanelles (Figure 1). In the cyanelles of *Glaucocystis nostochinearum* the electron-dense body localizes to polar regions. The function of this body likely is equivalent to that of the cyanobacterial carboxysome or of the pyrenoid of algae, both with an important role in the CCM. Direct analysis of the operation of a CCM and of the effect of ambient CO_2 concentration on its induction in *C. paradoxa* was missing at the onset of this doctoral thesis. In fact there was the opinion that *C. paradoxa* rather does not possess a CCM (A. Goyal, personal communication). Meanwhile, microarray data on CO_2–responsive genes were established and gas exchange measurements were performed. Mass spectrometric measurements of CO_2 uptake and external CA activity gave five-fold and three-fold increases, respectively, upon shift to low CO_2. (D. Sültemeyer, personal communication).

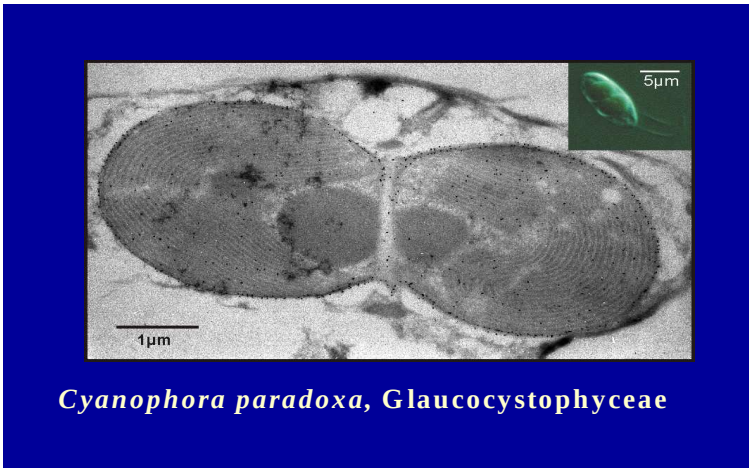


Fig.1. Cryosection of a chemically fixed *C. paradoxa* showing a dividing cyanelle. The septum cleaves the ”carboxysome”. Primary antibodies are directed against peptidoglycan from *E. coli*

The cyanelles of *Cyanophora paradoxa* are the best characterized among the glaucocystophytes: immunoelectron microscopy with anti-Rubisco antibodies identified the central bodies as the major if not exclusive location of the key enzyme for CO_2 fixation (Mangeney and Gibbs, 1987). It is not clear at present if the *C. paradoxa* "carboxysomes" are confined by a (proteinaceous) non-unit membrane but the polyhedral central bodies of cyanelles from the glaucocystophytes *Gloeochaete wittrockiana* and *Cyanoptyche gloeocystis* are enclosed by such a shell (Kies, 1992). It is likely that the polyhedral nature, observed infrequently, and the surrounding shell of the *C. paradoxa* central bodies are camouflaged by the condensed cyanelle DNA covering them and the closely adjacent concentric thylakoids. During cyanelle division, the newly formed septum constricts and finally dissects the cyanelle nucleoid and the photosynthetic apparatus, i.e. separates the DNA, thylakoids and putative carboxysome into two halves bound for the daughter cyanelles (Steiner and Löffelhardt, 2002; Figure 1). An interesting hypothesis (Raven, 2003) recently linked the two prominent characteristics of cyanelles together: the presumably carboxysomal type of the CCM involves accumulation of HCO_3^- in the cyanelles to such an extent that the cytosolic osmolarity of the fresh water organism would not be able to counterbalance it. The stabilizing peptidoglycan layer prevents the cyanelles from bursting. Thus, in the course of plastid evolution, both features, peptidoglycan and carboxysomes were abandoned or changed into pyrenoids, respectively.

The cyanelle central bodies could also be pyrenoids that perform functions similar to carboxysomes in lower phototrophic eukaryotes. There are a number of characteristics that allow a distinction between carboxysomes and pyrenoids for example, the intraorganellar distribution of carbonic anhydrase (CA).

1. 1. Cyanelle (muroplast) wall

The cyanelle wall is lysozyme-sensitive and has been shown to consist of peptidoglycan in *Cyanophora paradoxa* (Schenk, 1970; Aitken and Stanier, 1979) and *Glaucocystis nostochinearum* (Scott et al., 1984). The fine structure of this wall was completely resolved (Pfanzagl et al., 1996). It exceeds that from *E. coli* with

respect to thickness (7 nm) and crosslinkage and is distinguished by an unusual amidation of the D-isoglutamyl moiety by N-acetylputrescine (Pfanzagl et al., 1996). Cyanelles as well as their protoplastid ancestor had to face the problem of peptidoglycan wall permeability. Based on calculations of the mesh size of the peptidoglycan network in *E. coli*, proteins larger than 50 kDa should have difficulties to diffuse. The modification of the cyanelle peptidoglycan with N-acetylputrescine might serve the purpose to render the wall permeable for the numerous proteins that have to be imported across the cyanelle envelope (Löffelhardt, 1998). The biosynthetic pathway of cyanelle murein appears to be analogous to *E. coli* with respect to intermediates, the participating enzymes and their compartmentation. Penicillin-binding proteins (PBPs) possess transglycosylase and/or transpeptidase activity and perform the last steps of bacterial peptidoglycan biosynthesis by introducing new monomeric building blocks into the growing carbohydrate chain and crosslinking the peptide side chains. Seven PBPs in the size range from 110 to 35 kDa have been identified in the cyanelle envelope by labeling with a radioactive derivative of ampicillin (Berenguer et al., 1987).

In addition, data about the membrane-bound, intermediate steps of muroplast peptidoglycan synthesis were obtained. Cyanelle envelope preparations incorporated label from ^{14}Cputrescine or from ^{14}C-UDP-N-acetylglucosamine into modified lipid II (Pfanzagl and Löffelhardt, 1999).

1. 2. Cyanelle genome

From a comparative analysis, a standard set of approximately 80 genes emerged that are shared by almost all plastid types regardless of their pigment composition, their more primitive or more derived characteristics, or the fact that some (Euglena gracilis, Odontella sinensis) arose from a superimposed secondary endosymbiotic event. Among these common plastid genes are those for more than 30 rRNAs and tRNAs, 20 ribosomal proteins, 27 thylakoid proteins, 4 subunits of the RNA polymerase, and a few selected stromal proteins such as the LSU of Rubisco and the ClpP protease subunit. This minimal set is most closely displayed by *E. gracilis*

(143 kb, 97 genes), where 40% of the plastid genome is occupied by introns. The protoplastid, on the other hand, presumably contained genetic information higher by a factor of 2.5 - 3 than the standard set. This means that the major part (90%) of the gene transfer from the cyanobacterial endocytobiont (a phototrophic prokaryote comprising about 2500 genes) to the host cell nucleus had already taken place. A combination of the features of the extant cyanelles of *C. paradoxa* (136 kb, 193 genes) and the rhodoplasts of *Porphyra purpurea* would most closely describe the protoplastid, the large rhodoplast genome (191kb) harboring the highest number of genes (250) thus far reported for a plastid and the peptidoglycan wall of the cyanelles.

The surplus (as compared to higher plant chloroplasts) of more than 50 genes found on the cyanelle genome is most pronounced with respect to ribosomal proteins (36 vs. 21 in higher plants). This is paralleled and even surpassed in the *P. purpurea* and *O. sinensis* plastid genomes. To the surprise of the investigators the more than 20 genes for enzymes involved in murein biosynthesis reside on the nuclear genome of *C. paradoxa*. Only ftsW, a homolog to an *E. coli* cell division protein, was found on the cyanelle genome. Since cell division proteins of prokaryotic origin were also found in *Arabidopsis* and other algae one would assume that some "bacterial" features in organelle division still exist although the peptidoglycan wall has been abandoned in all plastid types with the exception of the cyanelles.

Interestingly, another gene directly involved in synthesis of peptidoglycan during septum formation, has been detected on the plastid genome of *Nephroselmis olivacea* (Prasinophyceae; Turmel et al., 1999). At the moment it is not clear if this alga has also retained a rudimentary layer of peptidoglycan or if the enzyme has been adapted for an unknown role in the plastid biosynthetic machinery.

1. 3. Cyanobacteria and carboxysomes

Carboxysomes are ubiquitous inclusions of the vegetative cells and akinetes (spores) of cyanobacteria. 3 to 12 of these polyhedral bodies are localized in the nucleoplasmic region of the cell. Nitrogen-fixing heterocysts of filamentous

cyanobacteria do not possess polyhedral bodies. Carboxysomes are 100-200 nm in diameter and are surrounded by a “shell”, a proteinaceous monolayer 3-4 nm thick (Shively, 1988). Carboxysomes were originally described in the autotrophic sulfur bacterium *Thiobacillus neapolitanus* (Shively, 1973). These structures were further isolated from *Anabaena cylindrica*, *Chlorogloeopsis fritschii*, *Thiobacillus thyasiris, Nitrosomas* spp., several *Nitrobacter* spp., *Prochloron* and *Prochlorothrix.*
Immunogold-labelling experiments provided evidence that carboxysomes contain the bulk of Rubisco (McKay et al., 1993; So et al., 2002b) making them the major sites of CO_2 fixation in cyanobacteria (Kaplan et al., 1998; Price et al., 1998). Besides carboxysomal particulate Rubisco, cyanobacteria possess a soluble form of the enzyme. The amounts present in either form are dependent on growth conditions and culture age (Shively, 1988).

1. 4. CCM in cyanobacteria

In cyanobacteria, the CCM allows for growth at low atmospheric CO_2 levels notwithstanding the relatively low affinity and selectivity of the cyanobacterial Rubisco for its substrate (Kaplan and Reinhold, 1999; Badger et al., 2006). The CCM comprises two phases: i) Uptake of inorganic carbon (C_i) from the environment and its accumulation within the cell, and ii) its utilization for photosynthesis through the carboxysomal microcompartment where the concentration of CO_2 around Rubisco is raised to near substrate saturation. Cyanobacteria are now classified according to the type of Rubisco they carry: alpha-cyanobacteria contain form IA and alpha-carboxysomes, beta-cyanobacteria form IB and beta-carboxysomes (Badger et al., 2006). The cyanobacterial plasma membrane may contain at least three transporters for HCO_3^-. First, an inducible (at very low C_i concentrations) ABC-type high-affinity bicarbonate transporter, BCT1, is encoded by the genes *cmpABCD* in *Synechococcus* sp. PCC 7942 (Omata et al., 1999). CmpA appears to be identical to the long known 42 kDa plasma membrane protein synthesized under C_i-limited conditions. This protein was shown to function as the membrane-anchored substrate-binding protein of the transporter (Maeda et al., 2000). The second is an inducible, Na^+-dependent, high-affinity bicarbonate transporter, SbtA (Shibata et al.,

2002), and a third one is represented by the recently identified low-affinity bicarbonate transporter, BicA (Price et al., 2004).

Passive diffusion of CO_2 occurs across the plasma membrane as suggested by the inhibitory action of a water channel blocker (Tchernov et al., 2001). In addition, two energized CO_2 uptake systems have been shown to operate on the cytosolic side of the thylakoid membranes in β-cyanobacteria: a constitutive protein complex, mediated by NdhF4, NdhD4, and ChpX, and an inducible (by low CO_2 concentrations), high-affinity complex consisting of NdhF3, NdhD3, and ChpY (Shibata et al., 2002; Maeda et al., 2002). Using light energy and mediated by cyclic electron transport around photosystem I, CO_2 is converted into bicarbonate which accumulates in the cytoplasm. Electrons from NAD(P)H are thought to generate a catalytic Zn-OH moiety as it is found in the active site of CAs (Kaplan and Reinhold, 1999, Maeda et al., 2002). The bicarbonate then diffuses into the carboxysomes where it is converted into CO_2 by carbonic anhydrases that are confined to these bodies and efficient fixation occurs. In this scenario, the carboxylase activity of Rubisco is maximized whereas the oxygenase activity is largely eliminated (Kaplan and Reinhold, 1999; Badger et al., 2006).

The carboxysomes of chemoautotrophic bacteria, especially *Halothiobacillus neapolitanus,* were the first to be studied. They contain most of the cellular Rubisco surrounded by a proteinaceous shell. The *cso* operon encodes the shell proteins (Shively et al., 1998). In the genomes of the cyanobacteria *Synechococcus sp.* PCC 7942, *Synechococcus* sp. PCC 7002, and *Synechocystis* sp. PCC 6803 a set of clustered *ccm* genes with sequence similarity to some of the *cso* genes has been detected (Ludwig et al., 2000). Recent structural studies allow us to imagine how such a polyhedral shell could be formed: carboxysomes are proposed to function not simply as a containment for Rubisco and CA but they appear to act as a micro-compartment with selective and controlled permeability for metabolites, perhaps including CO_2 and O_2 (Kerfeld et al., 2005). The 70 kDa CcmM protein was found to be essential for carboxysome assembly (Ludwig et al., 2000). This component of the carboxysomal shell shows sequence similarity to (archaebacterial) gamma-type CAs in its N-terminal region and similarity to Rubisco activase and, less pronounced, to Rubisco SSU in three or four (depending on the species) repetitive domains of the

C-terminal region. The *icfA* (*ccaA*) gene encoding carboxysomal beta-type (*E. coli*, higher plant chloroplasts) CA was characterized using high-CO_2-requiring mutants of *Synechococcus* sp. PCC 7942 (Badger and Price, 1994). The 31-kDa protein has a carboxy-terminal extension compared to the 24 kDa higher plant chloroplast enzyme. This extra domain was found to be responsible for aggregation of CA within the carboxysome (So et al., 2002). This is in accordance with the model that the dense packaging of Rubisco and CA inside the carboxysomes ensures a high concentration of CO_2 at the site of fixation which grants activation of the enzyme and compensates for its relatively low affinity for CO_2 and reduces loss of CO_2 by diffusion (Kaplan and Reinhold, 1999; Badger et al., 2006). In *Thiobacillus*, *Prochlorococcus*, and marine *Synechococcus* species, all containing alpha-carboxysomes, no gene encoding a typical carboxysomal CA has been found (Badger et al., 2006). Instead, one of the *Thiobacillus* shell proteins, CsoS3, was shown to possess CA activity ascribed to the new epsilon-class (So et al., 2004). Very recently, the structure determination of this protein, now renamed to CsoSCA, justified its positioning in a distinct beta-subclass (Sawaya et al., 2006). There is no CA *sensu stricto* in the cytoplasm of cyanobacteria. However, for an efficient operation of the CCM, a CA-like activity at the thylakoid membrane is postulated as a CO_2-scavenger. CO_2 leaking out from the carboxysomes as well as CO_2 entering from the medium will be converted into HCO_3^- against the thermodynamic potential (thus maintaining an inward diffusion gradient along which CO_2 enters passively) at the expense of membrane energization (Kaplan and Reinhold, 1999).

Rubisco activase plays an important role in photosynthetic carbon fixation of higher plants (Portis, 2003), but a corresponding gene has not been identified in the genome of *Synechocystis* sp. PCC 6803. Obviously, this enzyme is not absolutely necessary for a carboxysome-containing organism. However, heterocystous filamentous cyanobacteria (*Anabaena, Nostoc*) possess carboxysomes and Rubisco activase.

This chaperone that removes prematurely bound substrate as well as substrate analogs and inhibitors from the active center of Rubisco and also ensures full carbamylation of the enzyme (Portis, 2003) is indispensable in all phototrophic eukaryotes. In algae, Rubisco activase localizes to the pyrenoid (McKay et al. 1991)

and also plays a role in CCM (Pollock et al. 2003). The carboxysomal CCM provides an optimal environment for cyanobacterial Rubisco which is fully activated and also less susceptible to inhibition by the misfire product, xylulose-1,5-bisphosphate (Pearce, 2006), whereas the “natural” inhibitor in plants, carboxyarabinitol-1-phosphate is not formed at all in cyanobacteria. However, *rca*-like genes are found in the genomes of filamentous, nitrogen-fixing cyanobacteria (Li et al. 1999). Interestingly, a truncated form is contained in the *gloeobacter* genome. Now, one might speculate that filamentous cyanobacteria certainly are better candidates for the plastid ancestor than *Synechocystis* 6803. Anyway, there is convincing sequence identity (>50%) over a region of 300 AA between cyanobacterial and plant *rca* genes. Plant proteins contain a N-terminal extension missing in the cyanobacterial counterparts. On the other hand, only cyanobacterial proteins contain a C-terminal extension with distinct sequence similarity to the repeats of the shell protein CcmM (Portis, 2003). The localization of the cyanobacterial protein is unknown and a mechanism/function different from plant Rubisco activase is considered (Portis 2003, Pearce 2006).

In a genome-wide microarray analysis conducted with *Synechocystis sp.* PCC 6803, genes for the photosystems I and II, phycobilisome components, Calvin cycle enzymes, and proteins of the translation apparatus were shown to be down-regulated by a shift to low CO_2, whereas genes related to the CCM were up-regulated (Wang, Postier and Burnap, 2004). Inclusion of *ndhR* knockout mutants into this study revealed the important regulatory role of NdhR (now CcmR) in acclimation to low CO_2.

1. 5. CCM in green algae

Among eukaryotic microorganisms, the CCM is best investigated in *Chlamydomonas reinhardtii* (Giordano et al., 2005). Even there, much less is known about cellular import of Ci than in cyanobacteria. Rh1, a Rhesus protein homolog from *C. reinhardtii* was recently proposed as a CO_2 channel in the chloroplast inner envelope membrane (Soupene et al., 2004). Expression of Rh1 is down-regulated

by low CO_2, probably to avoid CO_2 efflux. Multiple CAs are a characteristic of eukaryotic CCMs (Mitra et al., 2004). Protein levels of all CAs increase upon a shift from 5% CO_2 to ambient CO_2 (Badger and Price, 1994; Kaplan and Reinhold, 1999). Periplasmic beta-type (animal) CAs and their genes containing amino-terminal signal peptides were characterized for *C. reinhardtii* (Cah1 and Cah2; Fujiwara et al., 1990). Two closely related genes (*Ca1* and *Ca2*) encoding mitochondrial CAs of *C. reinhardtii* were also identified (Villand et al., 1997). A recent model (Raven, 2001) suggested that the mitochondrial CA activity may reduce leak of respiratory CO_2 from the cells. Cytosolic forms of CA are less well defined. Their function would be necessary assuming the presence of a Ci-transporter in the chloroplast envelope with a distinct preference for either CO_2 or HCO_3^- (Badger and Price, 1994). A low-CO_2-inducible chloroplast envelope protein, (LIP-36) could have a role in CO_2 or HCO_3^- transfer into the chloroplast (Chen et al., 1997). In *C. reinhardtii*, the expression ratio of a novel gene, *LciA*, was particularly high among low-CO_2 inducible genes (Miura et al., 2004). *LciA* was predicted to encode a polypeptide with significant sequence similarity to the formate transporter, FdhC, from *Methanobacterium thermoformicicum* and identical to *Nar1;2* (Rexach et al., 2000), one of five *Nar1*-related genes. Nar1;1 is a chloroplastic nitrite transporter in *C. reinhardtii.*

A putative chloroplast transit peptide suggests that LciA localizes to thylakoids or chloroplast envelope membranes in *Chlamydomonas*. In addition, it was shown that *LciA* expression is concomitant with CCM induction, and that its function is not linked to nitrite transport (Miura et al., 2004). In cyanobacteria, a well-characterized bicarbonate transporter showed sequence similarity to a nitrite transporter (Omata et al. 1999). This led Miura et al. (2004) to conclude that LciA functions as a transporter for bicarbonate into chloroplasts.

An important issue is the sub-organellar localization of algal chloroplast CA. Pyrenoids are electron-dense structures that are found in the plastids of *C. reinhardtii* and other algae possessing a CCM, with the exception of some *Chloromonas* species (Morita et al., 1998), but the equivalent structure is absent in algae missing a CCM. It is accepted that the pyrenoids are functionally equivalent to carboxysomes (Kaplan and Reinhold, 1999; Giordano et al., 2005) but they lack the

quasi-crystalline appearance and the surrounding shell of the latter. In many algal species, unstacked thylakoid membranes traverse the pyrenoids. The pyrenoid is the sole location of Rubisco in *Chlorella pyrenoidosa*, irrespective of the light regime (McKay et al., 1991). The *C. reinhardtii* pyrenoid was shown to contain significant amounts of Rubisco that varied depending on growth conditions, reaching up to 90% of the total cellular Rubisco content when the cells were grown at ambient CO_2. In this condition a starch layer with pores surrounding the pyrenoid becomes apparent. The pyrenoid also harbors Rubisco activase (McKay et al., 1991; Villarejo et al., 1998). Insertion of a bleomycin resistance cassette into the Rca gene of *C. reinhardtii* led to a high-CO_2-requiring mutant (Pollock et al., 2003). Algal Rubisco likely is more prone to inhibition by substrate analogs than the cyanobacterial enzyme. Furthermore, due to the less pronounced enrichment of Ci in a pyrenoidal CCM, complete activation of Rubisco might not be achieved in the absence of its chaperone. In contrast to beta-carboxysomes, the immediate presence of CA in pyrenoids is questionable (Badger and Price, 1994). The gene for a precursor to a chloroplastic CA (Cah3) from *C. reinhardtii* was found by complementation of high-CO_2-requiring mutants, indicating the importance of CA for photosynthesis at ambient levels of CO_2 (Funke et al., 1997). Cah3 specifies an alpha-type CA that localizes to the thylakoid lumen. The bipartite pre-sequence consists of a transit peptide and a thylakoid transfer peptide with a twin arginine motif, which points towards a Delta pH-dependent pathway for thylakoid import. Another mutant (*cia3*) with amino acid exchanges in the vicinity of this motif was incompetent with respect to lumenal targeting of this CA (Karlsson et al., 1998) and also required high CO_2 for growth. Such sub-compartmentation of chloroplast CAs is crucial for a model that postulates passive transport of HCO_3^- through an anion channel into the thylakoid lumen, concomitant with light-driven H^+ transport. Due to the low pH in the lumen, the equilibrium is shifted towards CO_2 and its rapid attainment is catalyzed by CA. The CO_2 formed will diffuse across the thylakoid membrane (thereby facilitating further import of HCO_3^-) and will generate optimal conditions for CO_2 fixation by pyrenoidal Rubisco (Moroney and Somanchi, 1999). Experimental proof for such a role of Cah3 has been obtained (Hanson et al., 2003). Furthermore, an enrichment of Cah3 in thylakoids traversing the pyrenoid could be demonstrated (Mitra et al.,

2005). Recently, evidence for Cah6, a stromal CA (as found in higher plant chloroplasts) in pyrenoid-containing organisms was presented for *C. reinhardtii* (Mitra et al. 2004). Cah6 that localizes to the stroma at the periphery of the pyrenoid might have dual functions: direct supply of CO_2 to Rubisco and recapturing of CO_2 escaped from the starch-covered pyrenoid (Mitra et al. 2005). To render the situation in *Chlamydomonas* even more complex, an additional CA targeted to the chloroplast stroma by a signal sequence was reported (Villarejo et al., 2005).

A low-CO_2-inducible CA was found associated with PSI-containing thylakoid membranes traversing the pyrenoid in *Chlorella vulgaris* (Villarejo et al., 1998). Very limited data exist on CAs of algae other than chlorophytes. Recently, a plastid–targeted CA was reported to localize to particles observed at the stromal side of girdle band thylakoids in the diatom, *Phaeodactylum tricornutum* (Tanaka et al., 2005). These aggregates are different from the more centrally located pyrenoids. There is no evidence for an intra-thylakoidal localization of this CA, since a second signal sequence necessary for thylakoid translocation is missing from the bipartite pre-sequence typical for complex plastids. An (essential) function of this enzyme in C_i import into *P. tricornutum* plastids has been discussed (Tanaka et al., 2005), assuming an analogon to the postulated thylakoid–bound CA-like complex in cyanobacteria (Kaplan and Reinhold, 1999; Badger and Price, 2003).

Complementation of high-CO_2-requiring *Chlamydomonas* mutants unable to up-regulate the expression of low-CO_2-induced genes identified a gene, *Ccm1* (*Cia5*), involved in the acclimation to low CO_2 conditions. The Ccm1/Cia5 protein probably undergoes post-translational modification (phosphorylation) at the C-terminus in response to exposure to low CO_2 and activates transcription of several low CO_2-induced genes (Fukuzawa et al., 2001). Macroarray (Miura et al., 2004) and microarray (Wang et al., 2005) analyses confirmed the role of Ccm1/Cia5 as a master regulator in the expression of CCM-related genes. Very recently, the high CO_2-requiring *pmp1* mutation and the allelic *ad1* mutation were assigned to the *LciB* gene (Wang and Spalding, 2006). In the *C. reinhardtii* genome a family of three other genes, *LciC, LciD, and LciE* was found, with high sequence similarity to *LciB*. All these genes are CO_2-responsive (Wang and Spalding, 2006; Miura et al., 2004) and the gene products should be soluble proteins, likely targeted to the chloroplast.

Physiological measurements led Wang and Spalding to propose for LciB the role of a functional component somehow involved in Ci transport rather than that of a regulator.

1.6. Aims of the project

The aim of this project is to study the nature of the assumed CCM operating in the cyanelles of *C. paradoxa*. An isolation procedure for central bodies should be developed to identify components other than Rubisco via mass spectrometry. Two cDNA libraries, for conditions of low- and high-CO_2, should be established. High-throughput sequencing without subtraction should generate a collection of expressed sequence tags (ESTs). It was expected that genes of interest, i.e. involved in the CCM, would be among the abundant transcripts. While the respective types of carbonic anhydrase identified would allude towards either an pyrenoidal or carboxysomal kind of CCM, the presence or absence of other genes would also be conclusive in this regard. The following genes, for instance, which had not yet been identified for *C. paradoxa* at project commencement, would be highly indicative: carboxysomal shell proteins, Ci-transporter components, NADH dehydrogenase subunits (essential for cyanobacterial type CO_2 uptake) and CCM1 (bearing an important role in transcription regulation of low CO_2-dependent genes in green algae).

We also have to face the scenario that the cyanelle micro-compartments in glaucocystophytes are “carboxysomes” *en route* to their final conversion into pyrenoids that has already taken place in all other algae: number and size of the glaucocystophyte central bodies are more in line with pyrenoids. They show a polyhedral shape confined by a distinct “shell” in *Cyanoptyche* gloeocystis and *Gloechaete wittrockiana*, but not in *C. paradoxa* (Kies et al, 1992).

In view of these dual properties, we proposed to generate additional biochemical and molecular data that will allow us to definitely support or refute the hypothesis of "eukaryotic carboxysomes" or to establish a hitherto undescribed link between the two micro-compartment types.

1. Electron microscopy of high *vs.* low CO_2 grown cells should show a potential increase in size of the putative carboxysome. In prokaryotes which contain multiple carboxysomes, their numbers increase upon CO_2-stress (Reinhold and Kaplan, 1999). Isolated carboxysomes will be checked with respect to intactness, polyhedral appearance and recognizability of the ”shell” which might be camouflaged by surrounding cyanelle DNA and thylakoid membranes. Here, cryofixation should be superior, as chemical fixation often leads to the disappearence of the shell (Kaneko et al., 2006). Heterologous antisera directed against CA, rubisco activase, and shell proteins in combination with gold-labelled secondary antibodies should support their co-localization with Rubisco.

2. Central body isolation can be seen as a compromise between purity and intactness. Methods suitable for the (much smaller) carboxysomes of cyanobacteria were not directly applicable. We will continue with the Percoll step gradient/detergent treatment procedure, which we developed (Fathinejad et al., manuscript in preparation), and, eventually, also employ a new method adapted from pyrenoid purification protocols (Rodríguez-Buey et al., 2005). Peptide pattern and sequence information for components of isolated central bodies will be obtained after in-gel protease digestion of protein bands via MALDI-TOF- and nano-electrospray tandem mass spectrometry, respectively (collaboration with Günter Allmaier and Martina Marchetti, Technical University of Vienna). The protein components other than Rubisco of the cyanelle central bodies will be determined. keeping in mind the problems and pitfalls encountered with cyanobacterial carboxysomes (Long et al., 2005).

The expected increase in parallel in *Cyanophora* EST sequence information will aid in identification.

3. The well established *in vitro* import system with isolated cyanelles and the micro-fractionation techniques available will allow to test the assembly of labeled putative central body components despite the preponderance of Rubisco.

2. MATERIALS AND METHODS

2.1. Culture of Cyanophora paradoxa

Cyanophora paradoxa (Kies-strain, 1555; Breiteneder et al., 1988) was grown in mineral medium without an organic carbon source.

100-fold concentrated stock solutions, required for mineral medium, contained per liter:

Solution 1:	1.71 g	$K_2HPO_4.3H_2O$
	10 g	KNO_3
	10 g	NaH_2PO_4
Solution 2:	0.99 g	$MgSO_4.7H_2O$
	0.74 g	$CaCl_2.2H_20$
	0.20 g	$MnCl_2.4H_20$
Solution 3:	28 mg	$CoSO_4.7H_2O$
	140 mg	$ZnSO_4.7H_2O$
	2.4 mg	$Na_2MoO_4.2H_2O$
	2.5 mg	$CuSO_4.5H_2O$
Solution 4:	62 mg	H_3BO_3
Solution 5:	700 mg	$FeSO_4.7H_2O$
	730 mg	EDTA
Solution 6:	$2x10^{-4}$ M	Vitamin B_{12}

All medium storage containers and culture flasks were sterilized by autoclaving prior to use.

For the preparation of minimal medium, stock solutions 1- 4 were combined in a flask and distilled water used for adjustment to the total volume. After autoclaving, solutions 5 and 6 were added to the cold medium through a syringe equipped with a sterile 0.2 µm membrane filter.

Mass cultures of C. paradoxa were grown in 1.3 l cylindrical bubble-flasks. 100 to 300 ml of stock culture was added to each bubble-flask and filled up with the minimal medium. Flasks were closed with sterilized air-permeable sponge stoppers. Cultures grew at 26℃ under a light intensity of 15 00 lux and were aerated with a filtered mixture of air and CO_2 (2-5%). When cultures reached a higher density, they could be supplemented with a few ml of nitrate-rich mineral medium SA (50 mM $Ca(NO_3)_2.4H_20$, 30 mM $MgSO_4.7H_20$, 300 mM KNO_3) for continued logarithmic growth.

The generation time is about 24 h. Mass cultures grow to late logarithmic or early stationary phase in 5-7 days and can be harvested by centrifugal separation with a Westfalia separator. Only cells attached to the conical inner wall of the separator were used. The non-axenic stock cultures were kept at room temperature without aeration and reinoculated into fresh medium once a week.

2.2. Isolation of intact cyanelles from Cyanophora paradoxa

Mass cultures were harvested and cells suspended in 200 ml precooled SIM medium on ice. The cells were broken in a Waring Blendor: 5x 1 min full speed with 1 min cooling in ice water in between. Inspection under the light microscope should reveal more than 90% of broken cells. Following the addition of another 200 ml precooled SIM medium, the cyanelle suspension was filtered through a two layered Miracloth^{TM} filter funnel and centrifuged at 3,000 rpm, 2 min, 4℃ in a Sorvall RC5C centrifuge (GSA rotor) with the brake off. The pellet was gently resuspended in 8 ml 1x SRM buffer on ice using a cut 1 ml tip. 4 ml of 40% Percoll cushion (in SRM buffer) was prepared in 15 ml Corex tubes and 4 ml of cyanelle suspension layered

on top. Centrifugation at 3,000 rpm, 2 min, 4℃ (Sorvall SS34 rotor) rendered blue-green intact cyanelle pellets which were washed twice in 30 ml Corex tubes as follows: resuspension with a cut tip in 2 ml 1x SRM buffer, addition of 1x SRM to 30 ml total volume and gentle mixing by inverting, then centrifugation at 2,400 rpm, 2 min, 4℃. Pellets were resuspended in 1.5 ml 1x SRM buffer, pooled and the chlorophyll concentration measured. For this purpose, 10 µl cyanelle suspension was mixed with 990 µl 80% acetone, vortexed and sonicated for 2 x 20 impulses (50% power). Centrifugation in a table centrifuge at full-speed for 3 min rendered a blue phycobilisome pellet and a green chlorophyll supernatant which could be spectrophotometrically measured in a 1 ml microcuvette.
Chlorophyll a + b (µg/ml) = 20.2 x A_{645} + 8.02 x A_{663}

Cyanelles were kept on ice in the dark until used.

SIM (Sucrose-Isolation-Medium):
0.35 M sucrose
25 mM Hepes
2 mM EDTA
pH adjusted to 7.6 with KOH

5 x SRM (Sorbitol-Resuspension-Medium):
250 mM Hepes
1.65 M Sorbitol
pH = 8.0

2.3. Isolation of central bodies

Cyanelles were isolated as described in chapter 2.2.
Centrifugation steps were performed with a HB-4 swing-out rotor. 10% and 40% Percoll contained 1x SRM and 1x Protease-inhibitor (Complete EDTA-free protease

inhibitor cocktail tablets, Roche, Switzerland). Where indicated, homogenizing was performed using a potter. Final concentrations are noted.
Isolated cyanelles were incubated with 300 µg/ml lysozyme for 30 min at RT (dark) resulting in peptidoglycan wall digestion and then homogenized. 30 µg/ml DNase I and 1 mM $MgCl_2$ were added (for digestion of cyanelle DNA), the mixture incubated at RT (dark) for 15 min and subsequently homogenized.
3 ml 40% Percoll in a 15 ml Corex tube was overlaid with 3 ml 10% Percoll. 1.5 ml digested cyanelles were layered on the Percoll gradient and centrifuged at 16,500g for 3-10 min at 4℃. The organelle components were separated in the following order: phycobilisomes (top), thylakoids (at boundary 40%/10%), starch (bottom). This last procedure was efficient in removing starch, which could be seen as a white pellet (sometimes containing thylakoid fragments).
Recovered thylakoids could be washed for quantitative removal of phycobilisomes.
In this case, they were washed three times with 1x SRM (3000 rpm, 3 min, 4℃).
Thylakoids were treated with 0.1% Triton X-100, vortexed for 30 sec and layered on a 40%/10% (v/v) Percoll gradient. By centrifugation at 3,000 rpm for 3-10 min at 4℃, part of central bodies separated from thylakoids.

Collected fractions were incubated at 95℃ for 3 min with 2x SDS-sample buffer (see chapter 2.10.1), centrifuged at 13,200 rpm for 2 min at RT and could be stored at –20℃. For loading an aliquot on SDS-PAGE gel, samples were denatured once more at 95℃ and centrifuged.
“Total cyanelle extract”, cyanelles from C. paradoxa which were previously denatured in 2x loading buffer at 95℃ for 5 min, was loaded on gel as a control. Prior to loading, the “total cyanelle extract” was incubated at 60℃ for 10 min.

For the detection of starch, the following iodine solution was used:

3 g KI + 7 g I in 100 ml 92% ethanol (Jodtinktur, Gerlach, 1977)

2.4. Import

2.4.1. Isolation of import competent cyanelles

Cyanophora cells suspended in 25 mmol.L^{-1} HEPES buffer, pH 7.6, 0.35 mol.L^{-1} sucrose, 2mmol.L^{-1} EDTA were brocken in a Waring Blendor: five times for 1 min at full speed with 1 min of cooling in ice water in between blendings (Steiner et al. 2003). Inspection under the light microscope revealed more than 90% broken cells. Cyanelles were pelleted at 1500g (2 min in a Sorvall centrifuge, GSA rotor, with the brake off). The pellet was carefully suspended in 8 ml of SRM buffer (50 mmol.L-1 sorbitol, pH 8.0) using a fine brush. Cyanelle suspension (4 ml) was layered on top of a cushion of 40% Percoll (in SRM buffer, 4 ml).

After centrifugation for 7.5 min in a Sorvall SS34 rotor at 2000g, class II cyanelles (i.e., those not competent for protein import) and residual mithochondria remained on top of the cushion whereas intact cyanelles were pelleted.

The blue- green pellet was gently dissolved in 1 ml of SRM buffer, diluted to 40 ml with SRM buffer, and pelleted at 1000g for 2 min. This washing procedure was repeated twice.

2.4.2. Cyanelle Import Assay

The radiolabelled precursor of Rubisco activase was synthesized by in vitro transcription and translation of the respective cDNA cloned into the pBAT vector as described.

A cyanelle suspension in SRM buffer, equivalent to 40 µg of chlorophyll, was incubated with the translation mixture for 7-25 min at 25℃ in a total volume of 150µl. Cyanelle were then isolated again by centrifugation for 2 min at 800g and eventually treated with thermolysin to remove precursor adjacent to the envelope.

Cyanelle import assay:

x µl cyanelles (30-50 µg chlorophyll)
7.5 µl 100 mM ATP
5 µl 250 mM methionine
15 µl tranlation mix
SRM add 150 µl

2.5. RNA isolation and purification

Unlike DNAses, RNAses do not need metal ion co-factors and can maintain activity even after autoclaving. The ubiquitous presence of RNAses together with the chemical instability of RNA necessites special precautions for RNA work.
General working behaviour included: wearing gloves (hands are a major source of RNAses), using sterile plasticware, baking certain equipment such as spatulas at 180-200℃ for at least 4 hours and using untouched weigh paper. Twice autoclaved ddH_20 was used for making solutions. Electrophoresis tanks for RNA analysis were cleaned with 1% SDS, rinsed with ddH_20, rinsed with absolute ethanol and finally rinsed with ddH_20 before use. For cleaning, pH electrodes were immersed for 30 sec in 70% ethanol, 5 min in 1 M NaOH, then rinsed with ddH_20. In some cases, as described below, ddH_20 was treated with diethyl pyrocarbonate (DEPC) (0.1%) which is a strong, though not absolute inhibitor of RNAses. After DEPC addition, the ddH_20 was shaken at 37℃ overnight and subsequently autoclaved (for DEPC hydrolysis, releasing CO_2 and EtOH as reaction by-products) for 15 min at 15 lb/sq. in. on liquid cycle.

2.5.1. Isolation of total RNA

Total RNA was extracted as quickly as possible after culture harvesting to avoid RNA degradation. DEPC-treated ddH_20 was not used for any of the solutions

required for RNA isolation as remaining traces of DEPC might inhibit reverse transcription, the essential step in the construction of cDNA libraries.

Total RNA was extracted using the Qiagen extraction protocol for plant tissue (Qiagen, Inc., Chatsworth, CA) and Qiagen-tips 500 for a “Maxi-prep”.

2.5 to 4 l mass culture was harvested, cells were washed with 400 ml ice-cold mineral medium (chapter 2.1) and centrifuged in a GSA rotor for 5 min at 2,000 rpm at 4℃. The wet weight of the cell pellet was recor ded. Per preparation and QIAGEN-tip used, a maximum of 1 g cells was used. All following steps were carried out on ice. Solutions were prepared according to RNA standards described above and centrifugation tubes were twice autoclaved. The cell pellet was homogenized in a potter in 9 ml of ice-cold solution R1 (4 M guanidine thiocyanate (GIT), 100 mM Tris-HCl, 25 mM $MgCl_2$, 25 mM EDTA, pH 7.5) and 9 µl solution R2 (ß-mercaptoethanol). The homogenate was transfered in a Corex tube, 720 µl solution R3 (25% Triton X-100) added, mixed well by inversion and incubated for 15 min on ice. 9 ml ice-cold solution R4 (3 M NaAc, pH 6.0) was added, mixed well by inversion and incubated for further 15 min on ice. The homogenate was centrifuged in a SS34 rotor at 15,000x g for 30 min at 4℃. The supernatant was decanted into a clean centrifuge tube and 0.8 vol isopropanol added for RNA precipitation for 5 min on ice. After centrifugation in a GS-3 rotor at 15,000x g for 30 min at 4℃, the supernatant could be carefully removed and the transparent pellet dissolved thoroughly in 16 ml ice-cold solution R5 (20 mM Tris-HCl, 1 mM EDTA, pH 8.0) using a pipette with a cut-off blue tip. The undissolved particles were removed by centrifugation in a SS34 rotor at 20,000x g for 15 min at 4℃ and the supernatant transferred to a clean Corex tube. 4 ml solution R6 (2 M NaCl, 250 mM MOPS, pH 7.0, RT) was added. In the following steps, the QIAGEN-tip was always allowed to empty by gravity flow. The tip was equilibrated with 10 ml buffer QAT (400 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7.0, RT) and the sample applied to the tip. The tip was washed twice with 30 ml of buffer QA (400 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, RT) and the RNA eluted with 20 ml freshly prepared buffer QRU (900 mM NaCl, 50 mM MOPS, 15% ethanol, 6 M urea, pH 7.0, RT. Urea should be added to the buffer just prior to use). RNA was precipitated with 1 vol isopropanol for 10 min on ice and centrifuged in a SS34 rotor at 15,000x g for

30 min at 4℃. The pellet was washed with 80% ethan ol. Partial resuspension of the pellet could be carried out in approx. 1 ml 80% EtOH for stable storage at –70℃. After air drying the remaining pellet for 30 min, it could be resuspended in approx. 500 µl ddH_20.

2.5.2. Concentration measurement and quality control of total RNA

Total RNA was measured spectrophotometrically for the range 200 to 300 nm to determine concentration and purity. Quality control was further performed by loading 10 µg of total RNA, which was previously denatured at 65℃ for 10 min, on an agarose gel (1% in 1x formaldehyde gel-running buffer, FGRB). 1x FGRB was used as the running buffer.

5x formaldehyde gel-running buffer:

0.1 M MOPS pH 7.0
40 mM sodium acetate
5 mM EDTA pH 8.0

Solutions of sodium acetate and EDTA were made up with DEPC-treated ddH_20 and the prepared buffer was sterilized by filtration through a 0.2-micron Millipore filter.

2.5.3. Preparation of poly $(A)^+$mRNA

PolyA^+ mRNA was isolated from total RNA (preparation: chapter 2.4.1) using the Qiagen Oligotex-dT kit (Qiagen, Germany).
The Oligotex Suspension used consists of polystyrene-latex particles to which $dC_{10}T_{30}$ oligonucleotides are covalently linked. PolyA^+ RNAs hybridize via their poly-A tail to the dT oligomers in high-salt conditions. By lowering the ionic strength, mRNA is subsequently released. The Oligotex mRNA Batch Protocol (Oligotex Handbook 05/2002) was used. The concentration of starting total RNA was measured spectrophotometrically and RNA up to 0.75 mg adjusted to 500 µl with RNAse-free water. Oligotex Suspension was heated to 37℃, mixed by vortexing

and then placed at RT. All centrifugation steps were performed in a microcentrifuge at 13,200 rpm at RT.

For total RNA amounts 0.25 to 0.50 mg in 500 µl, 500 µl buffer OBB (20 mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM EDTA, 0.2% SDS) and 30 µl Oligotex Suspension were added. For total RNA amounts 0.50 to 0.75 mg in 500 µl, 500 µl buffer OBB and 45 Oligotex Suspension were added. The sample was incubated for 3 min at 70℃ on a heating block (disrupting RNA secondary s tructure) and then placed at RT for 10 min (for hybridization between mRNA and particles). Oligotex-mRNA complexes were pelleted for 2 min and the supernatant carefully removed. Loss of Oligotex particles could be avoided if 50 µl supernatant was left in the tube. The pellet was resuspended two times in 1 ml buffer OW2 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) by vortexing, centrifuged for 2 min and the supernatant removed. Complete supernatant removal was required the second time. 20 µl elution buffer OEB (5 mM Tris-HCl, pH 7.5) preheated to 70℃ was quickly added, the particles resuspended by pipetting 3-4 times and centrifuged 2 min. The supernatant containing eluted polyA$^+$ RNA was transfered to a fresh tube and for maximum yield, 20 µl buffer OEB was taken for a second elution. The eluates were pooled.

The mRNA concentration was measured spectrophotometrically for the range 200 to 300 nm.

For concentrating isolated mRNA by precipitation, 1/10 vol 3M Na-acetate and 2.5 vol ice-cold EtOH were added to the pooled eluate, mixed, incubated at –20℃ for 1 h, then centrifuged at high speed for 30 min at 4℃. Dried pellets were resuspended in 10 µl or less RNAse free dd$H_2$0.

2.6. Construction of cDNA libraries

In principal, isolated polyA$^+$ RNA may be converted to dsDNA and cloned into a plasmid vector for the construction of a cDNA library. Further, upon transformation and plating of E. coli on selective medium, bacterial colonies each contain a different cloned transcript. Transcripts can be sequenced (expressed sequence tag (EST) generation) and analyzed.

2.6.1. Culture conditions

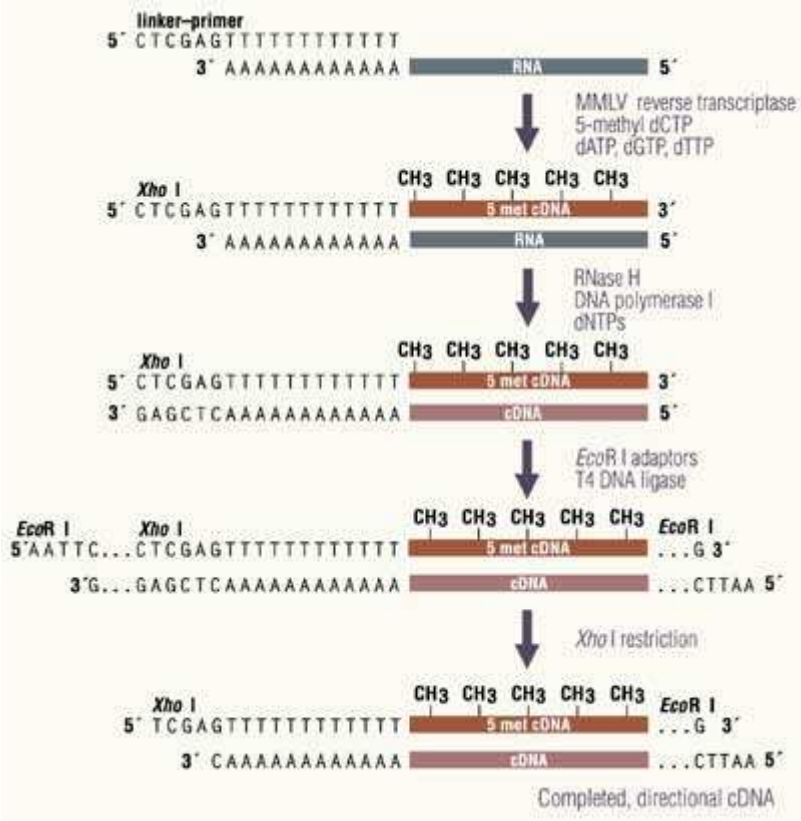
150 ml stock culture for each 1.3 l bubble flask was taken to start a mass culture (day 1). Cultures were grown at 26°C and aerated with air and 5% CO_2 from day 1 onwards. On day 2, light intensity was raised to 1500 lux. 5x 3 ml nitrate-rich mineral medium SA was added to each bubble flask over the first three days. On day 6, cultures generally reached an OD_{678} (chlorophyll peak) of 0.3 – 0.5 and the most dense cultures were harvested for timepoint 0 h (for the high-level CO_2 library). The additional CO_2 supply was cut immediately at timepoint 0 h, thus shifting parallel grown cultures to ambient CO_2 (0.04%). Individual cultures were harvested after 2, 12 and 24 h (for the low-level CO_2 library).

2.6.2. RNA isolation

Total RNA was extracted as described in chapter 2.4.1. Quality control was performed by loading 10 µg total RNA on gel. PolyA$^+$ RNA isolated (described in chapter 2.4.3) was on average only 0.5% of total RNA (while it was estimated that 1% of C. paradoxa total RNA is mRNA).

The cDNA libraries were constructed using the Stratagene cDNA Synthesis Kit (Stratagene, La Jolla, CA) using a modified protocol established by the group of Hans Bohnert. 3 µg of polyA$^+$ RNA was taken for construction of cDNA libraries, for the low-level CO_2 library 1 µg polyA$^+$ RNA each from timepoints 2, 12 and 24 h were pooled. A control was carried out in parallel with 2.5 µg Test polyA$^+$ RNA provided by the kit. Precipitation of nucleic acids was, when indicated, performed with 1/10 vol 3M Na-acetate and 2.5 vol ice-cold EtOH. The tube was subsequently centrifuged at 14,000 rpm for e.g. 30 min at 4°C. The pellet was washed with 500 µl 70% EtOH, centrifuged at 14,000 rpm for 5 min and air dried. Generally, following the addition of new components the tube was mixed gently and a quick spin-down was performed prior to the next incubation step.

Stratagene cDNA Synthesis Kit: schematic summary



2.6.3. First-strand synthesis

The polyA$^+$ RNA was brought to 18 µl with dd$H_2$0 and 1 µl linker-primer (1.4 µg/µl) was added. Incubation at 70℃ for 10 min denatured RNA secondary structure. 2.5 µl 10x first-strand buffer and 1.5 µl first-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP) were added for first-strand synthesis and incubated at 42℃ for 10 min. 2 µl StrataScript^{TM} reverse transcriptase (50 U/µl) was added and incubated at 42℃ for 90 min. The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, protecting it from digestion with certain restriction endonucleases such as Xho I.

2.6.4. Second-strand synthesis

The tube was placed on ice and the following components added: 10 µl 10x second-strand buffer, 3 µl second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP), 55.5 µl ddH_20, 1 µl RNase H (1.5 U/µl) and 5.5 µl DNA polymerase I (9 U/µl). After gently vortexing and spinning down, the reaction was incubated at 16℃ for 2.5 h and immediately placed on ice. RNase H nicks RNA bound to first-strand cDNA to produce fragments which serve as primers for DNA polymerase I.

2.6.5. Blunting

For blunting, 11.5 µl blunting dNTP mix (2.5 mM dATP, dGTP, dTTP, and dCTP) and 1 µl Pfu DNA polymerase (2.5 U/µl) were added, the mixture gently vortexed, spun down and incubated at 72℃ for 30 min. The mix ture was extracted once with an equal volume of phenol/chloroform/isoamylalcohol (PCI, 25:24:1, v/v) and once with chloroform. The aqueous layer was precipitated at –20℃ overnight, the tube centrifuged for 1 h and the washed pellet air-dried.

2.6.6. Adapter ligation

The pellet was dissolved in 2.5 µl ddH_20 and the following components added for adapter ligation: 4.5 µl Eco RI adapters (0.4 µg/µl), 1 µl 10x ligase buffer, 1 µl rATP (10 mM) and 1 µl T4 DNA ligase (4 U/µl). The ligation reaction was incubated at 4℃ for 4 days and subsequently, the ligase inactivated at 70℃ for 30 min. For the purpose of travelling, the mixture was precipitated and the pellet washed. The pellet covered with 70% EtOH could be kept at RT for several days.

2.6.7. Xho I digestion

The pellet was resuspended in 10 µl dd$H_2$0. The following components were added: 2 µl 10x ligase buffer, 1 µl T4 polynucleotide kinase (10 U/µl), 3 µl rATP (10 mM) and 4 µl dd$H_2$0. The tube was incubated at 37℃ for 30 min, then at 70℃ for 30 min (enzyme inactivation). 28 µl Xho I buffer supplement and 3 µl Xho I (25 U/µl) were added, incubated at 37℃ for 2 hours, another 3 µl Xho I added for further 3 h incubation. Endonuclease was inactivated by incubation at 65℃ for 10 min. One PCI and chloroform extraction each were performed with subsequent nucleic acid precipitation as described above.

2.6.8. cDNA size fractionation

Fragments larger than 500 bp were selected by cDNA size fractionation columns (Invitrogen, Carlsbad, CA) containing Sephacryl S-500 HR resin, eliminating residual adapters and shorter cDNAs. Columns were equilibrated according to instructions of the manufacturer. For quantification of cDNA eluate fractions, petri dishes were poured with agarose gel (0.8% in 1x TAE containing 1 µg/ml ethidium bromide) and dried at 37℃ for 45 min. DNA standards were prepar ed by diluting 500 µg/ml □DNA Bst EII digest (New England Biolabs, Beverly, MA) with dd$H_2$0 to 2, 1.5 and 1 ng/µl.
The cDNA pellet was dried at 37℃ for 10 min and di ssolved in 100 µl TEN buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 25 mM NaCl) on ice. The entire sample was applied onto the equilibrated column, the effluent collected in tube 1. 100 µl TEN buffer was applied and the effluent collected in tube 2. Beginning with the next 100 µl aliquot of TEN buffer, single drops (~ 35 µl) were collected into individual tubes. Using a yellow tip, the volume in each tube was measured and fractions collected until the cumulative volume 550 µl was reached (total of 9-10 tubes). It was expected that a higher percentage of small cDNA or adapters would be contained in fractions above this cut-off point.
1 µl of the prepared DNA standards and 1 µl of each collected fraction were pipetted onto a petri dish and the inversed plate photographed under UV light. By comparison, the fractions with the highest cDNA amounts were identified and pooled

for precipitation. Washed pellets were air dried for 10 min and resuspended in 10 µl ddH_20 at 4℃ for 1 h.

2.6.9. Vector preparation

4 µg pBluescript II SK (+) (Stratagene) (1 µg/µl) was digested with 20 U Eco RI and 20 U Xho I with 4 µl 10 x NEBuffer Eco RI (New England Biolabs) and 0.5 µl BSA (10 mg/ml) in a total volume of 40 µl. After incubation at 37℃ for 4.5 h, enzymes were inactivated at 65℃ for 20 min. The digested vector was separated on 0.8% TAE gel and a 3 kb band excised. DNA was purified from gel using the QIAEX II Agarose Gel Extraction protocol (Qiagen, Inc., Chatsworth, CA) according to the instructions of the manufacturer. 2 µg purified vector was dephosphorylated by addition of 2 µl shrimp alkaline phosphatase (SAP, 1 U/µl) (Promega, Madison, WI) and 10x SAP buffer in 50 µl total volume. Following incubation at 37℃ for 15 min, the reaction was inactivated at 65℃ for 15 min. A further step of purification was carried out by using the QIAquick PCR Purification Kit protocol (Qiagen, Inc.) according to the instructions of the manufacturer. The plasmid concentration of the eluate was measured by loading an aliquot on gel.

2.6.10. Vector-insert ligation

20 ng size-fractionated cDNA was ligated to 40 ng purified vector in 10 µl total reaction containing 200 U T4 DNA ligase (400 U/µl) (New England Biolabs) and 1 µl 10x ligase buffer with 10 mM ATP. Ligation reactions were incubated at 4℃ for 3 days. Enzyme inactivation was carried out at 65℃ for 10 min.

The total amount of size-fractionated cDNA was used for multiple ligations. The ligations which were not needed for bacterial transformation were pooled and precipitated for storage.

2.6.11. DNA clean up

StrataClean™ Resin was used for enzyme removal (Stratagene Inc., Cedar Creek, TX). Resin was vortexed for complete resuspension and 5 µl added to a ligation reaction. The mixture was vortexed for 15 sec and centrifuged at 15,000 rpm for 1 min. 10 µl supernatant was transfered into a fresh tube and stored at 4℃ until used.

2.6.12. Bacterial transformation

2 µl purified ligation was pipetted into an ice-precooled cuvette and 30 µl competent ElectroMAX™ DH10B™ cells (Invitrogen) added. Following two pulses at 1800 volts, 1 ml freshly prepared SOC medium (0.99 ml SOB including 10 µl 2 M glucose) preheated to 37℃ was added. The mixture was transfered to a fresh tube and incubated at 37℃ for 1 h without shaking. Aliquots of 50-100 µl were plated on S-Gal/LB Agar (Sigma-Aldrich, St. Louis, MO) containing 100 µg/ml ampicillin and plates were incubated at 37℃ for 14-16 h, then at 4℃ for 4 h for color development. Bacterial colonies containing an insert remained white while those without insert turned black.
SOB: 20g Trypton, 5g Yeast Extract per liter including 10 mM NaCl and 2.5 mM KCl were dissolved and autoclaved. 10 mM $MgCl_2$ and 10 mM $MgS0_4$ were added from sterile stock solutions.

2.6.13. Colony picking

White colonies were manually picked with sterile toothpicks and grown 16-18 h without shaking in 250 µl/well CIRCLEGROW® medium (Qbiogene, Inc., Carlsbad, CA) containing 125 µg/ml Ampicillin on 96-well plates covered with AirPore™ Tape Sheets (Qiagen Inc.).

2.7. DNA analysis

2.7.1. Isolation and purification of Cyanophora paradoxa nuclear DNA

C. paradoxa cultures were harvested, the cell pellet resuspended in 100 ml minimal medium (chapter 2.1) and centrifuged at 4,000 rpm for 5 min at 4℃. The supernatant was discarded and cells frozen at –70℃ for 1 h to cause cell lysis. Then, the pellet was resuspended in 10 ml TE (10 mM Tris-HCl pH 8.0, 10 mM EDTA) and homogenized in a potter. Following centrifugation at 4,000 rpm for 20 min at 4℃ in a J-6 centrifuge, the cyanelle pellet was discarded and buffer A (0.5% SDS, 10 mM NaCl, 10 mM EDTA, 10 mM Tris pH 8.0, 0.01 mol/l proteinase K (Merck)) added to the supernatant (nuclear DNA) to 20 ml total volume. Incubation of the mixture was performed at about 56℃ for 3 h with occasional agitation. Extraction with 10 ml buffer B-saturated phenol (buffer B: 0.5% SDS, 10 mM NaCl, 10 mM EDTA, 500 mM Tris, pH 8.0) at RT for 10 min with continuous inversion was performed. After centrifugation at 4,000g for 5 min at 4℃ in a megafuge, the extraction was repeated. The upper aqueous phase in each case, containing DNA, RNA and carbohydrates, was retained and further, transferred into a dialysis tube which had been pre-treated in boiling 10 mM EDTA, pH 8.0, for 10 min. Following dialysis against 2 l of buffer C (10 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 8.0) at 4℃ twice for 30 min, dialysis was performed against 4.5 l 1x TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4℃ overnight and then, against 4 l 1x TE for 2 h at 4℃.
1/10 vol of 3 M NaAc, pH 4.8, and 2.5 vol of ice-cold ethanol abs. were added to the dialysate for DNA precipitation at –20℃ for 4 h. After centrifuging at 16,500g for 10 min at 4℃ in a GSA rotor, the DNA pellet was washed with 100 ml 70% ethanol (RT), centrifuged at 16,500g for 5 min at 4℃ (GSA rotor), dried at RT for 20 min and resuspended in 7 ml dd$H_2$0. 9.273 g CsCl was added, mixed gently for complete dissolution, and 1x TE pH 8.0 added to a total volume of 10ml. Further, 2 µl ethidium bromide solution (10 mg/ml) was added. The mixture was poured into two Polyallomer Quick Seal centrifuge tubes (Beckman Coulter, Germany), equilibrated with CsCl solution (927 mg/ml) and sealed with a “quick-seal” device. Centrifugation

was carried out for 15 h in a L-80 ultracentrifuge (Beckmann Coulter) with a VTi 65 rotor at 50,000 rpm at RT. UV light (302 nm) was used to determine the location of DNA bands. Due to their different G-C contents, remaining cyanelle DNA is located at the top of the gradient while the nuclear DNA is seen in the middle. The nuclear DNA band was carefully removed by a sterile syringe with a wide gauge needle.

To the isolated DNA 1 vol ddH_20 was added, then ethidium bromide was extracted by adding approximately 0.5x vol isoamyl alcohol. The upper isoamyl alcohol phase attained a pinkish colour and was discarded. Extraction was continued until the upper phase remained colourless. The lower DNA phase was precipitated at RT for 1 h by addition of 2 vol 95% ethanol. After centrifugation at 10,000 rpm for 10 min at 4℃ with a SS-34 rotor, the pellet was resuspended in 500 µl TE and precipitated once more with 1/10 vol 3M NaAc and 2 vol 95% ethanol at –80℃ overnight. After centrifugation at 14,000 rpm for 10 min at 4℃, the pellet was washed with 70% ethanol (RT), centrifuged at 14,000 rpm for 5 min, dried under vacuum for 15 min and resuspended in 250 µl ddH_20.

The concentration and purity of DNA were determined spectrophotometrically at 260/280 nm.

2.7. 2. Plasmid quick preparation

4 ml sterile LB-medium containing 100 µg/ml ampicillin was inoculated with a single bacterial colony and bacteria grew overnight at 37°C while shaking. 1.5 ml of each culture was transferred into an Eppendorf tube and centrifuged at 8,000 rpm for 2 min at RT. After aspiration of the supernatant with a Pasteur pipet, further 1.5 ml of the same culture was transferred to the bacterial pellet; centrifugation and supernatant removal likewise performed. To the resulting pellet 200 µl lysis buffer was added (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1 µg/ml RNase A) and the pellet resuspended by vortexing. Following 20 min incubation on ice, 400 µl alkaline SDS (0.2 M NaOH, 1% SDS, freshly prepared, RT) was added for denaturation of proteins and DNA, the tube was inverted several times for mixing and placed on ice for 5 min. 300 µl cold high salt solution (3 M K-acetate, pH 4.8) was added for precipitation of chromosomal DNA and bacterial proteins, the tube

inverted several times and placed on ice for 10 min. All further centrifugations were performed at 13,200 rpm and RT. Following centrifugation for 10 min, the supernatant containing the plasmid DNA was transferred into a fresh tube. For DNA precipitation, 600 µl 2-propanol was added, vortexed for 30 sec, placed on ice for 10 min and centrifuged for 10 min. The supernatant was carefully removed. 400 µl 2.5 M NH_4Ac was added and the pellet removed from the tube wall with the pipette tip, vortexed and shaken at RT for 30 min for release of the plasmid DNA from the pellet. After centrifugation for 10 min, the supernatant was transferred into a fresh tube, 1 ml of cold (-20℃) 95% ethanol added for plasmid DNA precipitation, vortexed, placed on ice for 10 min and centrifuged for 10 min. The supernatant was discarded, 1 ml 70 % EtOH (RT) added, incubated for 5 min for RT, centrifuged for 5 min. After supernatant removal, the pellet was dried under vacuum for usually one hour and resuspended in 20 µl ddH20 containing 400 µg/ml RNase A.

This plasmid purification protocol was used to verify the identity of individual clones of the cDNA libraries by restriction analyses. Typically, 6 quick-preps were performed for one bacterial clone streaked out on a selective plate. The protocol was also utilized for probe synthesis from the plasmid insert for Southern and Northern analyses.

2.7.3. JETSTAR plasmid purification system

Using the JETSTAR kit (GENOMED Gmbh, Germany) plasmid yields up to 20 µg were obtained. This purification protocol rendering plasmid preparations of higher purity was usually taken for sequencing of individual clones by VBC-Genomics GmbH (Austria).
As described in chapter 2.8.2, 4 ml sterile LB-medium containing 100 µg/ml ampicillin was inoculated with a single bacterial colony for overnight growth at 37℃, E. coli cells were pelleted by centrifugation and the medium completely removed. All following steps were carried out according to the instructions of the manufacturer. A column was equilibrated by application of 2 ml solution E4 (600mM NaCl, 100 mM sodium acetate, 0.15% TritonX-100, acetic acid ad pH 5.0) and allowed to drain by

gravity flow. The bacterial pellet was resuspended in 0.4 ml solution E1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, containing RNase) by vortexing. Addition of 0.4 ml solution E2 (200 mM NaOH, 1.0% SDS (w/v)), thorough mixing by inversion and incubation at RT for 5 min resulted in a homogeneous cell lysate. Longer incubation, possibly denaturing plasmid DNA irreversibly, was avoided. For neutralization, 0.4 ml solution E3 (3.1 M potassium acetate, pH 5.5) was added (no surplus on pipette tip's outer side) and the tube immediately inverted 5 times. Following centrifugation at 16,000 g, 10 min at RT, the resulting supernatant was applied to the equilibrated column but the transfer of potassium dodecyl sulfate particles avoided. Allowing the column to drain by gravity flow completely each time, wash steps were subsequently performed with 2x 2.5 ml solution E5 (800 mM NaCl, 100 mM sodium acetate, acetic acid ad pH 5.0). Plasmid DNA was eluted by application of 0.9 ml solution E6 (1.25 M NaCl, 100mM Tris-HCl, pH 8.5). Addition of 0.63 ml isopropanol (RT) to the eluate, vortexing and centrifugation at 16,000 g for 30 min at 4℃ produced a pellet which was washed with 1 ml 70 % EtOH (RT), air dried for up to 30 min and resuspended in 20 µl ddH_20.

2.8. Hybridization methods

2.8.1. Southern analysis

2.8.1.1. Digestion of genomic DNA and gel run

Generally, handling of genomic DNA is performed gently; for mixing stirring with a yellow tip is recommended while vortexing should be avoided.

To 10 µl (20 µg) gDNA, 7 µl suitable 10x restriction buffer and 45.5 µl ddH_20 were added and the mixture incubated at 4℃ for 2 h. 7.5 µl (10U/µl) restriction enzyme was added in aliquots over a period of 12 h and the reaction periodically stirring. Digests were performed with Bam HI, Eco RI and Bam HI/Eco RI.

1% TAE agarose gel was prepared with wide slots. 35 µl digested DNA was loaded per slot and allowed to diffuse for 2 min. Bst EII digested DNA was loaded as marker. Electrophoresis was performed at 5V/cm for 4 h and a photo taken of the gel alongside a ruler.

Generally, a DNA gel was made with 1x TAE by adding the percentage of agarose indicated. Agarose was dissolved by heating in the microwave. 0.05 µg/ml ethidium bromide was added before pouring into a tray.
50x TAE (Tris-Acetate-EDTA): 2M Tris-Acetate, 0.05M EDTA pH 8.3

2.8.1.2. Gel blotting

The gel was shortly incubated in ddH_20, then in denaturation buffer for 30 min with gentle agitation. After a short incubation in ddH_20, gentle agitation for 30 min in neutralization buffer was performed. After a short incubation in ddH_20, a capillary blot was stacked on a support in the following order (bottom upwards): 3 sheets of Whatmann 3M paper, gel, Nylon membrane (Hybond-N^+, Amersham Biosciences), 3 Whatmann sheets, 15 cm height paper towels, beaker with 600 ml water. Blotting was done overnight with 20x SSC.

Denaturation buffer: 87.66g NaCl, 20g NaOH in 1 liter, autoclaved
Neutralization buffer: 87.66g NaCl, 60.5g Trizma base in 1 liter, pH 7.5, autoclaved
20 x SSC: 88.23 g Tri-sodium citrate, 175.32 g NaCl in 1 liter
The membrane was dried at RT for 2 h. Then, 3 µl of a dilution series (1/10) to 1:10,000 of the probe plasmid (positive control) and a different plasmid (negative control) was pipetted onto the membrane edge and the membrane UV cross-linked (GS Gene Linker™ UV chamber, Bio-Rad, Austria).

2.8.1.3. Probe synthesis and DIG labelling

5 µg plasmid was digested at 37℃ for 2 h with a co mbination of restriction enzymes (Roche) using 10 U enzyme per reaction volume (50 µl). Digested plasmid was loaded on 1 or 2% TAE agarose gel and electrophoresis performed at 80 V. Bands with insert fragments of the correct size were excised and DNA purified with the QIAEX II Agarose Gel Extraction kit (Qiagen, Inc.) according to the instructions of the manufacturer. DNA was eluted twice with 20 µl ddH_20.

The following enzyme combinations were used:

CY010012000e8: Eco RI, BamHI 850bp fragment

For labelling, the DIG DNA Labelling and Detection Kit from Boehringer Mannheim GmbH was used. 15 µl template DNA from the first eluate was denatured for 10 min in boiling water, quickly chilled on ice-water and the following components added on ice: 2 µl hexanucleotide mix, 2 µl dNTP mixture and 1 µl Klenow enzyme. The reaction was briefly centrifuged and incubated for 20 h at 37℃. To stop the reaction, 2 µl 0.2 M EDTA, pH 8.0, was added and labelled DNA precipitated by addition of 2.5 µl 4 M LiCl and 75 µl prechilled (-20℃) ethanol. Following incubation at –70℃ for 1 h, and centrifugation at full speed for 15 min at 4℃, the pellet was washed with 50 µl 70% ethanol (RT). After drying the pellet at RT for 15 min, it was dissolved in 50 µl TE-buffer.

2.8.1.4. Hybridization using a DIG labelled probe

The cross-linked membrane was incubated in 2x SSC for 5 min and pre-hybridized in a rotating chamber at 42℃ for 1 h with pre-warmed hybridization solution (20 ml/100 cm^2 membrane). 50 µl probe was denatured for 5 min by boiling, quickly chilled on ice-water and added to pre-warmed hybridization solution (2.5 ml/100 cm^2). The (pre-)hybridization solution was poured off and the probe solution added to the membrane for hybridization by rotation overnight at 42℃.

Hybridization solution: “High SDS” hybridization buffer
SDS, 7%
Formamide, deionized, 50%
5x SSC
50 mM sodium phosphate, pH 7.0
N-lauroylsarcosine, 0.1% (w/v)
Blocking reagent, 2% (Boehringer Mannheim)

The membrane was washed 2x 5 min in ample 2x SSC, 0.1% SDS at RT under constant agitation, then 2x 15 min in 0.1x SSC, 0.1% SDS at 68℃ in a rotating chamber.
A DIG-labelled probe in hybridization solution could be re-used and was stored at –20℃.

2.8.1.5. Immunological detection

The DIG DNA Labelling and Detection Kit from Boehringer Mannheim GmbH was used.
Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) was prepared.
The membrane was rinsed 5 min in washing buffer (maleic acid buffer, 0.3% (v/v) Tween 20), then incubated for 30 min in 100 ml 1x blocking solution (1% blocking reagent in maleic acid buffer, dissolved at 65℃). The anti-DIG-AP conjugate was diluted to 75 mU/ml (1:10000) in 1x blocking solution. The membrane was incubated for 30 min in 20 ml antibody solution, washed 2x 15 min with 100 ml washing buffer and equilibrated 5 min in 20 ml detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). The membrane was incubated in a box in the dark with about 10 ml freshly prepared colour solution (200 µl NBT/BCIP stock solution added to 10 ml detection buffer) without shaking. The color precipitate starts to form within a few minutes and the reaction usually reaches completion within 16 h. When desired band intensities were achieved, the membrane was washed under a flowing tap for 10 min and dried (RT, 30 min).

2.9. Protein techniques

2.9.1. Analysis of proteins by SDS-polyacrylamide gel electrophoresis

For separation and analysis of proteins, 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Mini-PROTEAN II apparatus from Bio-Rad.

Table 2.10.1.1. The components of two 12% SDS-PAGE minigels:

	12% separating gels (10 ml)	stacking gels (2 ml)
dd$H_2$0	3.3 ml	1.4 ml
30% acrylamide mix	4 ml	330 µl
1.5 M Tris-HCl (pH 8.8)	2.5 ml	
1.0 M Tris-HCl (pH 6.8)		250 µl
10% SDS	100 µl	20 µl
10% APS	100 µl	20 µl
TEMED	4 µl	2 µl

30% acrylamide mix: 29g acrylamide, 1g N,N'-methylenebisacrylamide in 100 ml $H_2$0

The 12% separating gel was prepared according to table 2.10.1.1. (APS and TEMED were added last), poured in between the glass plates clamped in the pouring stand and covered with isopropanol. After gel polymerization (30 min, RT), the isopropanol was discarded and the surface of the separating gel washed with dd$H_2$0. Excess water was removed with Whatmann 3M paper and stacking gel poured onto the separating gel. Immediately a comb was inserted into the stacking

gel. Following gel polymerization (30 min, RT), combs were removed, slots rinsed with ddH_20 and the apparatus assembled.

The gel was run in 1 x Laemmli running buffer. Samples with added 2x SDS-sample buffer were denatured and applied to slots using a yellow pipette tip. As a molecular weight standard, the Low Molecular Weight Marker (Amersham Biosciences) was used for subsequent Coomassie and silver staining; for blotting the Pre-stained Standard Low Range Marker (Bio-Rad) was loaded. Typically, electrophoresis was carried out at a constant current of 10 mA/gel for separation in the stacking gel and 40 mA/gel for the separating gel. The gel was run until bromophenol blue reached its end. The apparatus was disassembled for either staining or blotting.

10x Laemmli running buffer: 250 mM Tris-HCl, pH 8.4
1.92 M glycine
1% SDS

2x SDS-sample buffer: 250 mM Tris-HCl, pH 6.8
20 ml glycerol
4 g SDS
2 ml 2-mercaptoethanol
0.01 g bromophenol blue per 100 ml

Low Molecular Weight Marker (Amersham Biosciences)

LMW (14 000-97 000)

Protein	M_r (kDa)	Source	Amount (µg)
Phosphorylase b	97	rabbit muscle	67
Albumin	66	bovine serum	83
Ovalbumin	45	chicken egg white	147
Carbonic anhydrase	30	bovine erythrocyte	83
Trypsin inhibitor	20.1	soybean	80

2.9.2. Coomassie staining

For staining with Coomassie, the gel was covered with Coomassie staining solution and agitated gently at RT for 30 min on a shaker. Then, the gel was covered with Destaining solution and agitated 30-45 min, until bands were clearly visible. The gel was incubated for 10 min in Fixing solution, placed on Whatmann 3M paper, covered with a plastic wrap and dried at 60℃ for a t least 2 hours under vacuum.

Coomassie staining solution:	50% acetic acid
	10% ethanol
	0.25% Coomassie brilliant blue R250
Destaining solution:	500 ml dH_20
	400 ml methanol
	100 ml glacial acetic acid
Fixing solution:	10% acetic acid
	10% ethanol

2.9.3. Silver staining

The gel was incubated in following solutions in the given order. All solutions were prepared with ddH_20.

20 min	Fixer solution (50% methanol, 5% acetic acid)
10 min	Washing solution (50% methanol)
2h	ddH_20
1 min exactly	Sensitizing solution (0.03 g sodium thiosulfate.$5H_20$ in 100 ml)
3x 20 sec	ddH_20
20 min, 4℃	Silver solution (0.1 g silver nitrate in 100 ml, fresh)
2x 20 sec	ddH_20

0.5-5 min Developing solution (2 g sodium carbonate, 40 µl 35% formaldehyde in 100ml)
3x 1 min Stop solution (5% acetic acid)

2.9.4. Western Blotting

SDS-PAGE was performed as described above using the following marker:

Pre-stained SDS-PAGE Standard marker, Low Range (Bio-Rad):

Protein	M_r (kDa)
Phosphorylase B	113.0
Bovine serum albumin	91.0
Ovalbumin	49.9
Carbonic anhydrase	35.1
Soybean trypsin inhibitor	28.4
Lysozyme	20.8

Following electrophoresis, the gel was briefly rinsed in water and a sandwich prepared with the following:
- 3 layers Whatmann 3M paper, pre-wetted in blotting buffer
- 1 nitrocellulose membrane (PROTRAN, 0.2 micron pore size)
- gel
- 3 layers Whatmann 3M paper, pre-wetted in blotting buffer

The transfer was done at 140 mA, 5V-15V for 2-3 h. The following incubation steps were carried out at RT unless otherwise specified.
To verify if transfer of proteins succeeded, the membrane was reversibly stained with Ponceau S (10 min), surplus colour was removed by water and the membrane was washed 3x with TBS-T. For blocking (of unspecific binding sites), the membrane was incubated in 10% milk in TBS-T overnight at 4℃ without agitation.

Membranes were washed 3x 10 min in TBS-T and incubated with primary antibody for 1 hour. After washing 2x 10 min in TBS-T, incubation with the secondary antibody for 1 h was carried out, then washing 2x 10 min in TBS-T and 10 min in AP buffer. Incubation with freshly prepared detection solution (10 ml AP buffer containing 66 µl NBT (50 mg/ml; in 70% DMF) and 33 µl BCIP (50 mg/ml; in 100% DMF) was carried out in the dark until bands were visible. The reaction was terminated by washing with ddH_20.
For detection of bands for Rubisco LSU, rabbit -LSU, diluted 1:3000, was used as primary antibody and goat anti-rabbit IgG alkaline phosphatase (AP) conjugate (Promega), diluted 1:7500, as secondary antibody.

Blotting buffer: 1 x Laemmli running buffer, 10% methanol

Ponceau S: 2 g Ponceau S, 30 g trichloroacetic acid, 30 g sulfoalicylic acid, 100 ml ddH_20

TBS-T: 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20

AP buffer: 100 mM Tris-HCl, pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$, freshly made

2. 10. Mass Spectrometry

2. 10. 1. Protein analysis by mass spectrometry

The spots of interest from the SDS-PAGE gels were excised manually with a stainless steel scalpel and subjected to in-gel digestion. using trypsin (bovine pancreas, modified; sequencing grade, Roche, Mannheim, Germany). Extracted tryptic peptides were desalted and purified utilising ZipTip® technology (C_{18} reversed phase, standard bed, Millipore). Sample preparation for MALDI mass spectrometry was carried out on a stainless steel target, applying the dried droplet preparation technique using α-cyano-4-hydroxy-cinnamic acid (Fluka, Buchs, Switzerland) as matrix (3 $mg \cdot mL^{-1}$, dissolved in acetonitrile/0.1% trifluoracetic acid 7/3).

Positive ion mass spectra for peptide mass fingerprinting (PMF) were recorded on a MALDI-TOF mass spectrometer (Axima CFR^+, Shimadzu Biotech, Manchester, UK) equipped with a nitrogen-laser (λ=337 nm) by accumulating 200-500 single unselected laser shots. The instrument was operated throughout all PMF experiments in the reflectron mode, applying 20 kV acceleration voltage and delayed extraction (optimized setting for ions of m/z 2000). External calibration was performed using an aqueous solution of standard peptides (Bradykinin fragments 1-7 and 1-5, human Angiotensin I and II, Glu-1-fibrinopeptide, N-acetyl renin substrate and ACTH fragments 1-17, 18-39 and 7-38). The lists of monoisotopic m/z-values derived from the MALDI mass spectra of in-gel digested spots were submitted to the Mascot search engine for PMF search with a peptide tolerance of ± 0.03 Da. The search was run against all proteins and DNA sequence information from public databases (Swiss-Prot, NCBInr) without any species restrictions. The fixed modifications were set to carbamidomethyl and methionine oxidation was set as an allowed variable modification.

For sequence information seamless post source decay (PSD, AXIMA CFR^+) or low energy collision-induced dissociation (CID, Axima QIT, Shimadzu Biotech, Manchester, UK) experiments of at least 3 abundant tryptic peptides were performed by accumulating 1000 to 2000 single unselected laser shots. For PSD and CID experiments again the Mascot search engine was used, applying the same

settings for species and modifications as mentioned above and the addition of precursor (± 0.2 Da) and product ion tolerances (± 0.2 Da).

2.10.2. Coomassie staining of SDS – PAGE gels for subsequent MS analysis

Solution	Time	Volume	
Fixation	60 min	100 ml	45 % Methanol 5 % Acetic acid UHQ
Washing	2x 1 min	2x 100 ml	UHQ
Staining	Until bands become clear	100 ml	0,025 % Coomassie R 250 40 % Methanol 7 % Acetic acid UHQ
Destaining I	30 min	100 ml	40 % Methanol 7 % Acetic acid UHQ
Destaining II	Until backround become clear		7 % Acetic acid 5 % Methanol UHQ
Storage for subsequent analysis		100 mL	UHQ

2.10. 3. In-Gel Digestion

During In Gel Digestion the purest chemicals available should be used. Gloves should be worn to avoid contamination by human epidermal proteins (keratin). To beware of talcum powder or dust contamination the gloves should be carefully rinsed with water before use.
The razor should be washed with water and methanol before use.

2. 10. 4. Buffers and Solutions

50 mM NH_4HCO_3:	50 mM NH_4HCO_3 pH 8.5 0.395 g NH_4HCO_3, 100 mL UHQ, use NH_3 to adjust pH
Dithiotreitol solution:	10 mM 1.54 mg DTT, 1mL 50 mM NH_4HCO_3
Iodoacetamide:	55 mM 10.2 mg Iodoacetamide, 1mL 50 mM NH_4HCO_3
Digestion Buffer:	50 mM NH_4HCO_3, 12.5 ng trypsin / µL 125 µg trypsin, 10 mL 50 mM NH_4HCO_3 100 µL aliquots can be stored at –20 ℃

2. 10. 5. Procedure

Wash the gel slab with UHQ (2 changes, 10 min each)
Use a clean scalpel to excise the spot of interest from the gel. Cut as close to the protein band as possible to reduce the amount of background gel. Excise a gel piece of roughly the same size from a gel region which does not carry any protein to use it as a control.

Cut the excised piece into roughly 1 mm^3 cubes, and transfer them to a clean 0.5 mL microfuge tube.

Wash the gel particles with UHQ and UHQ/ACN 1:1 (v/v) – one or two changes each, 15 min/change. Solvent volumes used in the washing steps should roughly equal to the gel volume.

Remove all liquid and add enough ACN to cover the gel particles.

After the gel pieces have shrunk (they become milky and stick together) remove the ACN and rehydrate the gel pieces in 50 mM NH_4HCO_3 for 5 min.

Add an equal volume of ACN (to get 50 mM NH_4HCO_3 / ACN, 1:1) and incubate for 15 min.

Remove all liquid and dry gel particles in a vacuum centrifuge.

Swell the gel particles in 10 mM DTT / 50 mM NH_4HCO_3 and incubate for 45 min at 56 ℃ to reduce the protein.

Chill tubes to room temperature. Remove excess liquid, and replace it quickly with roughly the same volume of freshly prepared 55 mM iodoacetamide / 50 mM NH_4HCO_3 solution. Incubate at room temperature for 30 min in the dark.

Remove iodoacetamide solution and wash the gel particles with 50 mM NH_4HCO_3 for 5 min.

Add an equal volume of ACN (to get 50 mM NH_4HCO_3 / ACN, 1:1) and incubate for 15 min.

All the Coomassie stain should be removed at this time. The gel particles should appear completely transparent. If a large amount of protein is analysed (more than 10 pmol) remaining Coomassie staining may still be visible. In this case, an additional 50 mM NH_4HCO_3 / ACN washing cycle should be performed.

Remove all liquid and dry gel particles in a vacuum centrifuge.

Rehydrate gel particles by adding digestion buffer. Add more buffer if all the initially added volume is soaked up. Incubate for 45 min.

Remove remaining enzyme supernatant and replace it with 5-20 µL of the same buffer without enzyme.

Digest overnight at 37 ℃.

The first peptide analysis can be performed already after 3-4 h of digestion. If some liquid has evaporated and condensed on the side or on the lid of the microcentrifuge tube, centrifuge briefly to gather the liquid at the bottom of the tube.
After overnight digestion add a sufficient volume of 25 mM NH_4HCO_3 to cover the gel pieces and incubate for 15 min.
Add the same volume of ACN. Incubate for 15 min and recover the supernatant.
Repeat the extraction two times with 5 % HCOOH and ACN (1:1, v/v).
Pool all the extracts.
Dry the sample in a vacuum centrifuge.
Redissolve peptides in 10-30 µL of 0.1 % TFA, sonicate briefly and analyse an aliquot after Zip-Tip purification.

2. 10. 6. Zip-Tip purification

Wet the Zip-Tip three times with 0.1 % TFA/ACN (1:1)
Equilibrate the Zip-Tip by wetting the Zip-Tip three times with 0.1 % TFA
Bind the peptides by sucking in the redissolved peptides three times
Wash the Zip-Tip three times with 0.1 % TFA
Elute the peptides with 5 µL 0.1 % TFA/ACN (1:1)

2. 11. Electron Microscopy

2. 11. 1. Chemical Fixation and Epoxy Resin Embedding of Algae

Algae were centrifuged at 1000g for 5 min to form a pellet at the bottom of a 50 ml Falcon tube. Cells in the pellet were fixed in 3% glutaraldehyde in 0.15M, pH 7.4, for 2 h. After washing thrice with Sorensen`s buffer the pellets were postfixed in 1% OsO4 in Sorensen`s buffer for 1 h. Subsequently they were washed again, and transferred in glass vials for dehydration in a series of ethanol. Step-wise infiltration with epoxy resin (Agar 100) was mediated by propylene oxide. Finally, the pellets were transferred in embedding molds. After infiltration in pure resin over night the samples were polymerization in the oven at 60ºC.

2. 11. 2. High-pressure freezing, Low-temperature Fixation and Epoxy Resin Embedding of Algae

For cryopreparation of algae a protocol adapted to the needs for processing of cell suspensions was used, which has been described previously (Reipert et al. 2004; Nowikovsky et al. 2007). Algae were centrifuged at 2000 rpm for 2 min at a bench centrifuge and re-suspended in 20% bovine serum albumin in phosphate buffered saline (PBS) for repeated centrifugation. The supernatant was discharged and the enriched cells were taken for high-pressure freezing (HPF) with a LEICA EMPACT machine (LEICA Microsystems, Vienna, Austria). For loading of the flat sample carriers (inner diameter of 1.2 mm, 200 µm in depth) cells were transferred with a Gilson pipette. Transfer and fitting of the sample carrier into a specimen pod, supported by a loading station, took about 10-15 s. Further 10-15 s are required for mounting of the specimen pods onto the loading device and starting of the freezing process at a high pressure of about 1990 bar. Cryoimmobilization of the living state occurred within microseconds.

Freeze substitution with 2% OsO4 in dried acetone was performed in an automatic freeze substitution unit, LEICA AFS (LEICA Microsystems, Vienna, Austria). While still in the flat sample carriers, samples were transferred from liquid N2 onto the frozen substitution medium in Sarstedt tubes. To avoid cracks in the frozen samples the AFS was gradually warmed up (35 ºC/hr) from -160º to -90ºC. Freeze substitution was performed at -90 ºC for 72 hrs. Low temperature-fixation was activated by gradual warming up of the solvent by 2 ºC/hr up to -54 ºC. This temperature was kept for 8 hrs before continuing the warming up at a rate of 3 ºC/h up to -24 ºC. Samples remained at -24 ºC for 7 hrs. Subsequently, they were contrasted more intensely by keeping them at 0 ºC for 1hr. Contrasting in OsO4/ acetone was followed by washing of the samples with acetone.

For embedding, samples were infiltrated with epoxy resin/ acetone mixtures (1/3 volume Agar 100 and 2/3 volume acetone for 1 hr at 10 ºC; 1/2 volume Agar 100 and 1/2 volume acetone for 1 hr at 10 ºC; 2/3 volume Agar 100 and 1/3 volume acetone for 3 hr at 20 ºC). Under the stereo microscope, samples were separated from their carriers and transferred into embedding molds with pure resin Agar 100. After infiltration over night, samples were polymerized in the oven at 36 ºC for 36 hrs.

2. 11. 3. Thin sectioning and Contrasting for Transmission Electron Microscopy

Thin sections (60-80 nm) were cut with an Ultracut S ultramicrotome (LEICA Microsystems, Vienna, Austria). They were mounted on copper grids, contrasted by uranyl acetate and lead citrate and viewed at 80 kV in a JEM-1210 (Jeol Ltd Tokyo, Japan) electron microscope. Images were acquired using a digital camera Morada for the wide-angle port of the TEM and analySIS FIVE software (Soft Image System, Münster, Germany).

2. 11. 4. Buffer Preparations for electron microscopy

The most commonly used buffers for EM are phosphate buffers (either sodium phosphate or a mixture of sodium and potassium phosphate) and buffers made from cacodylic acid. Both can buffer solutions in the pH range 6.5-7.5 quite effectively (physiological pH range of most cells and tissues).

Phosphate buffers are more physiological than any other buffer, bcause they are found in the living systems in the form of inorganic phosphates and phosphate esters. However they are negatively charged and can thus interact with some cytochemical reagents or salts in the medium. In our lab we use Sorensen´s Phosphate buffer exclusively.

Cacodylate buffers do not possess highly reactive groups and thus are the buffer systems of choice when working near neutral pH with materials that contain positively charged meoities capable of reacting with phosphate buffers. Cacodylate buffers are toxic (contain arsenic) and more expensive than phosphate buffer.

Note: Buffer stock solutions will be stored in Duran bottles. They have to be labeled with the molarity, the name of the buffer, the pH, the date of preparation, and the name of the person who made it. (If not noted otherwise the pH is measured at room temperature.)

2. 11. 5. Preparation of Sorensen`s buffer pH 7.4 (osmolarity: 440 mosm)

Prepare solution A: Dissolve 5.94 g Sodium phosphate dibasic (Na2HPO4 2H20) in 500 ml ddw.

Prepare solution B: Dissolve 1.82 g Potassium phosphate, monobasic (KH2PO4) in 200 ml ddw.

To prepare 500 ml Sorensen`s pH 7.4 take 409 ml Solution A and add 91 ml of the solution B.

(For other desired pHs see Hayat: Principles and techniques of EM, 3. d ed., page 23)

2. 11. 6. Preparation Epoxy Resin Agar 100 and Infiltration Mixtures for Embedding

Agar 100 kit from Agar (supplier Gröpl) consisting of small bottles will be used. This avoids long term storage of opened bottles and ensures that water content of the air will not jeopardize the components.
The resin will be prepared by always using the same procedure to obtain the defined quantity of 40 ml. It can be used to prepare resin-solvent mixture (2:3 and 1:2)
To avoid trouble with this frozen left-overs stay with the following rules:
For warming up, place it in the oven (warmed at 60 Cº) while the baker is still covered with aluminum foil.
Warm the resin up for about 10 min until it reaches room temperature.

2. 11. 7. Preparation of solvent-resin mixtures

The number of infiltration steps may vary in dependence of the sample to embed. For easily to infiltrating samples, three infiltration steps will be performed:
1:3 resin-solvent volume ratio, 2:3 solvent-resin volume ratio and pure resin. Other applications may require an additional 1: 2 solvent-resin infiltration between the first and second step.
(Explanation: 1:3 resin-solvent volume ration means mixing of 1 part resin (e.g. 5ml) with 2 parts of the solvent (e.g. 10 ml).)

2. 11. 8. Epoxy Resin Embedding of Glutaraldehyde-fixed Cell Pellets

The protocol was suitable for mammalian suspension cells or trysinized adherent growing cells. It might require modifications in centrifugation to obtain a stable pellet for algae or bacteria. The cell material provided should allow the formation of a clearly visible pellet (2-3 million mammalian cells).

Take the cells without washing and spin them down in the bench centrifuge (10 min at 800-1200 rpm). Decide whether or not cooling during centrifugation is required for the experiment. Use a tube with a conical bottom for this purpose (e.g. Greiner tube 15 ml, Sterilin tube 15 ml).

Pre-fixation: Remove the supernatant and add about 2 ml 3% glutaraldehyde (GA) in Sorensen´s buffer. Allow the fixative to stabilize the pellet for about 15 min.

Take a wooden stick and form one of its ends to a spatula by using a razor blade. (Alternatively you might use a long metallic spatula with a U-shaped end.) Use the wooden stick to detach the pellet from the wall of the tube. This allows the fixative to infiltrate more easily underneath the pellet. Allow fixation for further at least further 1 h 45 min. Alternatively, you might fix the pellet over night in the fridge.

Wash the pellets 3 times for 10 min in Sorensen´s buffer (alternatively cacodylate buffer). OsO_4 infiltration (toxic- use hood and gloves): Prepare 1.5 % OsO_4 by dilution of a 4% OsO_4 stock solution provided in an ampoule (see guidelines for the use of OsO_4). Replace the buffer by 1-2 ml OsO_4 in Sorensen´s or cacodylate buffer. Incubate for 1-2 hours.

Wash the pellets again 3 times for 5 min in Sorensen´s buffer (alternatively cacodylate buffer). After the first wash, transfer the sample into a glass vial. Use a plastic Pasteur pipette fro this purpose with its tip widened.

Ethanol Dehydration: Perform a gradual dehydration:

10 min each 30%, 50%, 70%, 95% ethanol

2x 10 min 100 % ethanol

Propylene oxide (PO): Replace 100% ethanol by propylene oxide: 2x 10 min PO

Resin infiltration:

The preparation and use of Agar 100 epoxy resin

15 min 1:3 resin : PO (use old resin from the freezer)

45 min 2:3 resin : PO (use freshly prepared resin)

Transfer the pellet into an embedding mould and fill this mould with pure resin. Infiltrate pure resin for 2-3 h or, alternatively, overnight.

Polymerization for 36 hours in the oven at 60℃.

2. 11. 9. Lead Citrate Staining

The grid with its shiny side facing up onto the LC droplet in the Petri dish and close the lid.

Stain for 5 minutes (epoxy resin) or 3 minutes (Lowicryl).

Stop the staining by taking the grid with the tweezers and 10-fold dipping into beaker 3 filled with ddw. Continue by dipping the grid 10 times into beaker 4.

Remove the excess water by using Whatmans filter paper and place the grid into the slot of the grid box. Support its release by using the filter paper to keep in the slot. Ensure that the grid deep enough in the slot before closing the box. Do not come close with your gloves since electrostatic charging might cause grids to “jump” out of the box.

Wait until the grids are dried before you start microscopy.

2. 11. 10. Preparation of Uranyl Acetate

Fort staining of sections uranly acetate will be dissolved either in ddw.. Usually, 1% or 2% UA solution in ddw will be used. However, to increase the contrast, sometimes it becomes necessary to prepare 2% UA in methanol. While UA should be kept over night to dissolve in ddw, it dissolves quickly in methanol.

2. 11. 11. Staining by using Uranyl Acetate

Staining by UA usually precedes staining by lead citrate. This combined combination of these stains is named Reinhold`s staining.

Staining of epoxy resin sections	5-8 min on a drop of 1-2% UA
Staining of Lowicryl sections	3 min on a drop of 1-2% UA

Droplets of UA solution will be placed on Parafilm by means of a glass Pasteur pippette (Use every droplet just one times). Grids with sections attached to the tanished side will be placed with the sections facing the droplets (shiny side of the grid facing up). This is in particular important for staining of sections on formvar.

Application of UA in methanol requires permanent monitoring and larger drops to prevent the complete evaporation of the solvent. The surface tension of such a droplet is low and the grid will not float.

Staining will be followed by 10 dips in a 50 ml beaker with ddw followed by 10 dips in a further beaker with ddw. Access water will be removed by contacting both grid and the tip of the tweezers with a piece of Whatman filter paper. After that, staining might be continued by using lead citrate.

3.RESULTS

3. 1. Central body isolation and identification of its components

To study the composition of the central body in *C. paradoxa* cyanelles, a method for central body isolation was developed. The various approaches to finding an optimal method are described in this chapter. Isolated cyanelles of *C. paradoxa* are osmotically stable due to their peptidoglycan wall which is thinner than the walls of extant cyanobacteria. It is therefore plausible that carboxysome isolation techniques developed for cyanobacteria should also work for *C. paradoxa* central bodies if modified. Since lysozyme treatment has already been investigated for the glaucocystophyte, it was integrated into our method of carboxysome isolation.

Cells of *C. paradoxa* were broken in a Waring Blendor and cyanelles isolated as described in section 2.2., treated with lysozyme (digestion of the peptidoglycan wall) and DNase (digestion of cyanelle DNA) for subsequent central body isolation: we isolated central bodies occluded by thylakoid membranes while excluding other cyanelle components through a Percoll gradient, and then aimed to separate central bodies from the thylakoid membranes (3.1.2.). Alternatively, we attempted to collect a pure central body fraction by chromatography using Sepharose CL-2B (3.1.1.). Fractions of isolated central bodies were loaded on SDS-PAGE gels for Coomassie- or Silver staining or Western analysis. The Rubisco large subunit (LSU) was used as a marker for the central body (band of 52 kDa). It had been previously demonstrated with Immuno-EM that antisera directed against Rubisco LSU primarily decorated central bodies (Mangeney and Gibbs, 1987).

For carboxysome isolation from (cyano)bacteria which are surrounded by a stable peptidoglycan wall, cells may be broken with a French press (e.g. 110 Pa). Then whole cells and large membrane fragments are removed by centrifugation and carboxysomes are purified on a Percoll-sucrose gradient, as was demonstrated with *Thiobacillus neapolitanus* (Shively, 1988). An alternative method for *Synechococcus* PCC7942 includes incubating cells with lysozyme with subsequent use of the French press and then pelleting the polyhedral bodies with 20% Percoll (v/v) in the presence of 27 mM Mg^{2+} and 0.1% Triton X-100. It was argued that Percoll beads

aggregate with carboxysomes in the presence of Mg^{2+} while Triton partially solubilizes thylakoid membranes, which are thus left behind in the supernatant after centrifugation (Price et al., 1992). As described, an isolation procedure for central bodies via two successive Percoll step gradients was developed. The size of the central body and its tight connection to cyanelle DNA and thylakoids necessitated a step involving mild detergent treatment of the thylakoid fraction to release part of the bound microcompartments. Isolated carboxysomes offered the potential for a proteomics approach and for the identification of carboxysome proteins other than Rubisco.

3.1.1. Chromatography over Sepharose CL2B

Bohnert et al. (1983) previously demonstrated that cyanelle DNA could be isolated associated with a large electron dense particle, which according to its size and often polyhedral shape was assumed to be the central body. This agglomeration was contained in a slightly yellowish fraction of the leading front collected when chromatography of lysed cyanelles over Sepharose CL-2B was performed. An electron micrograph of the authors shows cyDNA directly attached to the central body. We applied lysed cyanelles on a Sepharose CL-2B column following this method and attempted to isolate central bodies: low amounts were found in almost all collected fractions (yellowish; green-thylakoids; blue-phycobilisomes) but we failed to collect an essentially pure fraction.

3.1.2. Centrifugation over a Percoll gradient

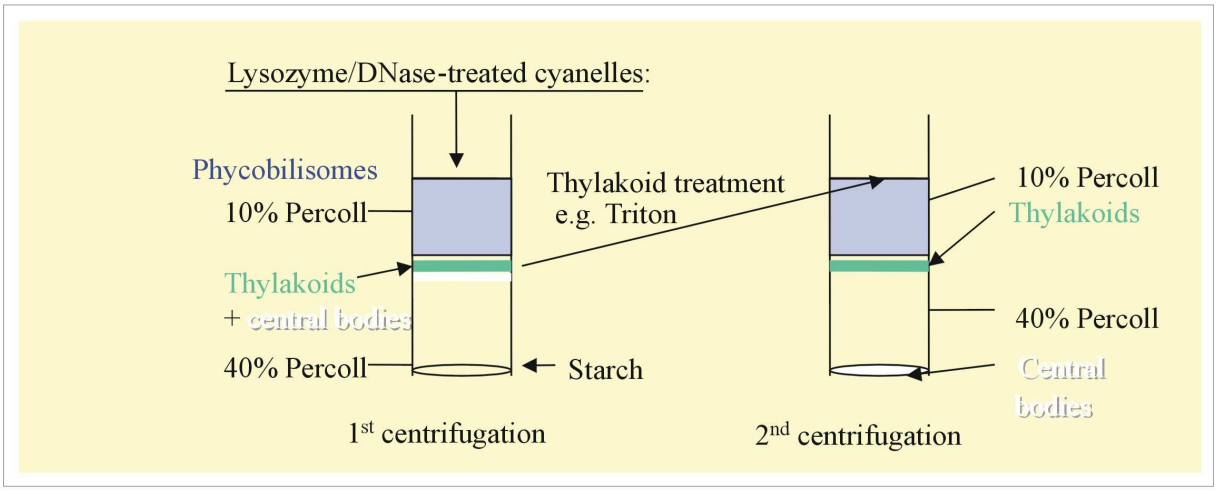


Figure 3.1.2.1. An isolation method for central bodies of *C. paradoxa* using two consecutive centrifugation steps on Percoll gradients.

A central body isolation method consisting of two subsequent Percoll gradients was established (Fig. 3.1.2.1.). Lysed cyanelles were layered on a gradient of 40% and 10% Percoll. A first centrifugation step separated thylakoids and attached central bodies (boundary 40%/10%) from the bulk of phycobiliproteins and starch. The thylakoid-central body fraction was aspired, treated with a detergent e.g. Triton or sonicated, and layered on a second Percoll gradient. Centrifugation achieved the separation of part of the central bodies from thylakoids. The central body pellet was collected.

Collected central body pellets appeared white while thylakoid contamination was visible as green specks. Thylakoid contamination of the pellet was avoided by e.g. reducing the concentration of detergent or centrifuging at lower rpm. The beneficial effect of Mg^{2+} (Price et al., 1992) on aggregating the (much smaller) cyanobacterial carboxysomes with Percoll could not be demonstrated with *C. paradoxa*.

Starch contamination (the main cytoplasmic reserve product) of central body pellets was considered problematic due to the attachment of Q-enzyme (branching enzyme) and starch synthase to starch granules. The presence of starch could be

determined by addition of iodine solution to sample fractions: brown starch particles were easily detected under the light microscope. Starch could be removed through a sucrose gradient (cyanelles are collected at the 55/50% sucrose interface) as demonstrated by Bohnert et al. (1982) but the reproducibility was not convincing. Using the method shown in Fig. 3.1.2.1., starch contamination could be eliminated to a large extent.

Only a part of central bodies can be separated from thylakoid membranes. We aimed to improve our isolation method to increase the proportion of separated central bodies while avoiding harsh treatment. Central bodies, quantified by the Rubisco LSU 52 kDa marker band, may be separated from thylakoids purely by centrifugation (Fig. 3.1.2.2.A, control). Central body pellet size was found to vary with centrifugation speed (data not shown). Concluding from the majority of isolations performed, the pellet size may be increased noticeably by including a low amount of detergent. Thylakoids were treated with Triton in combination with vortexing: 0.5% Triton was comparable to use of 0.1%, whereby vortexing for less than 30 sec was less effective for attaining a central body pellet (Fig. 3.1.2.2.A & B). Sonication is another possibility for central body - thylakoid separation (Fig. 3.1.2.2.B), rendering large central body pellets. As this method is likely to resolve protein complexes, it was decided not to utilize it. Antibody detection of Rubisco LSU revealed its presence in the pellet collected from treated thylakoids (Fig. 3.1.2.2.C). It is interesting to note that Rubisco, besides being localized to the central bodies, is commonly found also in its soluble form in *C. paradoxa* (Fig. 3.1.2.2.A & B, lanes c). Likely this is the result of central body disintegration during the isolation process.

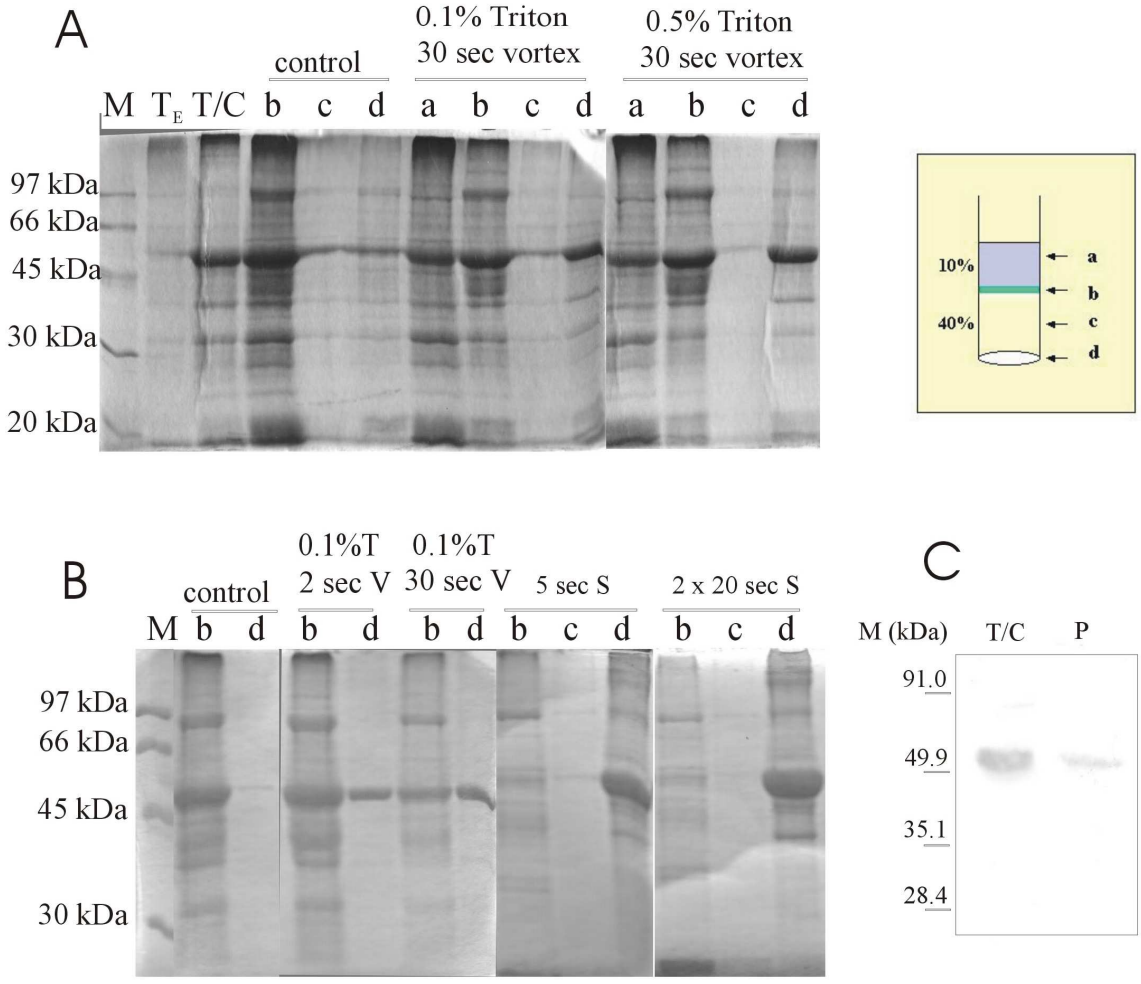


Figure 3.1.2.2. Treatment of isolated thylakoids with Triton in combination with vortexing as well as sonication separates part of attached central bodies.

Isolated cyanelles were digested with lysozyme, layered on a Percoll gradient and following centrifugation (HB-4, 16500g, 10 min, 4℃) thylakoid membranes were collected from the 40/10% boundary. Thylakoids were treated as indicated. Individual samples treated consisted of 150 µl thylakoids equivalent to 100µg chlorophyll a : Triton X-100 was added in various concentrations and samples vortexed or sonication performed as indicated. Treated samples and the untreated control were layered on 1 ml 40% Percoll and centrifuged at 3000 rpm for 3 min at 4℃. Aliquots *a-d* (40 µl of a-c; 30 µl of d) were taken from resulting layers and denatured in 2 x SDS-sample buffer.

A: *M*, Marker; T_E, total cyanelle extract; *T/C*, untreated thylakoids; *Control*, no treatment of thylakoids; Layers *a*, phycobilisomes (bands 14-21 kDa); *b*, thylakoids (noticeable band 83 kDa, PS I reaction center subunit PsaB); *c*, clear phase; *d*, central body pellet (marker band Rubisco LSU 52 kDa).

B: *M*, Marker; *Control*, no treatment of thylakoids; Treatment of thylakoids: *T*, triton; *V*, vortex; *S*, sonication.

C: *M*, Marker; *T/C*, untreated thylakoids; *P*, central body pellet from thylakoids treated with 0.1% Triton and vortexed 30 sec. After electrophoresis and gel blotting, a primary antibody was directed against Rubisco LSU was usedfor Western analysis.

Treatments with SDS (sodium dodecyl sulphate) (0.1-0.5%) and vortexing for 30 sec as well as with Hepes pH 8.0 (e.g. 5 mM) were compared and showed no advantage to Triton.

The nature of the interaction between central body and thylakoids is probably influenced by the cyanelle DNA located around the central body. During thylakoid isolation, digested cyanelles are incubated with 30 µg/ml DNase. Part of cyanelle DNA should thus be digested. Speculating that remaining DNA still could hamper central body isolation, DNase I (up to 100 µg/ml) was further added to thylakoids. Unfortunately, this had no noticeable effect (data not shown).

Silver staining of cyanelle components separated on SDS-PAGE gels revealed bands of potential interest which were, in part, predominantly present in central body pellets compared to total cyanelle extract and untreated thylakoids (Fig. 3.1.2.3.).

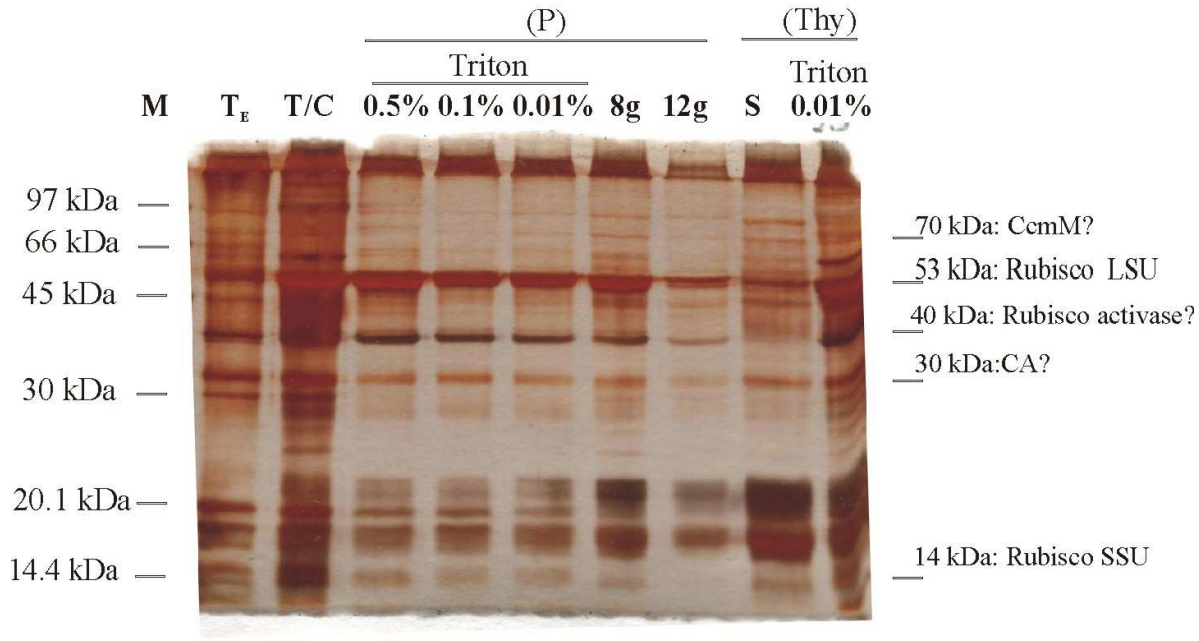


Figure 3.1.2.3. Silver staining reveals bands predominantly present in central body pellets.

Thylakoids were isolated from cyanelles as described for Fig. 3.1.2.2. and washed with 1 x SRM for removal of phycobilisomes. Individual samples treated consisted of 150 µl thylakoids equivalent to 100µg chlorophyll (a) : Triton X-100 was added in the concentrations indicated and samples vortexed for 30 seconds or sonication was performed for 5 seconds. Treated samples were layered on 1 ml 40% Percoll and centrifuged at 3000 rpm for 3 min at 4℃. Untreated thylakoids were centrifuged at indicated rpm for 3 min at 4℃. 40 µl of thylakoid fractions and 30 µl of collected pellets were denatured in 2 x SDS-sample buffer. 5 µl of each pellet and 2.5 µl T_E, T/C and B was loaded on 12% SDS-PAGE gel. The gel was subsequently silver-stained.

M, Marker; *T_E*, total cyanelle extract ; *T/C*, untreated thylakoids; *P*, central body pellets collected from centrifugation of thylakoids layered on 40%/10% Percoll; *0.5%, 0.1%, 0.01%,* thylakoids previously treated with Triton in indicated concentrations and 30 sec vortexing; *8g* and *12g*, 8000g and 12000g, centrifugation speeds for untreated thylakoids; *Thy*, thylakoids; *S*, sonicated 5 sec; *0.01%*, Triton and 30 sec vortexing.

Indicated on the right side are sizes of peptides, which upon comparison of lanes, are putatively localized to the central body.

3. 2. Rubisco activase

Rubisco activase from *C. paradoxa* was first obtained as an EST for the microarray project. It corresponds to the type found in plants and *C. reinhardtii* as vealed if by the sequence of the full-lengh cDNA which was completed through 5′ RACE (Fig.3.2.). Transcription of the gene is upregulated upon shift from high to low [CO_2] (Burey et al. 2007). The protein cross-reacts with antisera directed against Rubisco activase from tobacco and is thus a *bona fide* component of the “carboxysome” as corroborated by mass spectrometry (Fathinejad et. al., 2007).

5’-

MAFVGTPVAALSSAPALATSSKICKVAQEVKSAKAAAFGEKKSSFFYNPIAG
AKAAAGKVEFSVQA↓**GLNPDGSFSPDGGYSAPAPAKKQGGYSDSWGSALAND**
SSAGNDQLDIRRGRGMVDKKFQGAGMGLGSTHVVIQDSIEYYNTAKRTFGNI
QGDFYICPTFMDKIVLHITKNFLNLPKVKVPLILGIWGGKGQGKSFQCELVY
KSLGIEPILMSSGELEDASAGEPAKLIRQRYREAAEVIKKGKMCVLHINDLD
AGAGRMGGTTQYTVNNQMVNATLMNIADNPTNVQMPGMYNAEELPRVPIVVT
GNDFATLYAPLIRDGRMEKFYWNPTREDRIGVCWGIFKEDGISEQDVAALVD
AFPDRSIDFFGSLRSRVYDDEIRKFIEKTGVENLSKRIVNTKDPLPEFTKPH
ITLQTLMTYGQRLSNEMRLVQEVKLAEEYVANLADERTIAADLRARGVKSRM
AGLNDDGSDDY

Figure 1. Pre-Rubisco Activase from *Cyanophora paradoxa* showing the N-terminal transit sequences typical for cyanelles.
The putative processing site is indicated by an arrow. The mature protein is given in bold letters.
MS sequencing of two peptides (red color) from the 47 kDa band. Peptide map of the 47 kDa band: 45% Coverage to above sequense.

3. 3. Import

Cyanelles can be used as “honorary Cyanobacteria”: microcompartment assembly processes, e.g. the incorporation of linker polypeptides into isolated, intact phycobilisomes (Steiner et al, 2003) can be studied *in vitro*.
Import of the labelled pre-Rubisco activase into isolated cyanelles and subsequent fractionation revealed an incorporation of 25-30% into the central body fraction within an incubation time of 30 min. This indicates a dynamic structure for the microcompartment which is expected to expand and shrink according to CO_2 availability.

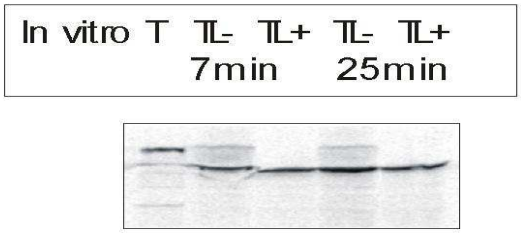
3.3.1. Small scale isolation of central bodies after import experiment

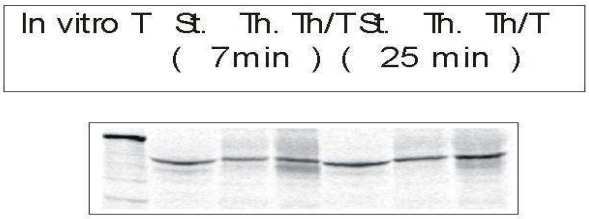
The import reaction was stopped by the addition of 1 ml ice-cold SRM buffer followed by centrifugation at 800g and 4℃ for 2 min. The cyanelle pellet (without thermolysin treatment) was washed in SRM, resuspended in 1 ml 2x SRM, and incubated for 25 min at room temperature with 30 µl of a 10 mg.ml-1 lysozyme stock solution, which led to digestion of the peptidoglycan wall and cyanelle lysis. After DNAse treatment, the lysate was layered on top of a step gradient consisting of two percoll layers (10% and 40% respectively) and centrifuged. Thylakoid membranes with bound central bodies banded at the interface. The thylakoid layer was carefully removed and after that was treated with SRM buffer containing 0.1% Triton X-100, and loaded on top of a second gradient of analogous composition. After centrifugation, a whitish pellet was obtained indicating that mild detergent treatment resulted in partial release of the central bodies from the thylakoid membranes.

3. 3. 2. Gel electrophoresis

Proteins from intact cyanelles and from the stroma, thylakoid, and central body fractions were separated on SDS-polyacrylamide gradient gels (10-18%). Import

data were analyzed using a PhosphoImager and the Molecular Dynamics IMAGE QUANT version 3.3 program, ensuring that all the signals remained in the linear detection range.





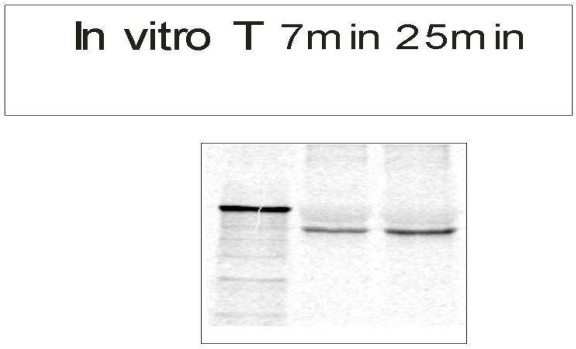


Figure: 3.3.2. *In vitro* assembly of Rubisco activase in the central body.Tr- translation mix

1. Cyanelle import of Pre-Rubisco activase +/- with and without thermolysin
2. Cyanelle lysis and fractionation

St. Stroma, Th. Thylakoids, Th/T. Thylakoids treated with Triton X-100

3. Cyanelle lysis and fractionation

Central body pellet

3. 4. Mass spectrometry

Fractions of isolated central bodies were loaded on SDS-PAGE gels for Coomassie and Silver staining. After Coomassie staining and destaining the gels were sent for analysis to the MALDI-MS/MS lab. Bands of interest,i .e. those that were enriched during central body preparation or that yielded signals on western blots were further investigated.

Peptide masses from the (major) 52 and 103 kDa bands and partial amino acid sequences generated via MS/MS of selected peptides corresponded to Rubisco large subunit (LSU), and to the phycobilisome core-membrane linker polypeptide ApcE, respectively. ApcE, together with a 50 kDa band assumed starch synthase, were contaminants of the isolated fraction. However the presence of shell proteins, encoded by *ccm* genes, thus far could not be shown for *C. paradoxa*. They would be profound evidence for a “eukaryotic carboxysome” in this glaucocystophyte. It could be argued that the use of Triton effectuates the (partial) loss of the shell of these delicate microcompartments. Due to their relatively large size and tight association with the thylakoids, their isolation has to be a compromise between intactness and purity.

The sensitivity of the mass spectrometric method is sufficient to characterize even minor bands. Nevertheless, we estimate that more EST data for *C. paradoxa* will enable the discrimination between *bona fide* components of the central body and contaminating proteins. 2D-gel analysis could also be carried out. The identification of a cyanelle CA would be of prime importance in this respect.

In the case of *Cyanophora*, we could unequivocally demonstrate a tight association of Rubisco activase with its substrate, Rubisco, in the cyanelle microcompartment. Interestingly, while Rubisco LSU is highly conserved, i.e. the identity scores between the cyanelle protein and its cyanobacterial counterparts lie between 83 and 84%, a BLAST search with the cyanelle Rubisco LSU against Cyanobase nevertheless produced top ranking hits for the filamentous, nitrogen-fixing and activase-containing cyanobacterial species *Anabaena* 7120, *Nostoc punctiforme* and *Trichodesmium erythraeum* (data not shown). The catalytic mechanism of cyanobacterial Rubisco activase might somewhat differ from that of the eukaryotic

enzyme (Portis, 2003), which raises the question about the catalytic mechanism of the plant-type *Cyanophora* activase vis-à-vis its cyanobacterial-type substrate.

In a first series of experiments, several bands ranging in size from 30 to 103 kDa were subjected to in-gel digestion (see figures). Peptide masses from the (major) 52- and 103-kDa bands and partial amino acid sequences generated via MS/MS of selected peptides corresponded to Rubisco and to the phycobilisome core-membrane anchor polypeptide ApcE (a contaminant), respectively.

45% peptide coverage was reached and sequenced peptides proved to be identical to Rubisco activase sequences (see figures).

This is the first demonstration of Rubisco activase in a carboxysome-like microcompartment.

No conclusive results have been obtained from database searches either with respect to peptide pattern or to partial sequences for the other bands investigated. In particular neither a carbonic anhydrase nor a shell protein could be identified.

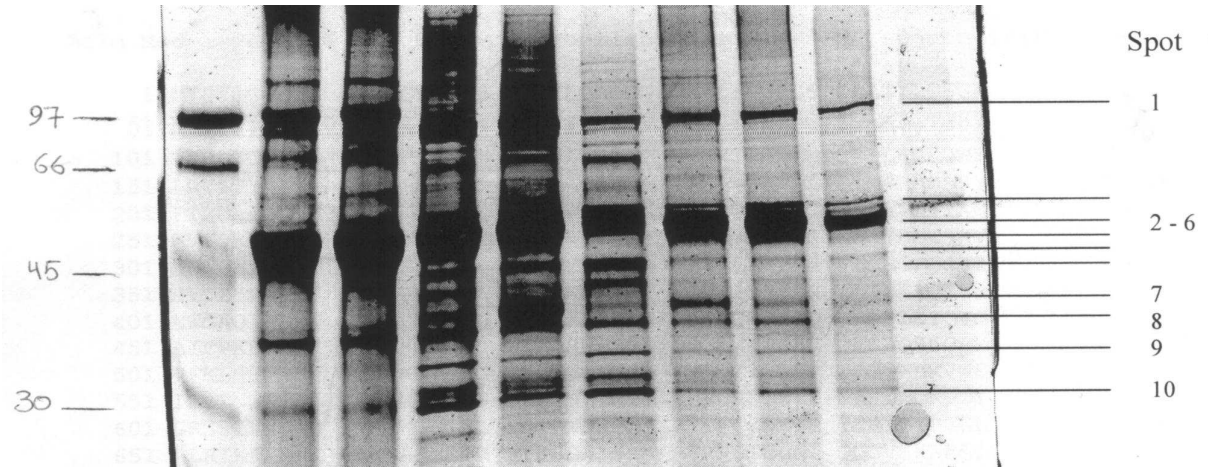


Figure 3.4.1. Coomassie Staining

SDS- PAGE of the central body pellet, several bands ranging in size from 30 to 97 kDa were subjected to in-gel digestion.

In a second series of experiments, similar conditions were adopted and some bands were reinvestigated, together with some new determinations (Fig. 3.4.2.) in the hope to identify a carbonic anhydrase. This goal was not achieved, a second slightly smaller band was also correlated with Rubisco activase. Glyceraldehyde phosphate dehydrogenase (contaminant) was made likely. RbcL and ApcE could be confirmed, all other bands gave in part nice peptide patterns but at present identification via existing databases was not possible (Table 3).

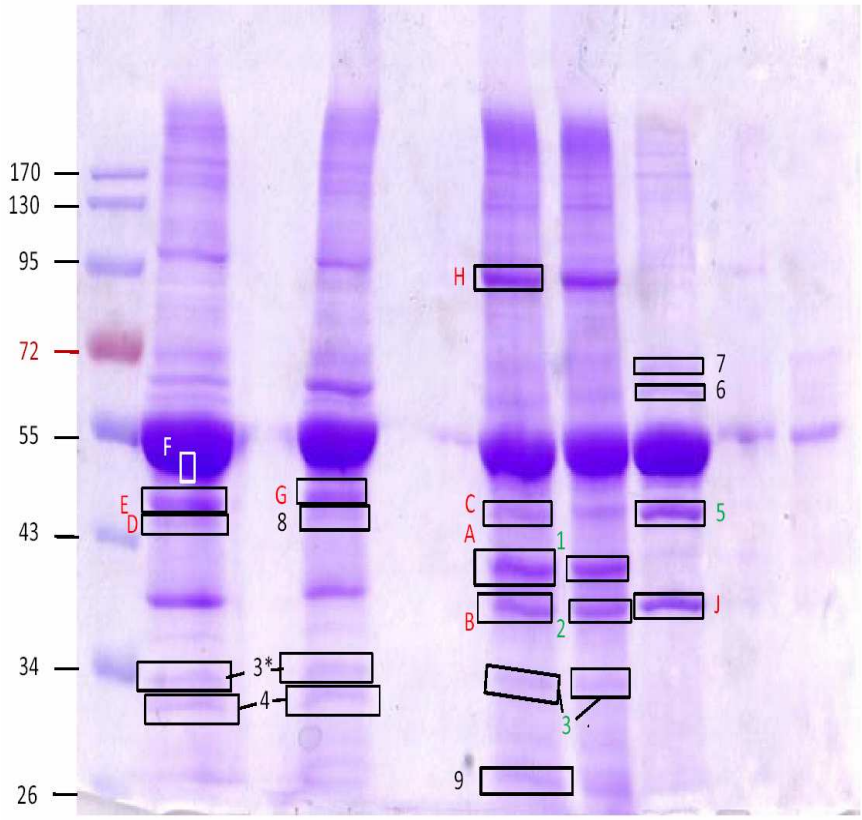


Figure 3.4.2. Coomassie Staining

SDS- PAGE of the central body pellet, several bands ranging in size from 30 to 130 kDa were subjected to in-gel digestion.

Thylakoids were isolated from cyanelles as described for Fig. 3.1.2.2. and washed with 1 x SRM for removal of phycobilisomes. Individual samples treated consisted of 150 µl thylakoids equivalent to 100µg chlorophyll (a): Triton X-100 was added in the concentrations indicated and samples vortexed for 30 seconds or sonication was performed for 5 seconds. Treated samples were layered on 1 ml 40% Percoll and centrifuged at 3000 rpm for 3 min at 4℃. Untreated thylakoids were centrifuged at indicated rpm for 3 min at 4℃. 40 µl of thylakoid fractions and 30 µl of collected pellets were denatured in 2 x SDS-sample buffer. The gel was subsequently Coomassie-stained.

1, Marker; *2*, 2xSRM without DNAse with Triton ;3, 2xSRM with DNAse and with Triton ; *4*, 2xSRM washed central body; 5, Empty
6,and 7, pellets collected from centrifugation of thylakoids layered on 40%/10% Percoll (Treatment with sodiumcarbonate and sonication), 8, pellets collected from centrifugation of thylakoids layered on 40%/10% Percoll (Treatment with carbonat and 2xSRM- DNAse + Mg^{2+}), 9, 2xSRM sonication, 10, 2xSRM Triton and DNAse + Mg^{2+}

Spot	MW	Protein	Comment
Spot F	50kD	RUBISCO	Confirmed
Spot A Spot 1	38kD	?	both bands have same level and size at the Gel, PMF identical, as 1 Spot treated, Not compatible to Carbonic Anhydrase
Spot B Spot J Spot 2	36kDa	Glyceraldehyde-3-phosphate dehydrogenase ?	1 peptide sequence indicated by BLAST GAP-Dehydrogenase with E-value 2.3 3 further peptids not compatible from PMF – More than 1 Protein?
Spot 8 Spot 5 Spot C Spot D	45kDa	RUBISCO Activase	All bands from the same size level, measured at 2 days, slightly different PMF (8=5=C, D), D had 1 more peptid but not compatible to Activase Confirmed
Spot G Spot E	>45kDa	RUBISCO Activase	Separately treated from 8,5,C,D , because of location above them 2 peptides not contained in Activase
Spot 3	34 kDa	?	PMFs of the three smaller proteins not compatible to Carbonic Anhydrase No distinct identification possible
Spot 9	26 kDa	?	
Spot 6	68 kDa	?	
Spot 7	70 kDa	?	
Spot 4	32 kDa	?	
Spot H	95 kDa	Phycobilisome Linker	Confirmed

Table 3. Mass spectrometric interpretation of isolated bands of the central body from *C. paradoxa* cyanelles.

Third series: This time a gradient gel was used to achieve better separation in the low MW range (Fig. 3.4.3.). Comparison of the results with individual bands from series 1 and 2 were done where ever possible (Table 4). Rubisco activase and Rubisco LSU were confirmed again and the 10-12 kDa band could be clearly identified as Rubisco SSU. Lysozyme, used for cyanelle lysis, was shown to be the 14 kDa contaminant. The other bands did not allow the identification of Carbonic anhydrase (CA) or shell protein candidates.

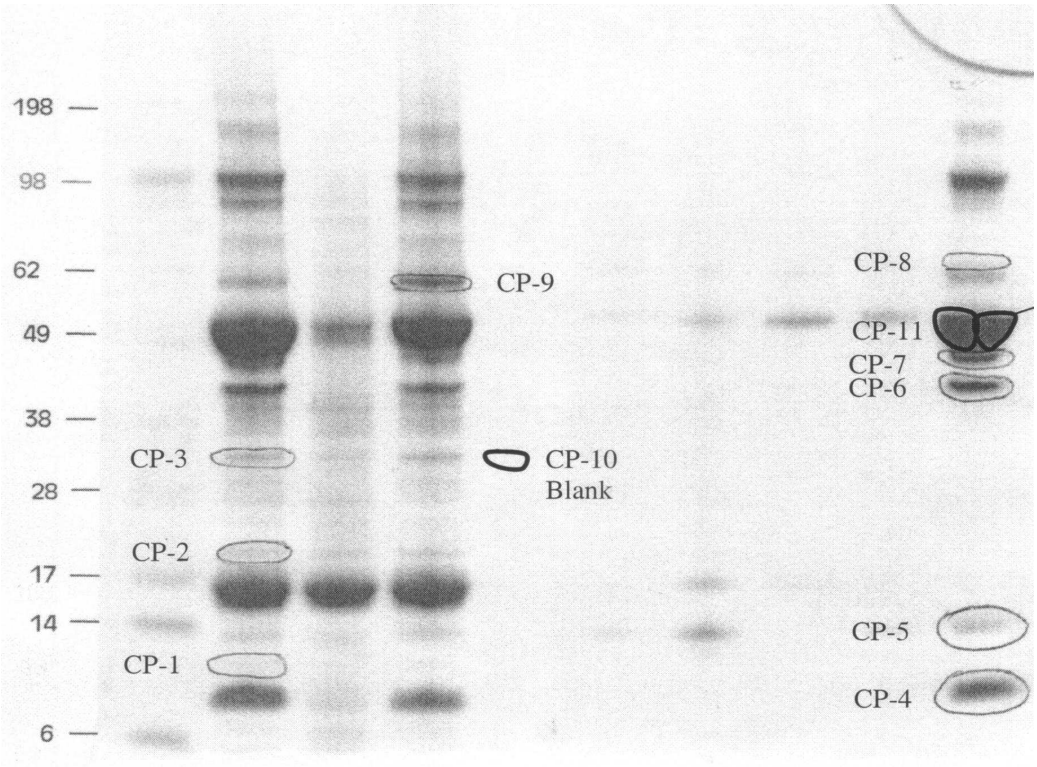


Figure 3.4.3. Coomassie Staining

Gradient-Gel (4-12% , Bis-Tris). The central body pellet, several bands ranging in size from 30 to 130 kDa were subjected to in-gel digestion.

Thylakoids were isolated from cyanelles as described for Fig. 3.1.2.2. and washed with 1 x SRM for removal of phycobilisomes. Individual samples treated consisted of 150 µl thylakoids equivalent to 100µg chlorophyll (a): Triton X-100 was added in the concentrations indicated and samples vortexed for 30 seconds or sonication was performed for 5 seconds. Treated samples were layered on 1 ml 40% Percoll and centrifuged at 3000 rpm for 3 min at 4℃. Untreated thylakoids were centrifuged at indicated rpm for 3 min at 4℃. 40 µl of thylakoid fractions (lanes2-4) and (lanes 6-9) 30 µl of

collected pellets were denatured in 2 x SDS-sample buffer. The gel was subsequently Coomassie-stained.

1, Marker; *2*, 2xSRM without DNAse with Triton ;3, 2xSRM with DNAse and with Triton ; *4*, 2xSRM washed central body; 5, Empty
6,and 7, pellets collected from centrifugation of thylakoids layered on 40%/10% Percoll (Treatment with sodiumcarbonate and sonication), 8, pellets collected from centrifugation of thylakoids layered on 40%/10% Percoll (Treatment with carbonat and 2xSRM- DNAse + Mg^{2+}), 9, 2xSRM and sonication, 10, 2xSRM Triton and DNAse + Mg^{2+}

Spot	MW Gel	Protein	Comment	Comparison with other gels
CP-11	49 kDa	Ribulose biphosphate carboxylase large chain RUBISCO LSU	as control a digestion without PSDs	PMF (Peptid Mass Fingerprint) equivalent Spot F
CP-4	10 kDa	Ribulose biphosphat carboxylase small chain RUBISCO SSU		Nonexistent- MS very small (undersized)
CP-5	14 kDa	Lysozym		Nonexistent- MS very small (undersized)
CP-7	45 kDa	Activase		Peptid Mass Fingerprint comparable with Spot 8/5/C/D/G/E
CP-1	12 kDa	Possibly RUBISCO SSU rates		Nonexistent- MS very small (undersized)
CP-2	20 kDa	?		Nonexistent- MS very small (undersized)
CP-6	42kDa	?		No possiblity for comparison of Peptid Mass Fingerprint with Spot 8/5/D/C/G/E (Activase) and also not with Spot A (n. id).
CP-8	62 kDa	?	Peptid Mass Fingerprint similar zu CP-9	2 peaks from Peptid Mass Fingerprint accord to peaks of Spot 6
CP-9	60 kDa	?		2 peaks from Peptid Mass Fingerprint accord to peaks of Spot 6
CP-3	32 kDa	?	GAVTNQST[278]F or F[278]TSQNTVAG	Peptid Mass Fingerprint not comparable with B/J/2

Table 4. Mass spectrometric interpretation of isolated bands of the central body from *C. paradoxa* cyanelles.

3. 5. Carbonic anhydrases

For attaining an improved depiction of the *C. paradoxa* CCM, isolation and characterization of carbonic anhydrase (CA) genes was focused on.

Provided a cyanelle CA could be found for *C. paradoxa*, a ß-type enzyme would imply carboxysomal origin while a β-type would point to an algal chloroplast CA. A N-terminal transit sequence would allude to a cyanelle localization of the protein while a transit sequence with an additional thylakoid transfer domain would indicate localization in the thylakoid lumen, as is the case for *Chlamydomonas reinhardtii* (Hanson et al., 2003).

Evidence for a “eukaryotic carboxysome” in *C. paradoxa* would be co-packaging of Rubisco and carbonic anhydrase whereby the CA would be distinguished by a C-terminal extension.

PCR amplification of a potential *C. paradoxa* cyanelle CA from genomic DNA using degenerate primers designed according to consensus regions of all comparable carboxysomal CA sequences available to this date (*Synechococcus* PCC7002, *Synechococcus* PCC7942, *Nostoc punctiforme* and *Synechocystis* PCC6803) was not successful and it was made by a different PhD student.

Various fractions of lysozyme-digested cyanelles of *C. paradoxa* including isolated carboxysomes were loaded on SDS-PAGE gel and blotted to a nitrocellulose membrane. Western analysis was performed with CcaA (IcfA) directed against the *Synechocystis* PCC6803 carboxysomal ß-type CA (received from George S. Espie) as primary antibody and Anti-Rabbit IgG (Fc) conjugate as secondary antibody using the method according to So and Espie, 1998. A protein of approx. 28 kDa should be detected. As a positive control *Synechococcus sp.* PCC6301 (gift from Georg Schmetterer) was used. Unfortunately, many unspecific signals e.g. from Rubisco LSU were obtained. Using higher dilutions of primary and secondary antibody, results were not improved. To date, a cyanelle CA for *C. paradoxa* has not been identified.

3. 6. Electron microscopy

The morphology and size of the central bodies and the distribution of Rubisco throughout the cyanelles were compared for high- and low-CO_2 cells using a JEOL 1210 transmission electron microscope. In addition to conventional methods, high pressure cryofixation (Empact HP-freezer, LEICA Microsystems) in combination with freeze substitution (AFS, LEICA Microsystems) was employed to preserve the fine structural details. The “carboxysome” pellets obtained after (modified) purification were also inspected by EM.

3.6.1. The central body of glaucocystophyte cyanelles in EM

The number of existing electron micrographs (EMs) of *C. paradoxa* is relatively small. We wanted to fully confirm that no thylakoids penetrate the central body (CB) and to find indications for a polyhedral structure. If possible, we would like to demonstrate a shell also for *C. paradoxa* cyanelles. A major goal was to define eventual differences in CB morphology between cells grown under high and low [CO_2].

In no case are these microcompartments traversed or penetrated by thylakoid membranes (see e.g. Fig.3.6.1). They remain stable during cyanelle division and are neatly halved through the peptidoglycan septum. In *C. paradoxa* the microcompartment mostly has a rounded shape though sometimes polyhedral elements become apparent (Fig 3.6.2.). Under high [CO_2] the contours of the CBs are not clearly defined. Electron-dense material (storage carotenoids?) is a bundant among thylakoids proteinal to the center. Cyanelle ribosomes appear around the central body (for Rubisco synthesis) and between the thylakoid (for phycobiliprotein synthesis) membranes. (Figs. 3.6.1. and 3.6.2.).

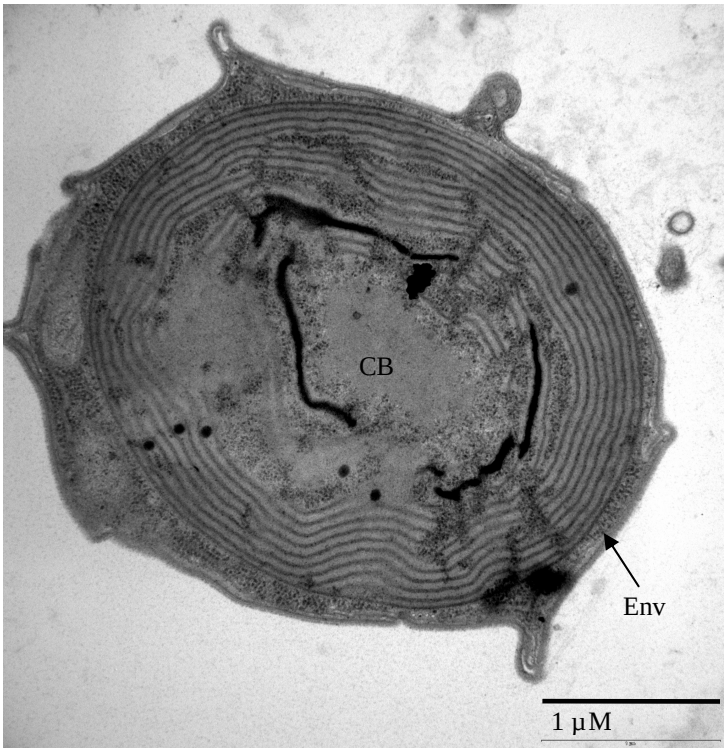


Figure 3.6.1. Transmission electron micrograph of a *Cyanophora* cell grown under high [CO_2]. Electron-dense regions around the innermost thylakoids are notable. (scale bar, 1µM). Chemically fixed and epoxy resin embedding. CB, central bodies; Env, envelope.

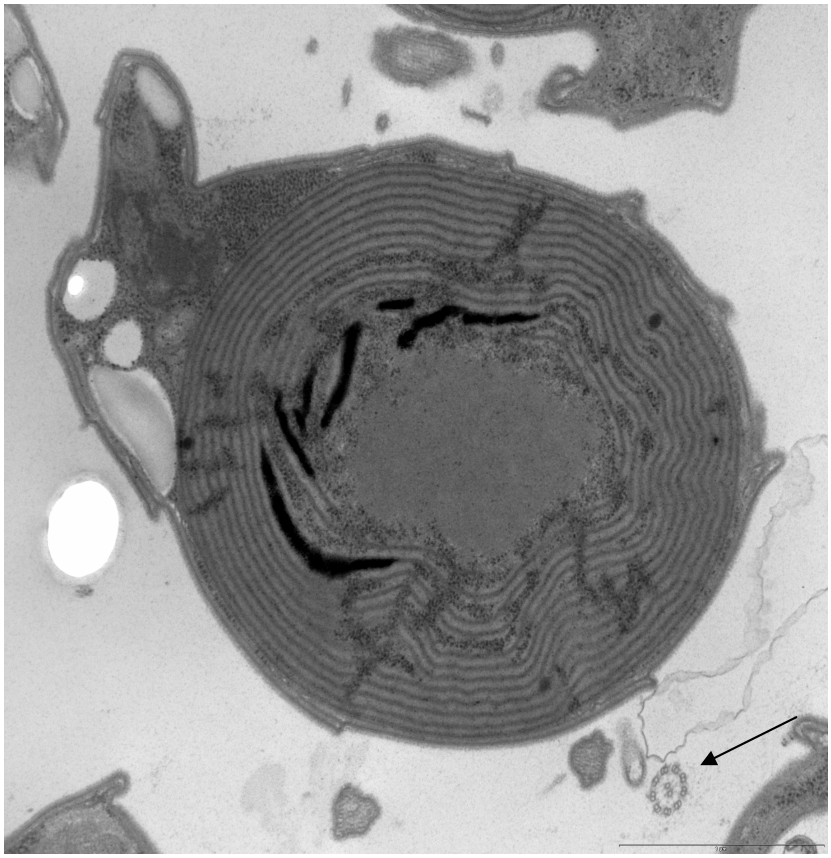


Figure 3.6.2. Transmission electron micrograph of a *Cyanophora* cell grown under high [CO_2]. Chemically fixed and epoxy resin embedding. Electron-dense regions around the innermost thylakoids are notable. (scale bar, 1µM). Ribosomes around central body and between thylakoids. A flagellar basal body was accidentally cut showing the (9+2) structure (arrow).

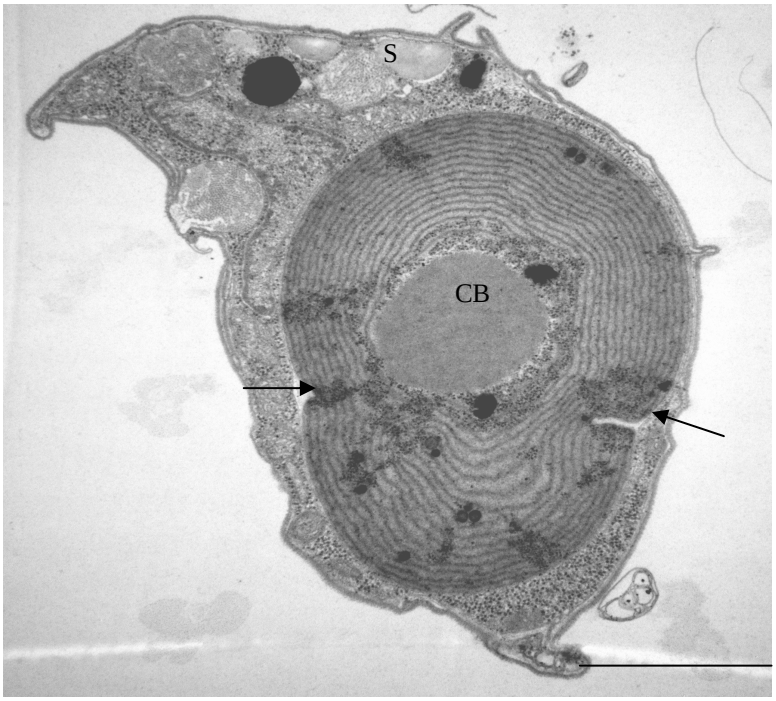


Figure 3.6.3. Transmission electron micrograph of a *Cyanophora* cell grown under low [CO_2]. (scale bar, 1µM). Chemically fixed and epoxy resin embedding. CB, central body; S, starch granule; arrows indicate the onset of septum growth. Indication for a polyhedral shape of CB. Cyanelle ribosomes concentrated around CB.

For low [CO_2] cells, the average size of the central bodies is increased (Figs. 3.6.3. and 3.6.4.).

The concentration of ribosomes around the central body indicates their main occupation with Rubisco biosynthesis whereas ribosome density in the thylakoid region is less than under high [CO_2]. Also, the CB is better defined and the number of starch granules seems to be higher, compared to high [CO_2] cells. With chemical fixation, no proof for the existence of a shell could be obtained, regardless of the [CO_2].

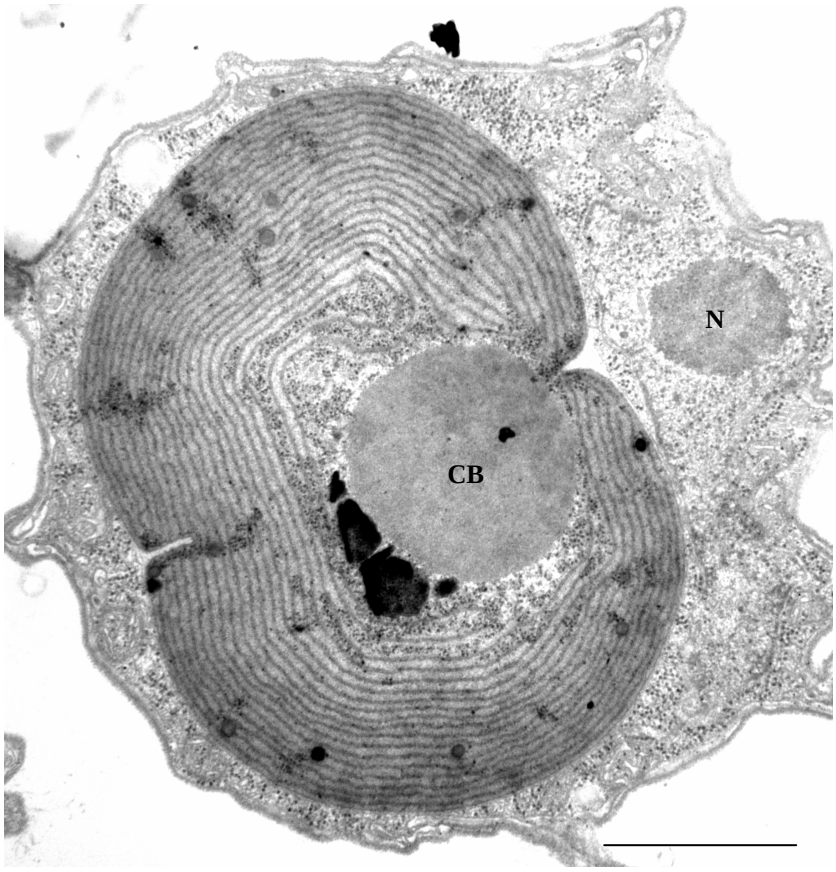


Figure 3.6.4. Transmission electron micrograph of a *Cyanophora* cell grown under low [CO_2]. (scale bar, 1µM).

Chemically fixed and epoxy resin embedding. CB, central body; N, nucleus; The onset of septum growth is observed. Indication for a polyhedral shape of CB (arrows).

Under cryofixation vacular structures are better preserved.

Also the periplasmic space is exposed. Polyhedral elements of the CB are seen sometimes. The boundaries of the CB are some what better defined under low [CO_2], although shell-like contours could not be demonstrated (Figs. 3.6.5. and 3.6.6.).

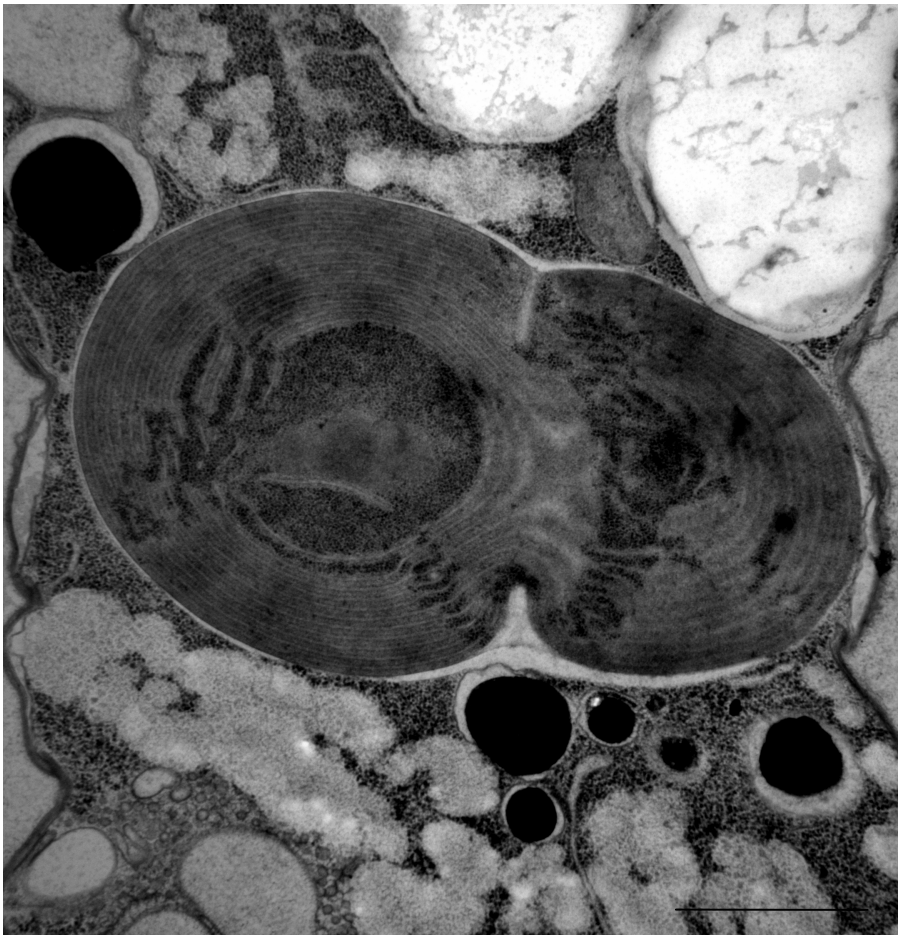


Figure 3.6.5. Transmission electron micrograph of a *Cyanophora* cell grown under high [CO_2]. (scale bar, 1µM). High-pressure freezing. (Cryofixation)

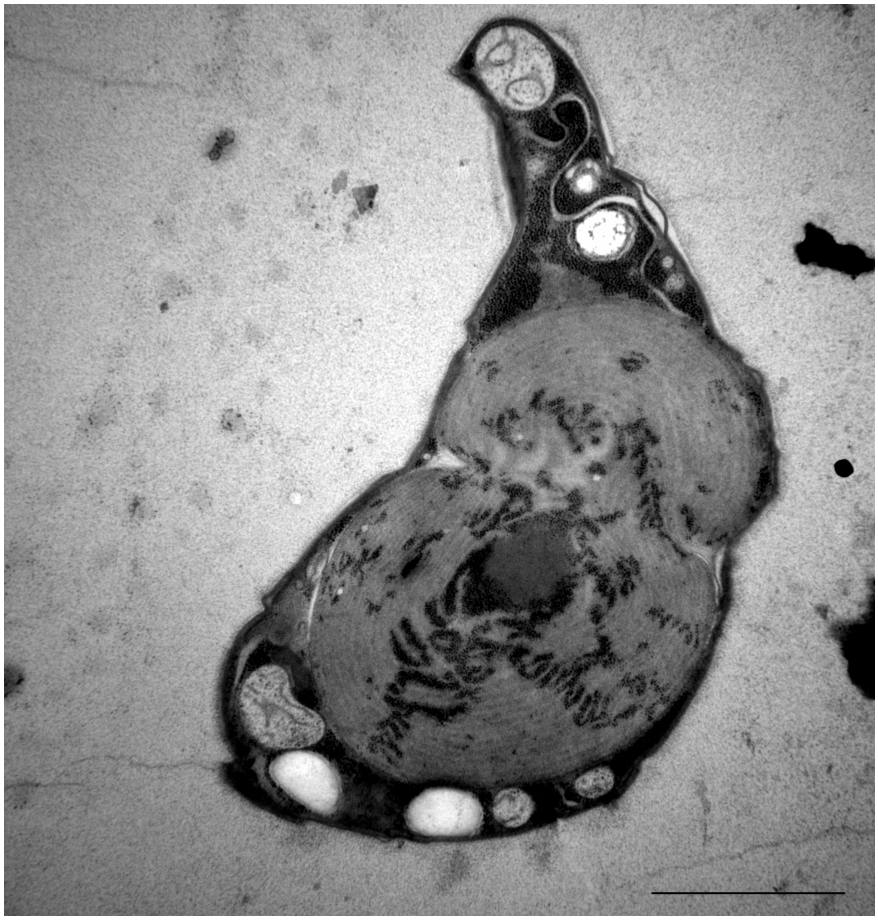


Figure 3.6.6.Transmission electron micrograph of a *Cyanophora* cell grown under low [CO_2]. (scale bar, 1µM). High-pressure freezing. (Cryofixation)

It was also important to inspect the state of the purified CBs. Carboxysomes as well as pyrenoids are known as delicate structures and with detergent treatment was inevitable to get the CBs off the thylakoid membranes. Indeed, fig 3.6.7. shows a CB with an electron translucent center without the usual smooth bondaries.

This preparation also contained membranous contaminations. The cleaner preparation in fig 3.6.8 depics the isolated CB of a dividing cyanelle. The higher magnification allows to better recognize the granular, quasi-crystalline substructure known from cyanobacterial carboxysomes (Kaneko et al., 2006).

Negatively stained CB preparations (figs, 3.6.9. and 3.6.10) appear to be collapsed, showing the individual Rubisco molecules with some tendencytowards self-assembly. This largely parallels reports on negatively stained cyanobacterial carboxysome preparations (Orus et al., 1995).

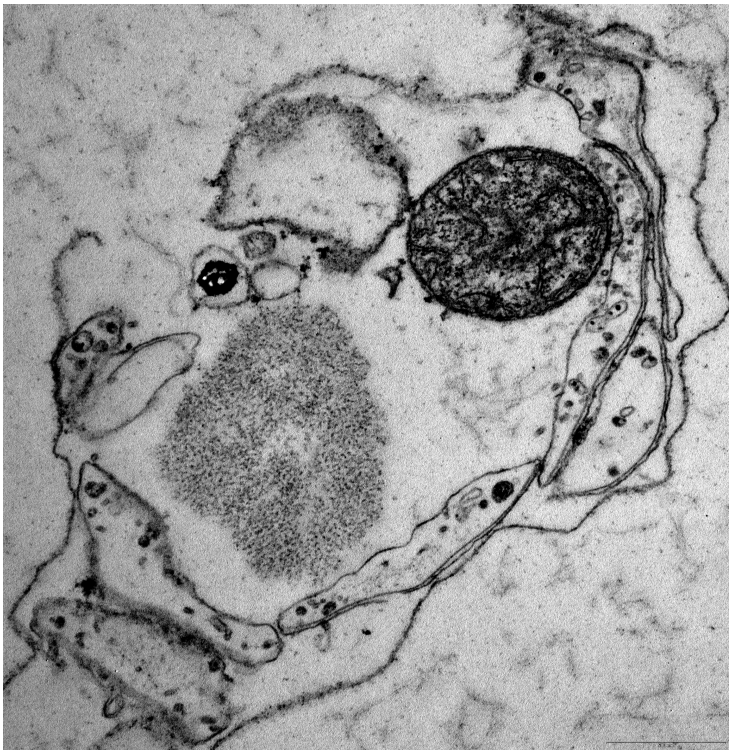


Figure 3.6.7. Transmission electron micrograph of a central body preparation from *Cyanophora*. Chemically fixed and agar-resin embedded.

Some contaminations are contained. The central body (CB) shows a granular substructure. Scale bar 500 nm.

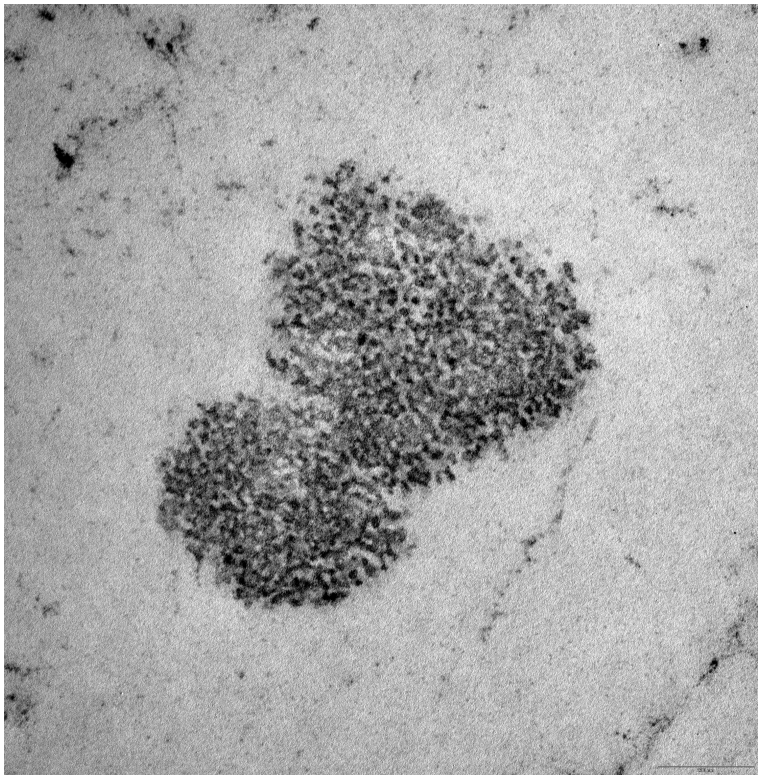


Figure 3.6.8. Transmission electron micrograph of a central body preparation from *Cyanophora*.
Chemically fixed and agar-resin embedded.
Central body (CB) from a dividing cyanelle: partially disintegrated, granular substructure.
scale bar 250 nm.

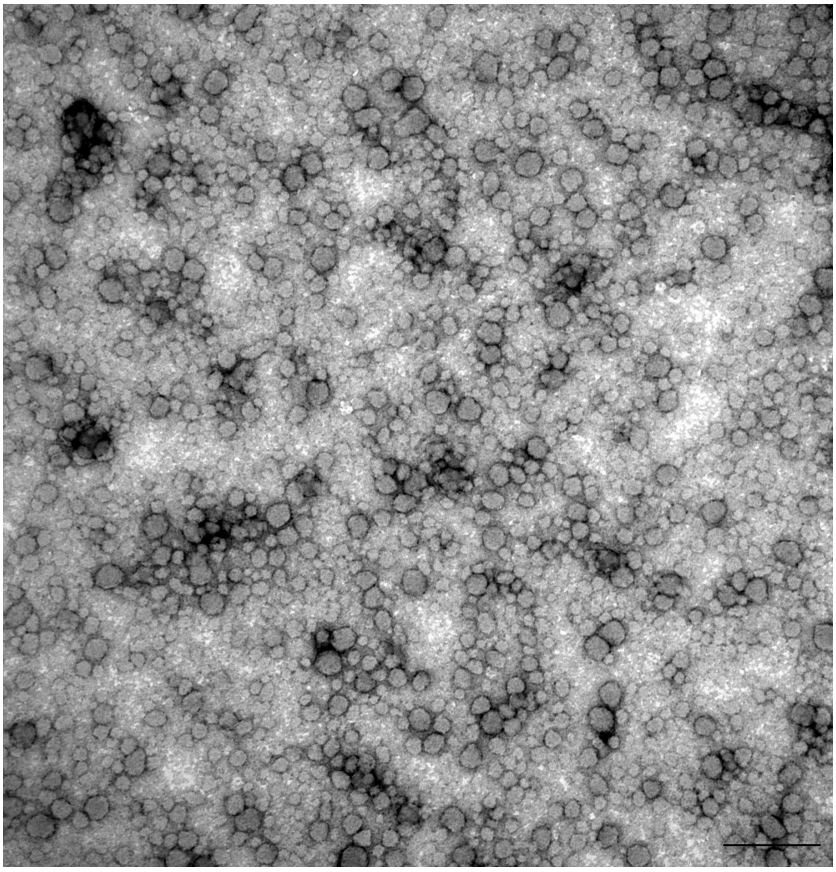


Figure 3.6.9. Negatively stained central body preparation from *Cyanophora paradoxa*.
Samples of isolated central bodies were negatively stained by floating carbon- coated grids onto a sample drop for 4 min, followed by refloating onto a 2% solution of uranyl acetate for 50 seconds. Grids were observed in a Jeol transmission electron microscope at higher magnification than in figures 3.6.7. and 3.6.8. (scale bar 100 nm).

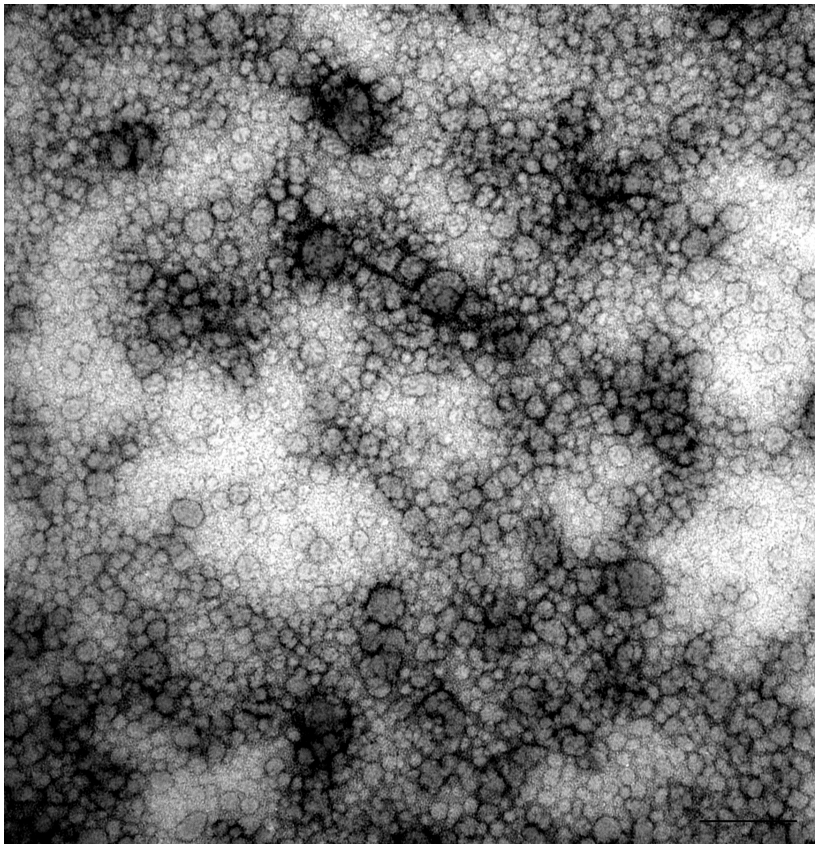


Figure 3.6.10. Negatively stained preparation of central bodies from *Cyanophora paradoxa*.
Quasi crystalline aggregates (Rubisco?) become apparent.
Samples of isolated carboxysomes were negatively stained by floating carbon- coated grids onto a sample drop for 4 min, followed by refloating onto a 2% solution of uranyl acetate for 50 seconds. Grids were observed in a Jeol transmission electron microscope. (scale bar 100 nm).

4. DISCUSSION

4.1. Proteomics:

4.1.1. Central body isolation and identification of its components (Mass Spectrometry)

The only carboxysomal proteins identified werde the large (52 kDa) and small subunits (12 kDa) of Rubisco and Rubisco Activase (45 kDa).

For comparison, proteomics of cyanobacterial carboxysomes was not very successful either: One-dimensional ELPHO and N-terminal sequencing identified Rubisco SSU only (Rodriguez-Buey et al, 2005) whereas two-dimensional separation and MS yielded three genuine components, RbcL, RbcS, and CcmM (Long et al., 2005). It could be argued that the use of Triton effectuates the (partial) loss of the shell of these delicate microcompartments as indicated by EM of central body preparation. Due to their relatively large size and tight association with the thylakoids, their isolation has to be a compromise between intactness and purity.

Also, cyanelle DNA sticks to the CB, therefore DNAse treatment is necessary, too.

The sensitivity of the mass spectrometric method is sufficient to characterize even minor bands, which in most cases will represant contaminating material (Long et al, 2005). Nevertheless, we estimate that more EST data for *C. paradoxa* will enable in the future the discrimination between *bona fide* components of the central body and contaminating proteins. The identification of a cyanelle CA would be of prime importance in this respect. Also, the presence of shell proteins, encoded by *ccm* genes, could not yet be shown for *C. paradoxa*. They would be profound evidence for a “eukaryotic carboxysome” in this glaucocystophyte.

4.1.2. Rubisco activase

MS/MS also clearly identified Rubisco activase as a central body component in *C. paradoxa*. Its gene was in the EST library established and was completed by 5´-RACE of a truncated cDNA, revealing a typical cyanelle transit sequence. Moreover, when ^{35}S-labelled pre-Rubisco activase was imported into cyanelles *in vitro*, incorporation of the mature protein into central bodies was demonstrated to increase over time (7 min: 10%, 25 min: 25%). Among cyanobacteria, Rubisco activase is found in filamentous, nitrogen-fixing species (the likely ancestors to chloroplasts) but not in unicellular ones, with the exception of *Gloeobacter violaceus* PCC 7421 (Cyanobase). The *Cyanophora* enzyme possesses the N-terminal extension of higher plants and green algae while the C-terminal extension, bearing sequence similarity to cyanobacterial CcmM repeats, is absent. The central part is well conserved for all of the oxygenic phototrophs with Rubisco activase that have been examined. The location of the cyanobacterial activase is assumed extra-carboxysomal (Friedberg et al., 1993), which is questioned by others (A. Portis, personal communication), whereas immunoelectron microscopy showed an association of this enzyme with pyrenoids in algae (McKay et al., 1991). In the case of *Cyanophora*, we could unequivocally demonstrate a tight association of Rubisco activase with its substrate, Rubisco, in the cyanelle carboxysome-like microcompartment. Interestingly, while Rubisco LSU is highly conserved, i.e. the identity scores between the cyanelle protein and its cyanobacterial counterparts lie between 83 and 84%, a BLAST search with the cyanelle Rubisco LSU against Cyanobase nevertheless produced top ranking hits for the filamentous, nitrogen-fixing and activase-containing cyanobacterial species *Anabaena* 7120, *Nostoc punctiforme* and *Trichodesmium erythraeum* (data not shown). The catalytic mechanism of cyanobacterial Rubisco activase might somewhat differ from that of the eukaryotic enzyme (Portis, 2003), which raises the question about the catalytic mechanism of the plant-type *Cyanophora* activase vis-à-vis its cyanobacterial-type substrate. MS data point towards the possibility of two enzyme forms of slightly different size in *Cyanophora paradoxa* (Portis et al. 2003).

4.2. Carbonic anhydrases

The identification of the gene(s) for cyanellar CA in *Cyanophora* could not be achieved. In the case of incorporation of CA into the central body, the latter could be considered a carboxysome. Instead, two isoforms of a putatively mitochondrial CA (with highest homology to mCA2, Cah5 of *C. reinhardtii*) and a putatively cytosolic CA (lacking a N-terminal transit sequence), all β-type carbonic anhydrases, were identified for *C. paradoxa.*

Microarray results (from s. Burey) of the putatively cytosolic carbonic anhydrase of *Cyanophora* point towards a late (24 h) induction of <2-fold (20G10, Fig. 3.2.4.2 A, B), while Northern analysis indicates an almost ten-fold up-regulation at 1 h upon shift. It is unclear whether this CA is involved in the CCM. A cytoplasmic CA would be necessary if we assume the presence of a Ci-transporter in the chloroplast envelope with a distinct preference for either CO_2 or HCO_3^- (Badger and Price, 1994) such as the putative cyanelle bicarbonate transporter encoded by CPL00000417. For *C. reinhardtii*, a cytoplasmic carbonic anhydrase was recently identified: CAH9 is related to bacterial CAs (Moroney and Ynalvez, 2007).

C. paradoxa was grown under continuous light of low intensity. Under these conditions, mitochondrial respiration plays a minor role only (though this has been questioned), explaining the much lower effect on expression of mitochondrial CA (Fig. 3.2.4.2.) compared to *C. reinhardtii* (*Mca*) where a factor of about 100 was reported (Miura et al., 2004). Also, high light was shown to increase the expression of CO_2 responsive genes.

Northern analyses of the putatively mitochondrial CA isoforms I and II indicated an early (1 h) two-fold induction upon low-CO_2 with a subsequent gradual reduction (microarray results show slight continuous induction of isoform I). It is thought that two isoforms of CA in *C. reinhardtii*, Cah4 and Cah5, which were shown to be highly dependent upon changes in the ambient [CO_2] (Moroney and Chen, 1998) recycle both respiratory and photorespiratory CO_2 by converting it to HCO_3^- in the mitochondrial matrix (Raven, 2001), which is subsequently available for the CCM by

transport into the chloroplast stroma. The finding that Ci limitation induces both putatively mitochondrial CA isoforms in *Cyanophora* allows for a similar scenario.

4.3. Electron Microscopy

It is not clear at present if the *C. paradoxa* “carboxysomes” are confined by a (proteinaceous) non-unit membrane but the polyhedral central bodies of cyanelles from the glaucocystophytes *Gloeochaete wittrockiana* and *Cyanoptyche gloeocystis* are enclosed by such a shell (Kies, 1992). It is likely that the polyhedral nature, observed infrequently, and the surrounding shell of the *C. paradoxa* central bodies are camouflaged by the condensed cyanelle DNA covering them and the closely adjacent concentric thylakoids.

Alternatively, a (partial) conversion from carboxysome to pyrenoid could have occurred within the glaucocystoohytes. The CBs of *C. gleocystis* and *G. wittrockiana* still represent (eukaryotic) carboxysomes whereas those of C. paradoxa and *G. mostochinearum* might have arrived of an intermediate state between the two microcompartment types. Electron microscopy of high *vs.* low [CO_2] grown cells showed a potential increase in size of the putative carboxysome. In prokaryotes which contain multiple carboxysomes, their numbers increase upon CO_2-stress (Reinhold and Kaplan, 1999). The increase in starch granules observed for low CO_2–grown cells parallels the appearance of a starch sheath around the pyrenoid in *C. reinhardtii* (Mitra et al., 2005). Ribosomes are found distributed through the thylakoid system under high [CO_2], likely occupied with phycobiliprotein synthesis which are major proteins in the cyanelles. In low [CO_2] cells ribosomes are concentrated around the CB: this is in accordance with the results of Wang et al. (2003) who observed a decrease in phycobiliprotein and PSI and II protein synthesis and an increase in Rubisco synthesis upon shift of *Synechocystis sp.* 6803 to low [CO_2].

Isolated carboxysomes were checked with respect to intactness, polyhedral appearance and quasi-crystalline substructure. It was obvious that delicate structures were subjected to stepwise disassembly which was less pronounced

when chemical fixing and embedding was adopted but complete when the preparations were treated with negative staining. The emerging substructure of individual, ordered Rubisco molecules very much reminds to the situation with isolated carboxysomes from cyanobacteria. However, it must be kept in mind that such experiments have not yet been with isolated pyrenoids.

4.4. Summary of the *C. paradoxa* project

The question of a ,,eukaryotic carboxysome,, *in C. paradoxa* still has to be await a cocclusive answer. Raven's hypothesis (2003) is appealing to us since it offers a "raison d'etre" for the eukaryotic peptidoglycan of glaucocystophytes. However, it remains to be shown that bicarbonate is indeed enriched in cyanelles by a factor of 1000 or more.

High-throughput EST sequencing revealed a number of novel interesting genes, which in future can be investigated with diverse means. A large number of suitable ESTs contributed to an extensive phylogenetic analysis. A subset of largely unique ESTs were spotted on coated slides for microarray analysis. Genes showing differential regulation upon high and low CO_2 were identified, among them CCM candidate genes. In summary, genetic and biochemical data allowed the identification of homologues of cyanobacterial and algal proteins (putatively) involved in carboxysome and/or pyrenoid functioning, including carbonic anhydrases, Rubisco activase and a likely bicarbonate transporter. Nevertheless, highly indicative genes for determining the type of CCM such as a cyanelle CA, carboxysomal shell proteins and other Ci-transporter components are still unidentified. Thus, the present data does not allow us to fully support the hypothesis of "eukaryotic carboxysomes". The bulk of Rubisco and activase seems to be concentrated in the central body, which should play a major role in the CCM. Possibly, the cyanelle central body represents an intermediate step in the evolution from a carboxysomal-type CCM towards a pyrenoidal-type CCM.

4.5. Perspectives

The genome of *C. paradoxa* is expected to be completely sequenced in the near future. Transit sequence prediction is more straightforward for cyanelles than for chloroplasts (Steiner and Löffelhardt 2005). Thus a cyanellar CA should be easily identified once the gene or EST is available. *In vitro* import and fractionation should show an eventual assembly of the protein into the “carboxysome”. The presequence will tell us also if a localization to the thylakoid lumen is possible, though in this case the mechanism must be different from that in *C. reinhardtii*, since microcompartment and photosynthetic membranes are strictly separated in cyanelles. We will also continue mass spectrometric identification of individual bands in the hope to identify a CA-like protein by this way.

We will put more effort into electron microscopy to elaborate on specific features of (immuno-EM) low [CO_2] grown cells: There might be a higher number of starch grains which are deposited in the cytoplasm of *C. paradoxa* and, in the cyanelles, slight increases in microcompartment size and in the number of surrounding ribosomes. Also, the boundaries of the microcompartment from low [CO_2] conditions seem to be better defined with sometimes slight polyhedral features which might be camouflaged through the covering cyanelle DNA. Antibodies directed against novel carbonic anhydrases, such as Cah8, could be tested on cyanelle protein blots. Measurements of O_2 evolution and CO_2 consumption (e.g. with a portable gas exchange system) upon a shift to low-CO_2 would be beneficial as well as determining the HCO_3^- concentration within cyanelles at low CO_2.

Glaucocystophytes are niche organisms: *C. paradoxa* is the best–investigated species since it is the only one with a reasonable growth rate (generation time 20 hours). *G. nostochinearum* grows slower and all others at a very low rate. Thus it is no advantage to keep the organelle wall, unless there is an absolute necessity for that. Continued protein analysis of the central body of *C. paradoxa* cyanelles veering towards a classification of the CCM would be very valuable. Mass spectrometric determination of CA activity for isolated purified cyanelles should be performed. Absent enzymatic activity though does not always exclude enzymatic presence as there exist several cases of inactive enzymes.

The effect of higher light intensity on CCM induction could be investigated with microarray analysis. A future approach will be to further exploit the low-CO_2 library by subtraction (excluding in particular ribosomal proteins) and to follow the expression pattern of potentially interesting genes via RT-PCR. Another goal will be to find and characterize the gene(s) for the elusive cyanelle CA as well as for bicarbonate transport and CO_2 uptake systems.

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