Characterization of the Cyanobacterial Lyase CpcS from *Thermosynechococcus elongatus* & Implementation of a Microbiology Project in Secondary School

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This thesis is dedicated to the friendship treaty between
the University of Innsbruck and the University of New Orleans
and to all the friendships originating from it.

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ABBREVIATIONS

AP: allophycocyanin; BV: Biliverdin; DALI:Distance-matrix alignment algorithm; HO1: Heme Oxygenase1; HY2: phytochromobilin synthase; IPTG: isopropyl-β-D-thiogalactopyranoside; LB: Luria–Bertani medium; LPSN: List of Prokaryotic names with Standing in Nomenclature; MS: mass spectrometry; NIH: National Institute of Health; Ni-NTA: nickel nitrolotriacetic acid; NOS: Nature of Science; OD : optical density; PAGE: polyacrylamide gel electrophoresis; PBP: phycobiliprotein(s); PBS: phycobilisome(s); PC: phycocyanin; PCB: phycocyanobilin; PCR: polymerase chain reaction; PcyA: Phycocyanobilin:Ferredoxin Oxidoreductase; PDB: Protein Database; PE: phycoerythrin; PEB: phycoerythrobilin; PebS: Phycoerythrobilin Synthase; PEC: Phycoerythrocyanin; PϕB: phytochromobilin; PS: photosystem; PUB: Phycourobilin; PVB: Phycoviolobilin; RuBisCO: Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase; NCBI: National Center for Biotechnology Information; SDS: sodium dodecylsulfate; SOC: Super Optimal broth with Catabolite repression; *Te*: *Thermosynechococcus elongatus*; Tris: tris(hydroxymethyl)aminomethane

ABSTRACT

Cyanobacteria are the only known prokaryotes that perform oxygenic photosynthesis. Due to their light-harvesting structures, termed phycobilisomes, they are able to absorb the energy of a broad spectrum of light. Phycobiliproteins are the main moieties of phycobilisomes and their ability of light-harvesting is dependent on the covalent ligation of phycobilin chromophores. The attachment reaction of the bilins to the apo-proteins is catalyzed by phycobilin lyases. CpcS from the thermophilic cyanobacterium *Thermosynechococcus elongatus* is the first lyase with a known X-ray crystallographic structure. The structure revealed *Te*CpcS to have a 10-stranded beta barrel fold with two alpha helices. Structural alignments showed a very high similarity to proteins from the lipocalin protein family and to the bilirubin binding UnaG Protein from Japanese freshwater eel. Manual docking experiments of phycocyanobilin to *Te*CpcS, using the UnaG-bilirubin interaction as a template, suggested certain amino acid residues to form strong interactions with the bilin. Trp-73, Arg-151 and Ser-155 of *Te*CpcS showed hydrogen bond interactions with phycocyanobilin in the computer generated model. Additionally, this lyase contains two non-conserved cysteine residues Cys-2 and Cys-169 that showed covalent bilin attachment in earlier studies. By creating site-directed mutations within the gene encoding the *Te*CpcS lyase, the intrinsic bilin binding properties and the activity of bilin attachment to α- and β-subunits of phycocyanin and allophycocyanin were tested in this study. The covalent attachment of phycocyanobilin and phycoerythrobilin occurs predominantly at the Cys-2 position, which is situated at the α1-helix at the outside of the funnel-like bilin binding site and the substitution of the Cys-2 residue by a Serine increases the lyase activity in heterologous co-expression constructs in *E.coli*. Arg-151 seems to be the most important interaction for bilin binding inside the funnel-like beta barrel structure. The substitution of Arg-151 to a glycine decreases the chromophorylation efficiency of various subunits.

The didactic section of this thesis shows a way of implementing the topic cyanobacteria and colour absorption in the biology curriculum of secondary school. “Growing Cyanobacteria” provides a plan for a long-term project that involves students in hands-on activities connected to microbiology. The project is based on the fundamentals of constructivistic approaches to the science classroom and uses elements of inquiry-based teaching.

ZUSAMMENFASSUNG

Cyanobakterien sind die einzigen bekannten Prokaryoten, die oxygene Photosynthese betreiben. Aufgrund ihrer Lichtsammelkomplexe, genannt Phycobilisomen, sind sie in der Lage, die Energie eines breiten Spektrums von Licht zu absorbieren. Phycobiliproteine sind die wichtigsten Komponenten von Phycobilisomen und ihre Fähigkeit der Lichtabsorption ist abhängig von gebundenen Phycobilinen. Die Bindungsreaktion der Biline an die Apo- Proteine wird durch Phycobilin Lyasen katalysiert. CpcS aus dem thermophilen Cyanobakterium *Thermosynechococcus elongatus* ist die erste Bilin-Lyase mit bekannter Röntgenkristallstruktur. Die Struktur von *Te*CpcS besteht aus einem 10- strängigen beta-Barrel und zwei zusätzlichen alpha-Helices. Struktur Alignments zeigten eine sehr hohe Ähnlichkeit zu Proteinen aus der Lipocalin -Proteinfamilie und zu UnaG, einem Bilirubin-bindenden Protein aus dem Japanischen Süßwasser Aal *Unagi*. Anhand von Docking Experimenten, basierend auf der Interaktion zwischen Bilirubin und UnaG, wurde eine Bindung von Phycocyanobilin an *Te*CpcS simuliert. Bestimmte Aminosäurereste von *Te*CpcS scheinen starke Interaktionen mit dem Bilin auszubilden. Trp -73, Arg- 151 und Ser -155 von *Te*CpcS zeigten Wasserstoff-Brücken-Bindungen mit Phycocyanobilin im computer-generierten Modell. Darüber hinaus enthält diese Lyase zwei evolutionär nicht konservierte Cysteinreste Cys-2 und Cys-169, die in früheren Studien kovalente Bindungen zu Phycocyanobilin zeigten. Durch die Einführung von ortsspezifischen Mutationen in die *Te*CpcS Lyase, wurden in dieser Studie die intrinsischen Bilin - Bindungseigenschaften und die katalytische Aktivität der Bilin Bindungsbildung an α - und β - Untereinheiten von Phycocyanin und Allophycocyanin untersucht. Die kovalente Bindung von Phycocyanobilin und Phycoerythrobilin tritt vorwiegend an der Cys -2-Position auf. Diese befindet sich in der α1 - Helix an der Oberfläche des Proteins und nicht in der Nähe der trichterförmigen Bilin –Bindungsstelle. Die Substitution des Cys -2 durch ein Serin erhöht die Lyase- Aktivität in heterologen Co- Expressionskonstrukten in *E. coli*. Arg -151 scheint die wichtigste Interaktion für Bilin -Bindung innerhalb der trichterförmigen Beta- Barrel-Struktur zu sein. Die Substitution von Arg -151 durch ein Glycin verringert die Effizienz der Bindung des Chromophors an diverse Untereinheiten der Phycobiliproteine.

Der didaktische Abschnitt dieser Arbeit zeigt eine Möglichkeit der Umsetzung des Themas Cyanobakterien und Farbabsorption im Lehrplan des Biologieunterrichts. "Growing Cyanobakterien" stellt einen Plan für ein langfristiges Projekt zur Verfügung, das praktische Aktivitäten der Schüler im Bereich der Mikrobiologie beinhaltet. Das Projekt basiert auf den Grundlagen der konstruktivistischen Ansätze naturwissenschaftlichen Unterrichts und verwendet Elemente des fragend-forschenden Lernens.

I.RESEARCH SECTION

1. INTRODUCTION

1.1. CYANOBACTERIA

Cyanobacteria, also known as “blue-green algae“ are a diverse group of gram-negative photosynthetic prokaryotes. The clade consists of simple unicellular organisms with a frequent tendency for aggregation or colony formation and filamentous structures that either are plain unbranched or more elaborate and branched. [1]

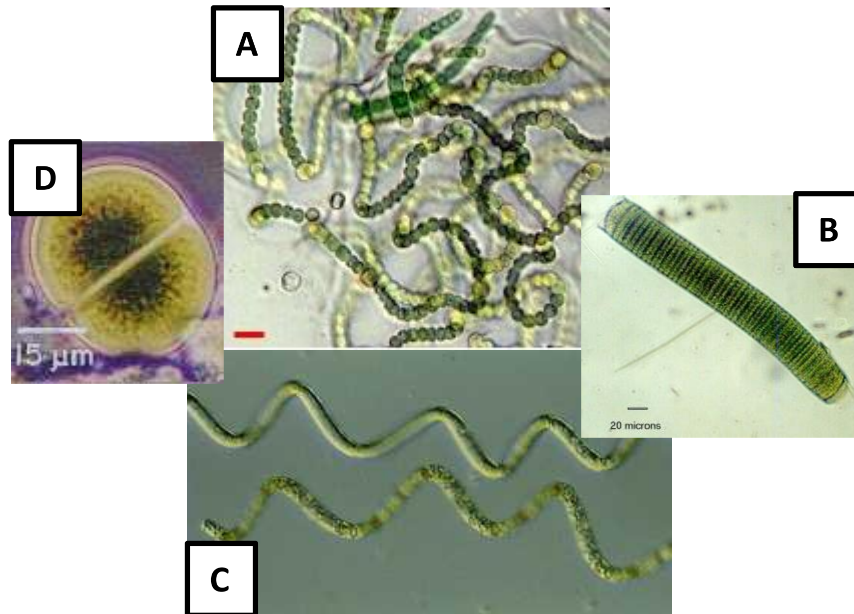


Figure 1 Different morphologies of cyanobacteria *Nostoc sp.*(A), *Oscillatoria princeps*(B), *Spirulina sp.*(C) and *Chroococcus sp.*(D). Images from Cyanosite, Purdue University. [2]

The first scientist to observe stromatolites was Kalkowsky and defined them as "a laminated benthic microbial deposit" [2] These layered sedimentary structures produced by phototrophic organisms (usually cyanobacteria) are considered the most ancient biological record and the earliest evidence of the emergence of life on earth. Stromatolites provide modern science with insights into evolutionary processes [3]

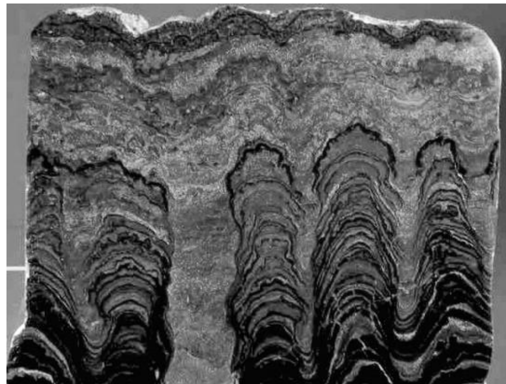


Figure 2 Proterozoic Stromatolite from Eastern Andes, Bolivia [4]

Among organisms cyanobacteria play an undisputed, important role in earth´s history. Not only that they contribute to the carbon cycle as primary producers and to the nitrogen cycle by the ability of some species to fix nitrogen, but especially their ability to use water molecules as electron donors guarantees them an important place in the redox history.[5]

Cyanobacteria are widely accepted as the progenitor of oxygenic photosynthesis, and they have evolved into one of the largest and most diverse groups of bacteria on this planet.[6] The evolution of the energy transduction pathway that transformed the earth´s atmosphere dates back approximately 2.3 billion years and ultimately facilitated the development of complex life forms that depend on aerobic metabolism and therefore on gaseous oxygen.[7,8] Although maybe no other clade of organisms had a greater impact on evolution of diverse life forms, its own development is yet not very well understood.

The origins of the essential machinery for conducting photosynthesis are not yet identified. The capability to perform oxygenic photosynthesis has been a “singular event“ in evolution as no other organism has developed this ability except those who inherited it from cyanobacterial ancestors. Although recent studies suggest the possibility that cyanobacteria were the first organisms to evolve photosynthesis in general, it seems to be more probable that cyanobacteria have adapted the photosynthetic apparatus from proteobacteria, who perfom anaerobic photosynthesis. These bacteria are able to use other electron donors than oxygen from water and have bacterio-chlorophyll instead of chlorophyll *a* or *b* in cyanobacteria. Horizontal (lateral) DNA transfer between cyanobacterial strains and other bacteria like *E.coli* has been demonstrated and likely played an important role in bacterial evolution. [5]

The evolution of eukaryotic photosynthetic oganisms is thought to have occurred by the uptake of cyanobacteria into unicellular eukaryotes to form predecessors of plant cells by „primary endosymbiosis“. It is commonly accepted that the photosynthetic plastids, i.e. chloroplasts, have entered the cell by a process of phagocytotic uptake. [9]

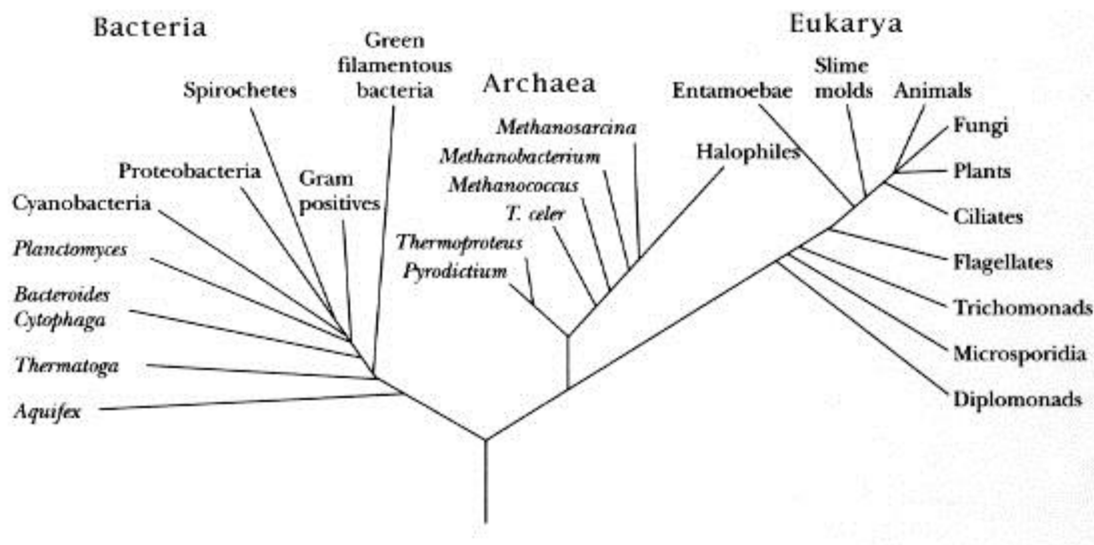


Figure 3 The "tree of life" Phylogenetic position of cyanobacteria in the 3-domain taxonomic system [10]

Taxonomy of cyanobacteria has historically been difficult because cyanobacteria historically have been classified as plants within the taxa of algae. According to Oren (2004) the systematic taxonomy of cyanobacteria within the bacteriological code is difficult because of the lack of minimal standards for the description of new species and genera.[11] The taxonomic system for cyanobacteria according to Cavalier-Smith (2002) below provides a general overview for the main taxa within the Division Cyanobacteria and describes the general phenotypes of the taxa.[12] This list has been compared to the "Taxonomic scheme according to NCBI Taxonomy Browser" (August 31, 2009) and to LPSN Bacterionet and shows a general consensus. [13, 14]

Division: *Cyanobacteria*	Oxygenic photosynthesis with chlorophyll *a*, flagella absent; often glide; ancestrally with phycobilisomes (sometimes lost)
Subdivision 1: *Gloeobacteria*	Without thylakoids
Class: *Gloeobacteria*	As for subdivision above
Order: *Gloebacterales*	Having phycobilisomes but no thylakoids
Subdivision 2: *Phycobacteria*	With thylakoids, gliding motility by slime secretion, classical Cyanophyceae and prochlorophytes. The five traditional orders already valid under the Code of Botanical Nomenclature, are here also formally validated under the Bacteriological (=prokaryotic) Code
Class: *Chroobacteria*	Unicellular, palmelloid, colonial or with filaments lacking heterocysts
Order: *Chroococcales*	Unicellular and colonial (non-filamentous) with phycobilisomes and prochlorophytes with chlorophyll *b* instead
Order: *Pleurocapsales*	Colonial or filamentous, reproducing by intramural multiple fission to yield smaller unicellular dispersal stages
Order: *Oscilliatorales*	Unbranched linear filaments without heterocysts; cells typically shorter than broad
Class: *Hormogoneae*	Filaments that multiply vegetatively by hormogonia, usually with heterocysts
Order: *Nostocales*	Unbranched filaments
Order: *Stigonematales*	Branched filament

The taxa prochlorophytes takes a special place within this taxonomic system as, like cyanophytes they are all clearly photosynthetic prokaryotes, but since they contain no blue or red bilin pigment they were assigned to a new algal sub-class, the Prochlorophyta. They are the only bacteria to possess chlorophyll *b* and have been considered to be the most viable ancestors for chloroplasts in plants.[15] However, since their possible phylogenetic relationships to ancestral green-plant chloroplasts have not

received support from molecular biology, it now seems suitable to consider them as aberrant cyanophytes. [16]

Blooms & Toxins

Food webs in freshwater ecosystems are highly dependent on suspended algae or phytoplankton. Increasing nutrient input in water bodies, much of it manmade, accelerates primary production or eutrophication. This leads to rapid growth of phytoplankton and subsequently to discoloration of affected waters. This is commonly called an algal bloom and decreases water quality drastically by causing foul odors, tastes, deoxygenation of bottom waters, toxicity, fish kills and food web alterations. Although a broad range of different species is able to form blooms, cyanobacteria are the most notorious bloomformers. Harmful toxic, surface-dwelling, scum-forming genera like *Anabaena*, *Aphanizomenon*, *Nodularia* and *Microcystis* and some subsurface bloom-formers like *Cylindrospermopsis* and *Oscillatoria* are frequently abundant in eutrophic water bodies. Rapid migration between radiance-rich surface waters and nutrient-rich bottom waters allows them to thrive very successfully. In addition to their ability to survive extreme environmental conditions including high light levels, temperatures, various levels of desiccation and periodic nutrient deprivation some of the occurring genera are capable of fixing atmospheric nitrogen. Additionally the symbiotic association with other organisms like fungi and higher plants enables them to survive under difficult circumstances. [17, 18]

Climate change including conditions of warming, increased vertical stratification of water bodies, salinization and intensification of extreme weather conditions like storms and droughts play a further interactive role in the frequency of cyanobacterial bloom formation. [18, 19]

Figure 4 Bloom at the shore of Lake Wannsee near Berlin in 2005 (pictures by Dittmann & Wiegand, 2006) [20]

In case of excessive growth such as bloom formation, cyanobacteria can produce inherent toxins in quantities causing toxicity in mammals, including humans. Toxic cyanobacterial blooms represent a serious hazard to environmental and human health, and the management and restoration of affected waterbodies can be challenging.[19]

Cyanotoxins include cyclic peptides and alkaloids. Among the cyclic peptides are the microcystins and the nodularins. The alkaloids include anatoxin-a, anatoxin-a(S), cylindrospermopsin, saxitoxins, aplysiatoxins and lyngbyatoxin.[21] The predominant producers of microcystins and nodularins are *Microcystis, Planktothrix, Anabaena* and *Nodularia*. Saxitoxins (also known as paralytic shellfish poisoning toxins) are produced by *Aphanizomenon* spp. and Anatoxin-a by *Anabaena* spp. but also by species of the genera *Aphanizomenon, Cylindrospermum, Planktothrix* and by *Microcystis aeruginosa*.[20]

From a toxicological viewpoint, the cyanotoxins span four major classes: the neurotoxins, hepatotoxins, cytotoxins, and dermatoxins (irritant toxins) [22]

Microcystin and Nodularin are hepatotoxic cyclic peptides consisting of seven or five amino acids, respectively. A common characteristic of both hepatotoxins is the rare amino acid Adda that is the responsible structure for the specific inhibition of protein serine/threonine phosphatases. The neurotoxins anatoxin-a and anatoxin-a(S) mimic

acetylcholine as a nicotinic agonist and inhibit Acteylcholine esterase, respectively. In both cases the constant activation of muscles leads to tetanus followed by fatigue. The neurotoxic Saxitoxins lead to paralysis by blockage of voltage-gated Na^+ channels in neuronal transmission. [20]

Symbiosis

Cyanobacteria form symbioses with a diverse group of eukaryotes that includes genera from mosses to angiosperms. The interactions between the cyanobacterium and the plants range from less intimate interactions to highly intricate symbioses such as the intracellular relation with the genus *Gunnera*. In *Azolla sp.* the relationship is perpetual and maintained between generations. With few exceptions the nitrogen-fixing cyanobacteria belong to the terrestrial widespread genus *Nostoc*, because this genus has a notable morphological plasticity and is therefore well suited to form symbiotic relationships.[23] Vegetative cells of *Nostoc* may differentiate into nitrogen-fixing heterocysts and filaments can fragment to form hormogonia that are a prerequisite for plant infection. The high frequency of heterocysts in symbiotic cyanobacteria is an indicator for inter-organism communication. [23]

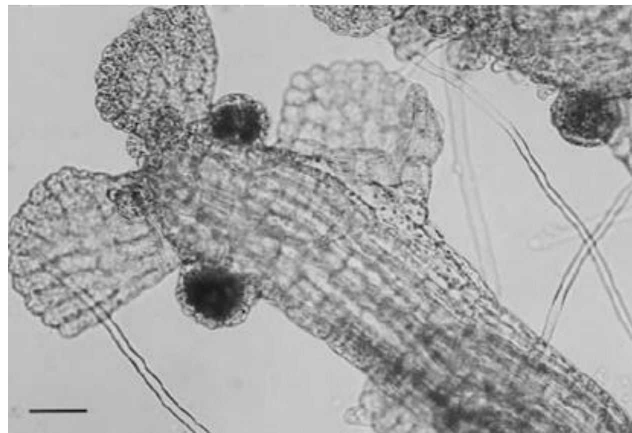


Figure 5 Symbiotic interaction of the liverwort Blasia pusilla containing cyanobacterial colonies from Nostoc in structures that are called auricles (picture from Adams & Duggan, 1999) [24]

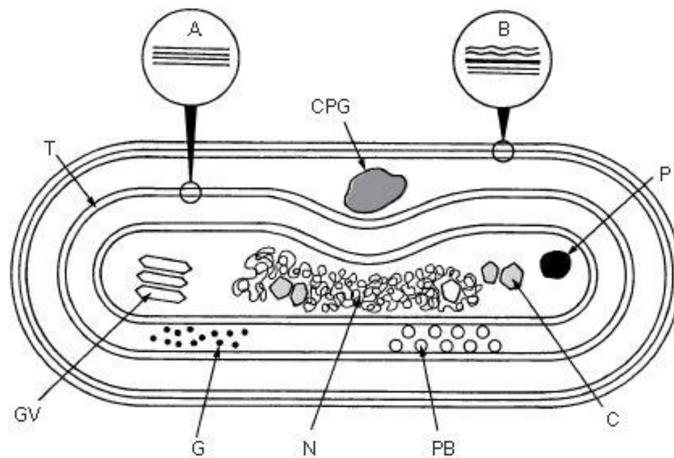


Figure 6 Schematic diagram of a thin section of a cyanobacterial cell showing carboxysomes (C), cyanophycin granules (CPG), thylakoids (T), polyphosphate granules (P), nucleoplasmic region (N), glycogen granule (G),phycobilisomes (PB), gas vesicles (GV). (Adapted from Adams & Duggan, 1999) [24]

Cyanobacteria are a diverse group of gram-negative organisms that vary in size from <1->100 µm. Compared to other prokaryotes cyanobacteria include a broad variety of structures. The cell envelope consists of an outer and an inner cytoplasmic membrane interspersed by a 1-10 nm thick peptidoglycan layer combined with covalently linked polysaccharides that are usually found in gram-positive bacteria. The peptidoglycan layer is usually traversed by pores to connect the outer and the inner membrane layer. The distribution of these pores varies significantly over the surface, especially in cross linked, filamentous cells that show a high abundance in the linking area. Many cyanobacteria possess an additional external sheath, mostly consisting of polysaccharide and up to 20 % of polypeptides. The consistency of this layer shows big variations, which explains the various names from glycocalyx, sheath, capsule, mucilage or slime. [24]

The prokaryotic cellular organization of cyanobacteria is characterized by the presence of intracellular membranes called thylakoids. These incorporate the photosynthetic

apparatus of the cell. The cytoplasm usually contains a variety of granular inclusions of diverse composition and function and the planktonic forms are distinct by the appearance of gas vacuoles that allow them to adapt their buoyancy according to their position in the water column.[1] Cyanophycin (multi- L-arginyl-poly- L-aspartate), a water-insoluble reserve polymer of cyanobacteria, is a product of non-ribosomal peptide synthesis and is stored in special cyanophycin granules to survive nitrogen and possibly carbon starvation.[25]

The carboxysome, a prototypical bacterial micro-compartment is composed of a protein shell and is found in cyanobacteria and some chemoautotrophs. It encapsulates ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase, and thereby enhances carbon fixation by elevating the levels of CO_2 in the vicinity of RuBisCO.[26]

Cell types - cell differentiation

Many cyanobacteria are able to create differentiated cells for reproduction, to outlast harsh circumstances or to perform certain metabolic ways. Filamentous strains are frequently able to form heterocysts, a special cell type that is specialized on nitrogen fixation. Additionally some species are able to form long lasting, thick walled cells to survive unfavorable environmental conditions called akinetes.

Heterocysts are enlarged cells that have enhanced activity for nitrogen fixation. Due to the sensitivity of the nitrogen fixation process to oxygen this cell type has a number of adaptations to exclude any interference. First, the lack of function of Photosystem II inhibits the photosynthetic creation of oxygen in heterocysts. Second, the thickened cell envelope decreases the diffusion of atmospheric oxygen into the cell. Third, the residual oxygen in the cell is removed by several molecular mechanisms including respiration. As heterocysts are lacking Rubisco and as mentioned before a functioning photosynthetic

apparatus, their survival relies on connections to vegetative cells. This is presumably also the reason why this cell type only occurs in filamentous cyanobacteria.[24] Heterocyst differentiation is a highly regulated process, resulting in a regularly spaced pattern of heterocysts in the filament. The evidence is most consistent with the pattern arising in two stages. First, nitrogen limitation triggers a non-randomly spaced cluster of cells (perhaps at a critical stage of their cell cycle) to initiate differentiation. Interactions between an inhibitory peptide exported by the differentiating cells and an activator protein within them causes one cell within each cluster to fully differentiate, yielding a single mature heterocyst. In symbiosis with plants, heterocyst frequencies are increased 3- to 10-fold. The physiology of symbiotically associated cyanobacteria raises the prospect that heterocyst differentiation proceeds independently of the nitrogen status of a cell and depends instead on signals produced by the plant partner.[27]

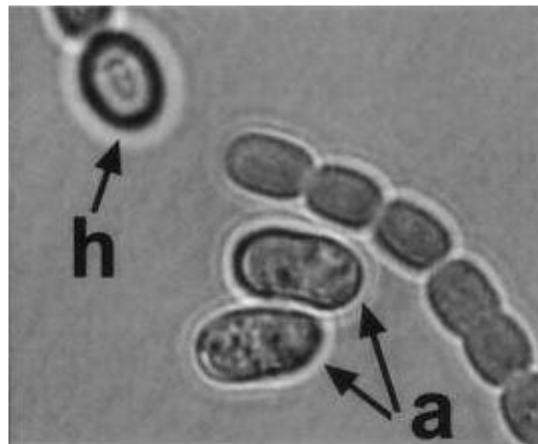


Figure 7 Akinetes(a) and heterocysts(h) in Anabaena variabilis (picture from Zhou et al. 2002)[28]

Under certain environmental stresses various genera of heterocystous cyanobacteria differentiate morphologically and ultra-structurally into resting-state cells for survival. Akinetes are resting-state cells of cyanobacteria whose formation is induced by a number of environmental factors including low temperature, desiccation, elevated levels of salt and iron depletion. [24] In nature, akinetes are believed to play an important role in allowing the producer strain to survive cold winters and to withstand adverse

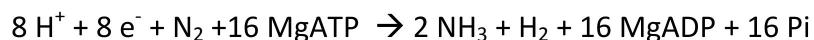
environmental stresses. Under optimized conditions, the akinetes germinate and vegetative-state cells are produced. The cyanobacteria can remain as akinetes for long periods of time without losing viability.[29]

Metabolism

Cyanobacteria are photoautotrophs, which means that they are using light as an energy source and carbon dioxide in air as their carbon source. This metabolism is associated with the inclusions called carboxysomes. These polyhedral structures contain the major enzyme for carbon dioxide fixation RuBisCO and are therefore the primary site of the Calvin cycle. However, there are not only photoautotrophic, but also chemoheterotrophic strains that are able of slow growth in the dark or in dim light. [30,24]

Diazotrophic cyanobacteria are the only oxygenic phototrophs among the nitrogen (N_2)-fixing organisms. Most unicellular diazotrophic cyanobacteria fix N_2 during the dark phase of light/dark cycles or in the night under atmospheric conditions.[31] Nitrogenases, which catalyze the biological reduction of di-nitrogen to ammonia, are complex metalloenzymes with conserved structural and mechanistic features. These enzymes contain two components that are named according to their metal composition. The smaller dimeric component, known as the iron (Fe) protein, functions as an ATP-dependent electron donor to the larger heterotetrameric component, known as the molybdenum–iron (MoFe) protein, which contains the enzyme catalytic site. [32]

Biological (di)nitrogen fixation follows equation 1: [33]



$$8\ H^+ + 8\ e^- + N_2 + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ Pi$$

As it is of interest for biotechnological processes and also related to the didactic part of this thesis, it should be mentioned that the production of dihydrogen in cyanobacteria involves several enzymes: nitrogenase(s) catalyzing the production of hydrogen (H_2) concomitantly with the reduction of nitrogen to ammonia as showed in equation 1

above, an uptake hydrogenase catalyzing the consumption of hydrogen produced by the nitrogenase, and a bidirectional hydrogenase which has the capacity to both take up and produce hydrogen. [34]

Photosynthesis

Unlike the anaerobic photosynthetic bacteria that use light as an energy source like purple bacteria, green sulfur bacteria, heliobacteria or green gliding bacteria, cyanobacteria evolved the ability to perform oxygenic photosynthesis (i.e. using water as electron source and producing oxygen by water splitting).

Some cyanobacteria can use sulfide, molecular hydrogen, and other compounds, instead of water, as electron donors in an anoxygenic type of photosynthesis that is dependent only on the Photosystem I reaction, as in other photosynthetic bacteria. [35]

Very similar to higher plants photosynthesis in cyanobacteria involves the operation of two distinct photosystems, photosystem I and photosystem II (PS I and PS II, respectively), which are linked in a series and interact through a chain of electron carriers.[1,36] Both photosystems communicate with one another via an electron transport chain known as the "Z-scheme" of photosynthesis. Electrons are initially extracted from water at the site of the OEC (oxygen evolving complex) at PS II by the conversion of the energy of one photon of light and finally released to a soluble ferredoxin complex after passing through PS I (where it is further re-energized by a second photon). Ferredoxin then provides electrons to Ferredoxin-$NADP^+$ oxidoreductase, which then reduces oxidized $NADP^+$ to form NADPH, a very important electron carrier in many reactions in metabolism.[37] Additionally the electorchemical gradient formed by protons on the thylakoid membranes is used by the ATP Synthase to generate the „universal intracellular currency" ATP.[38] Alternatively cyanobacteria can also use the reduced ferredoxin to perform cyclic photophosphorylation. In this case only PS I is involved and instead of the reduction of $NADP^+$ the electrons are used to reduce plastoquinone in the

Z-scheme and form an electrochemical gradient without splitting water and producing oxygen.[15]

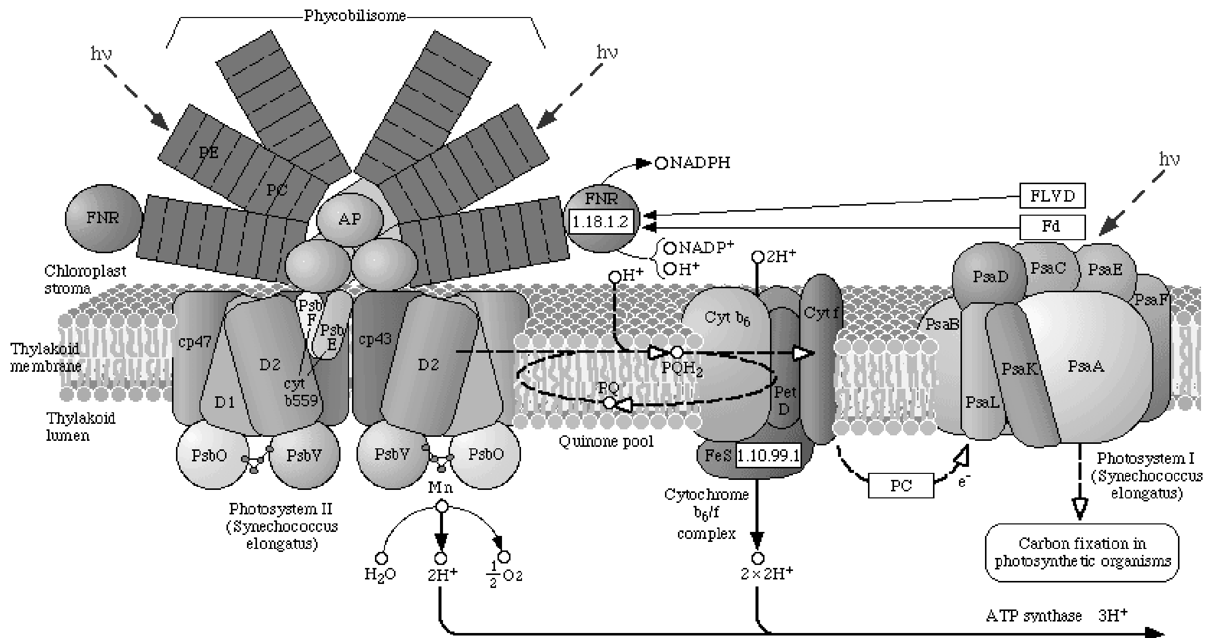


Figure 8 Subunit composition of PS II (left), Cytochrome b6/f complex (middle) and PS I (right) in cyanobacteria. The D1–D2 heterodimer forms the reaction center, which is surrounded by cytochrome b_{559} (E and F), PsbI, and the inner-antenna proteins CP47 and CP43. On the lumenal side, the Mn_4CaO_5 cluster is shielded by the extrinsic PS II proteins (*PsbO, PsbV*). Cyanobacteria have a peripheral-antenna system made of phycobilisomes, which are attached to the cytoplasmic side of PS II. The cytochrome b_6f complex or plastoquinol—plastocyanin reductase consists of the subunits cytochrome f with a c-type cytochrome, cytochrome b_6 with a low- and high-potential heme group, a Rieske iron-sulfur protein, a subunit IV along with four small subunits PetG, PetL, PetM, and PetN. Two main subunits of PS I, PsaA and PsaB, are closely related proteins involved in the binding of photorecetptors. [39, 40]

One of the major differences between chloroplasts in higher plants and cyanobacteria is the occurrence of phycobilisomes that cyanobacteria use as a light harvesting complex.[40] Unlike most light-harvesting antenna complexes, the phycobilisome is not an integral membrane complex but is attached to the surface of the photosynthetic membranes. It is composed of both the pigmented phycobiliproteins and the nonpigmented linker polypeptides. The former are important for absorbing light energy, while the latter are important for stability and assembly of the complex. [41, 42]

Phycobilisomes are composed of phycobiliproteins (PBPs). As shown in Figure 8 phycobilisomes are constructed from two main structural elements: a core substructure that is in contact with the thylakoid membrane and the photosystem II and peripheral rods that are arranged hemidiscoidally around that core. The core of most phycobilisomes is composed of three cylindrical subassemblies of allophycocyanin (AP). The rods are composed of stacked disks of phycocyanin (PC), phycoerythrin (PE) and phycoerythrocyanin (PEC). Usually AP and PC are always present, PE and PEC are only present in certain species. The two major subunits (α and β) of PBPs have a globin fold which contains helices A-B-E-F-G-H and two additional helices X-Y. These structures are homologous between variants (PC, PE, PEC, and AP). Typical PBPs covalently bind 1–3 molecules of bilin per subunit at an evolutionary conserved Cys residue(s). [43]

As shown in Figure 9, the α and β subunits are chromophorylated by the attachment of bilins. Although the formation of the biliprotein monomer (αβ) can occur without bilin attachment, the chromophorylation of the subunits usually enhances the dimerization and formation of the biliprotein monomer (αβ). Three of these monomers assemble to a disk-shaped trimer $(\alpha\beta)_3$. In the case of AP, four of the trimers form a cylinder that is stabilized by a linker-scaffold protein. In the case of the rod-building phycobiliproteins, two of the trimers form a hexamer $(\alpha\beta)_6$ and are subsequently stacked on one another to form rods of different compositions that are held together by linker proteins.[44]

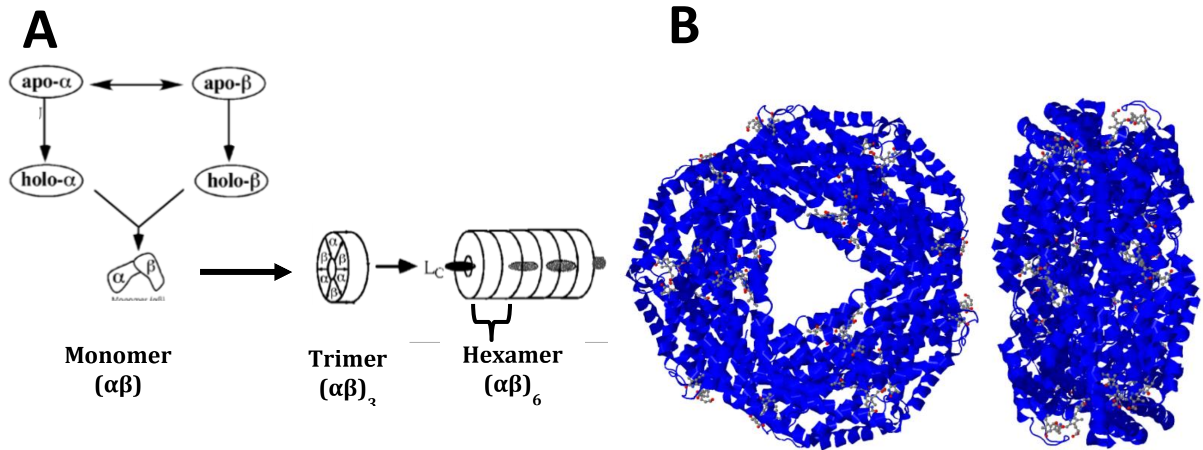


Figure 9 Phycobilisome assembly. (A) Model of the assembly of a rod in phycobilisomes (left) and (B) protein structure of one disk shaped trimer $(\alpha\beta)_3$ of phycocyanin from *Thermosynechococcus elongatus* (PDB id: 3L0F)[45]

Like chlorophyll *b* in plants, biliproteins expand the range of light energy that can service photosynthetic electron transfer but do so to a greater extent because of the spectral features of the three major biliprotein classes: phycoerythrin (PE, λ_{max} = 565 nm), phycocyanin (PC, λ_{max} = 617 nm) and allophycocyanin (AP, λ_{max} = 650 nm). In function, the phycobilisome absorbs light energy in the 500–650 nm range and transfers it to chlorophyll for photosynthesis at efficiencies that approach 100%.[45, 46] Absorbed light energy is transferred by very rapid, radiation-less downhill energy transfer from phycoerythrin or phycoerythrocyanin (if present) to phycocyanin and then to allophycocyanin that acts as the final energy transmitter from the phycobilisome to Chlorophyll a or the Photosystem II, respectively.[47, 48, 49]

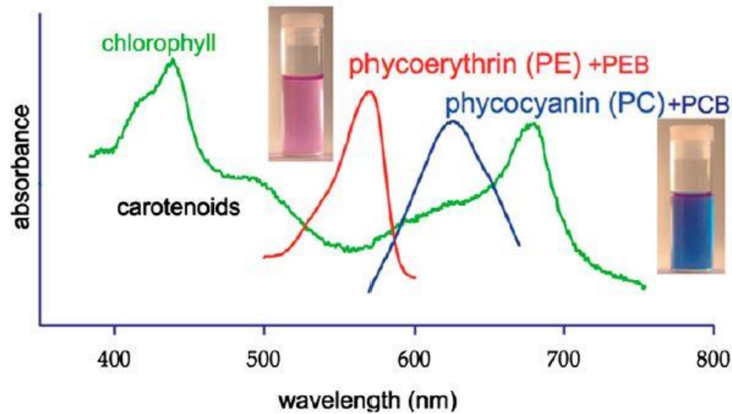


Figure 10 Absorbance spectra of cyanobacterial pigments. The area of low absorbance of chlorophyll at wavelengths from 500 – 650 nm is covered by the phycobiliproteins phycoerythrin (PE) and phycocyanin (PC). (Adapted from Kehoe, 2010) [50]

Phycobiliproteins may be present at very high concentrations (up to 30–40% of the cellular dry mass in some cases) and may also serve as reserve protein.[1] Phycobilisomes are completely dispensable because they are not absolutely required for the excitation of chlorophyll. Cyanobacteria have developed mechanisms for regulating phycobilisome synthesis and composition in response to external signals. Cyanobacteria regulate phycobilisome composition in response to both light intensity and light wavelength.[41, 45] The process of regulation in response to light quality is called chromatic acclimation, and cyanobacteria are divided into four groups of chromatic acclimators: Type I organisms do not change their PC or PE content when grown in light of different wavelengths. Type II organisms vary their PE content, Type III organisms affect both PE and PC levels, and Type IV chromatic acclimation involves changing the bilin composition of the PE present. [51, 50]

Bilins are open-chain tetrapyrrolic, biological pigments that are synthesized from porphyrines by many organisms. Phycobilins are bilins of plants and algae which act as the chromophores of phycobiliproteins where they function as light energy-harvesting pigments.[52] The most common phycobilins are phycocyanobilin (PCB) and phycoerythrobilin (PEB). While the bilin chromophores, that are attached to PBPs, adopt a quite flexible cyclo-helical conformation in solution, they are held in a rigid and extended conformation in native phycobiliproteins.[53] All bilins are attached by a thioether linkage to specific cysteine residues on PBPs. Most of them are attached at the C-3^1 position of the chromophore, however, additional binding at the C-18^1 has been shown for phycoerythrobilin and phycourobilin (PUB), as well as attachment at the C-3^2 position in rare cases.[54]

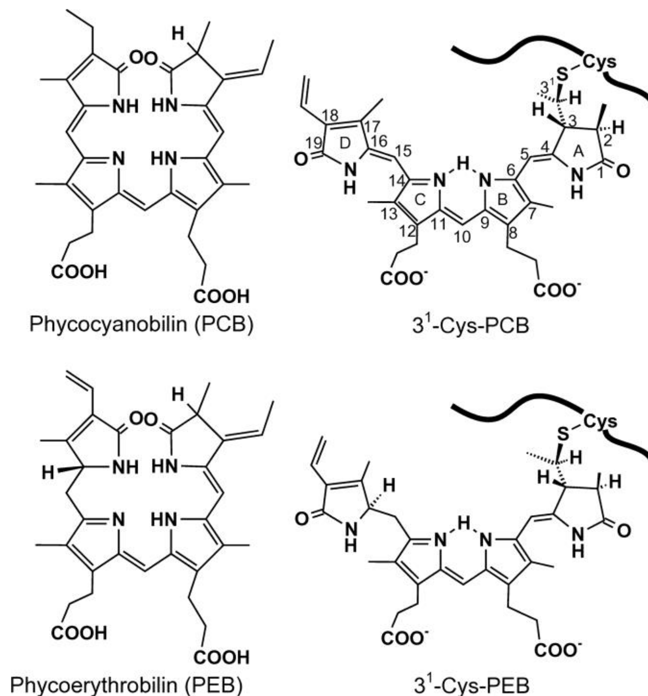


Figure 11 Bilin attachment to phycobiliproteins (PBPs) by thio-ether linkage at position C-3^1 (adapted from Zhao, 2006)[55]

Biosynthesis of phycobilins starts with the oxidative cleavage of heme by a heme oxygenase resulting into formation of biliverdin IXα (BV).[56,57,58] Biliverdin IXα is then reduced by ferredoxin dependent reductases to produce phycobilins. In plant cells Hy2 is responsible for the reduction of BV to phytochromobilin (PϕB).[59] PEB is synthesized in a two step reaction by PebA and PebB or alternatively in a single enzyme reaction by Phycoerythrobilin Synthase (PebS), a recently discovered enzyme found encoded in a virus that infects marine cyanobacteria.[60] The formation of PCB is catalyzed by the enzyme Phycocyanobilin:Ferredoxin Oxidoreductase (PcyA) by two consecutive reductions.[61] In addition to the occurrence of the most common bilins PCB and PEB, in some species these can be isomerized during attachment to the PBPs yielding in phycoviolobilin (PVB) and phycourobilin (PUB), respectively. [62, 63, 64]

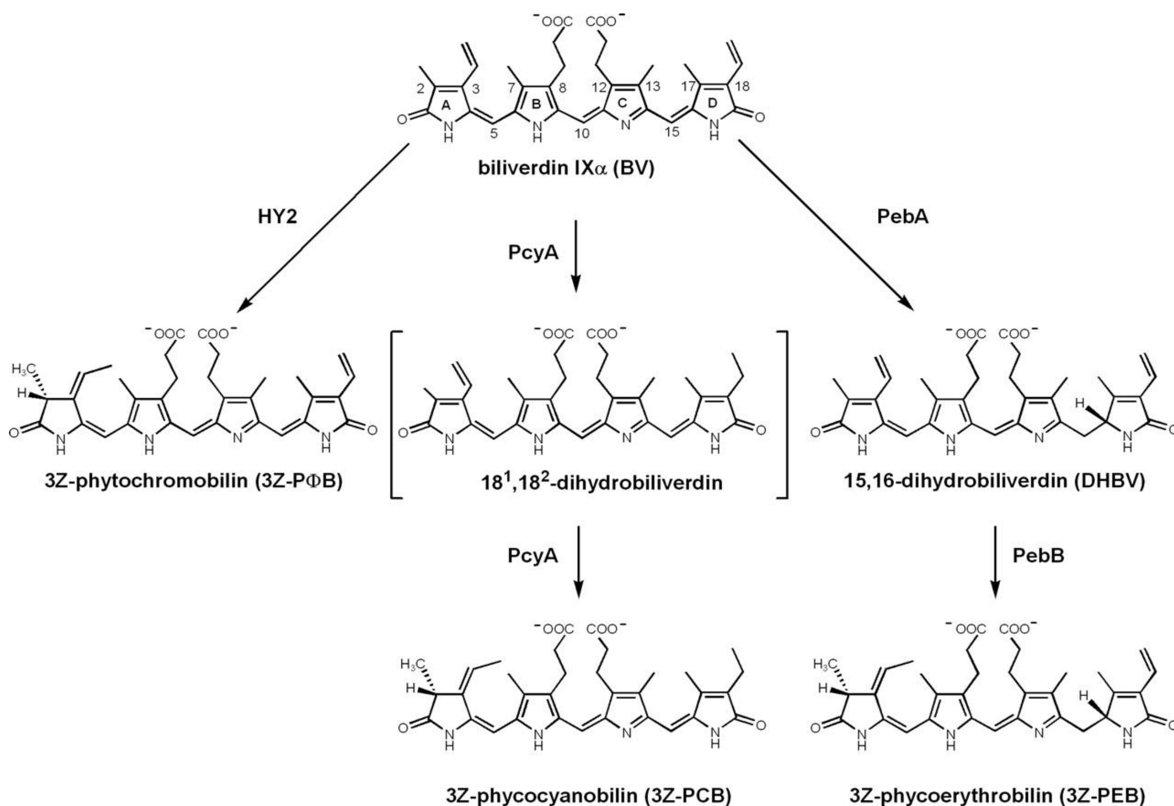


Figure 12 Phycobilin biosynthesis. Enzymatic reductions of biliverdin IXα to phytochromobilin (PϕB), phycocyanobilin (PCB) and Phycoerythrobilin (PEB) by ferredoxin dependent reductases HY2, PcyA and PebA + PebB, respectively.(adapted from Dammeyer, 2006)[65]

Bilin composition varies by type and number within the different biliprotein classes. AP has the simplest bilin content. Both subunits have a single chromophore, the central bilin, which is held between the E and F helices at Cys-81/82 (consensus position) and is common to all biliproteins.

The PC biliproteins add a second, peripheral phycocyanobilin to the β-subunit at a loop between helices G and H at Cys-155.[53] PE α has two chromophores, central and peripheral, at sites that are isologous to the PC β-subunit. The PE β-subunit incorporates a third bilin at a peripheral site on the A helix. The bilin network in the phycobilisome varies within different organisms as well as under different light conditions. However, it generally agrees with the following stoichiometry: 30 bilins/PE (αβ) hexamer, 18 bilins/PC (αβ) hexamer, 12 bilins/AP (αβ) hexamer.[45] Linker proteins are usually colorless, although at least two of them (L_{cm} and γ-PE-II) are chromophorylated.[53]

1.4. LYASES

Although spontaneous attachment of bilins to phycobiliproteins is observed *in vitro*, this process seems to be of very low fidelity and generally results into product mixtures *in vivo*. [66] True autocatalytic attachment that leads to spectroscopically, biochemically and functionally indistinguishable products from native forms isolated from organisms is very unusual for phycobiliproteins. So far it can only be found in plant phytochromes and in the linker protein ApcE.[67, 68]

Efficient chromophore binding to apoproteins is catalyzed by enzymes called bilin lyases, of which only few have been characterized in detail.

Lyases follow a four letter nomenclature. The first letter indicates the taxonomic division of the organism. The second and third letters indicate the phycobiliprotein substrate and the fourth letter indicates the type of lyase. CpcS therefore is a cyanobacterial lyase of the type S that chromophorylates phycocyanin.

Attempts to classify lyases according to their structure and phylogenetic relationships have lead to the following types of cyanobacterial lyases:[69]

E/F-TYPE LYASES

This group of lyases is variable in length, but all of these lyases possess HEAT repeats and an alpha-alpha superhelix fold and belong to the Armadillo repeat superfamily. This group contains the first described lyase, a heterodimeric lyase (CpcE/F) in *Synechococcus* PCC 7002 that is required for correct ligation of PCB to Cys-84 on α-PC[70] and catalyzes both the forward (binding) and the reverse (releasing) reaction.[71]

This group contains CpeY and CpeZ families that are involved in the attachment of PEB at Cys-82 α-PE (CpeA). CpeY alone, but not CpeZ can ligate PEB to CpeA, but with a reduced yield than CpeY and CpeZ together.[72]

This group also includes phycobilin lyases-isomerases PecEF from *Mastigocladus laminosus* [63], RpcE *Synechococcus* sp. strain WH8102 [64] and MpeZ from marine *Synechochoccus*[73] which isomerize bilins during attachment.

T-TYPE LYASES

Given the absence of any obvious companion sequence in phyletic profiles, members of this group are likely not forming heterodimeric complexes. CpcT has been characterized in two species to attach a PCB at Cys-153 of β-PC [74,75]. *Nostoc sp.* PCC 7120 CpcT also attaches PCB to Cys 155 of PecB [75]

S/U-TYPE LYASES

According to the only solved X-ray crystal structure and protein structure prediction, lyases in this group possess a 10-stranded anti-parallel β-barrel structure with an α-helix.[76] The S/U type of lyases comprises a family of proteins that are unrelated to the

E/F-type lyases and exhibit rather large and characteristic variations. The main feature of the S/U lyases is high binding site specificity, but a very low specificity for the chromophore and the receptor apoprotein. For example CpcS from *Nostoc* PCC 7120 is a nearly universal lyase for PCB attachment at Cys-84 of apo-phycobiliproteins.[77] S-type lyases can either act as monomers, homodimers or heterodimers with U-type lyases.[55,53] For example, CpcS from *Nostoc* PCC 7120 has been shown to be active as a monomeric lyase whereas CpcS from *Synechococcus* PCC 7002 is inactive on its own and requires the formation of a heterodimer with CpcU for lyase activity.[55,74,78] The former is phylogenetically classified as a CpcS type III lyase, the latter as a CpcS type I layse.[69,79] The CpcS and CpcU type II subfamilies are only known from marine *Synechococcus* and *Cyanobium* and their co-occurrence suggests a heterodimer formation.[80] Attachment of PEB to both subunits of phycoerythrin by CpcS from the PE-less *Nostoc* PCC 7120 has been reported *in vitro* and indicates the broad substrate specificity. [53]

In cyanobacteria that lack PE, only few lyases seem to be sufficient to attach all phycobiliprotein chromophores: CpcS(/U) attaches PCB chromophores to both subunits of AP at Cys-81.[81] CpcS(/U) and CpcT attach PCB to Cys-β84 and Cys-β155, respectively, of PC and PEC. CpcE/F and PecE/F catalyze attachment of PCB to Cys-α84 of PC and of PEC, respectively, and in case of PEC the isomerization of PCB to PVB. The chromophore of L_{cm} is attached autocatalytically to the apoprotein, ApcE. [53, 80]

Mechanism of lyase activity

So far there is very little information available about the mechanism of chromophore attachment by lyase activity. The mechanism for E/F type lyases seems to be more complex than for S(/U) type lyases.[53] However, Chaperone-like action has been proposed for both phylogenetically independent classes of lyases.[82, 83, 84, 85] The fast binding of the chromophores by the lyases can be interpreted as a way of reducing the degradation of the rather labile chromophores.[82] In PecE/F from *Nostoc* PCC 7120 both

subunits are required for a correct attachment and isomerization of the chromophore to form PEC, which includes a phycoviolobilin (PVB) chromophore. PecE is able to attach PCB to the PecA subunit alone, but only in combination with PecF the isomerization is conducted correctly.[84]

CpcS1 from *Nostoc* PCC 7120, that has been classified as a lyase of the phylogenetic type CpcS-III and compared to E/F type lyases, it seems to follow a quite simple mechanism.

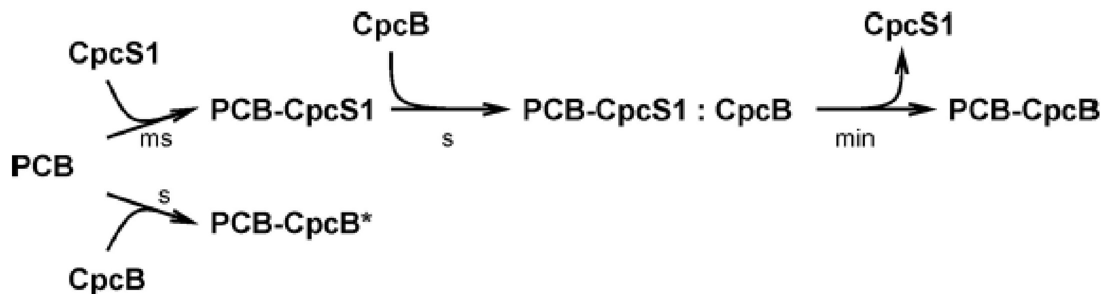


Figure 13 Catalytic scheme of CpcS1. The fast association of PCB with CpcS1 leads to the final phycobiliprotein CpcB with correctly attached PCB. In the absence of a lyase a slower spontaneous addition of PCB to CpcB leads to the incorrectly attached chromophore PCB, here PCB-CpcB*. (adapted from Kupka, 2009)[82]

1.4. *Te*CpcS (tll1699): CpcS from *Thermosynechococcus elongatus*

Thermosynechococcus elongatus BP-1 is a unicellular rod-shaped cyanobacterium, and grows at an optimal temperature of 55°C.[86] Due to its thermostability of proteins and determination of the entire genome sequence,[87] the organism has gained various interests of research[88] *T. elongatus* does not synthesize PEB and contains only PC and AP as major PBPs.[87] The protein *Te*CpcS (gene: tll1699) shows strong sequence similarity to the identified lyases *S*CpcS-I and *S*CpcU from *Synechococcus sp.* PCC 7002, *N*CpcS from *Nostoc sp.* PCC 7120, *P*CpeS-II from *Prochlorococcus marinus* and *Fd*CpeS-I from *Fremiella diplosiphon*.[89] Therefore it was proposed to be a lyase and according to the

CyanoLyase database (http://cyanolyase.genouest.org/) *Te*CpcS belongs to the CpcS-III subfamily.[69]

The lyase activity of *Te*CpcS was demonstrated by Kronfel et al. (2013). *Te*CpcS is able to attach either PEB, PCB or PϕB to the Cys-82 position of α- and β-subunit of allophycocyanin and the β-subunit of phycocyanin.[89]

*Te*CpcS is the first lyase whose X-ray crystal structure has been determined at a resolution of 2.8 Å due to the NIH Protein Structure Initiative. This initiative tried to find representatives from large domain families that were structurally undescribed thus far.[90] The detailed structure coordinates of TeCpcS can be found in the Protein Data Bank (PDB id: 3BDR).[76] According to the database PFAM,[91] *Te*CpcS belongs to the protein family of CpeS-like proteins (PF09367) that contains 209 proteins. However, if the Hidden Markov Model that is used by PFAM is applied to the UniProt database (2013_4) the domain signature can be found in 288 sequences. This discrepancy is due to the fact that PFAM only considers certain genomes.[89]

From a structural perspective *Te*CpcS belongs to the family of lipocalins. Although earlier thought to be only present in eukaryotes, the lipocalin structural motif can be found in a very broad spectrum of organisms.[92, 93] The sequence conversation between different members of this protein family is generally very low. Functionally lipocalins share similarities by transporting small hydrophobic molecules like steroids, bilins, retinoids and lipids.[94]

The structure of *Te*CpcS contains two α-helices and one 10-stranded, antiparallel β-sheet with a beta-barrel fold. One side of the beta-barrel seems to be capped by one of the α-helices, whereas the other side is wide open, presumably to be the entry side for the bound ligand. *Te*CpcS has been crystallized as a homodimer and also the retention time of size exclusion chromatography confirmed a dimerization in the purification process. Additionally the gene encoding *Te*CpcS is the only open reading frame that shows similarity to a CpcS-type in the *Thermosynechococcus elongatus* genome, which

decreases the possibility of a heterodimer formation with a CpcU-type lyase as seen in other cyanobacteria.[89]

Figure 14 X-Ray crystall structure of the *Te*CpcS momomer. Derived from Protein Data Base (PDB id: 3BDR)[77] and displayed from the side (left) and from top down (right). The helix on the lower end of the beta-barrel seems to act as a capping of the funnel-like structure

Cysteine residues

An earlier study on the intrinsic bilin binding of *Te*CpcS revealed a covalent attachment of PCB and PEB to the lyase. By mass spectrometric analysis two cysteine residues at position 2 and 169 of the lyase, were shown by MS/MS to be the binding sites of the bilin. These cysteine residues are not conserved in the CpcS-I/U lyase family. However, the covalent PCB ligation was of a much lower extent than in the case of biliproteins.[89] Interestingly, in the crystal structure the Cys-2 residue is located at the outside of the protein surface, where the N-terminal helix is putatively capping the beta-barrel, whereas the Cys-169 is positioned right at the center of the cavity.

The first goal of this diploma thesis is to investigate the influence of the above described cysteine residues on the intrinsic bilin binding of *Te*CpcS as well as their contribution to the lyase activity of bilin attachment to phycobiliproteins.

Through structure comparison using the Distance-matrix alignment algorithm (DALI)[95] numerous similar structures to TeCpcS could be found in the PDB. Although most of the structurally related proteins are fatty acid binding proteins, the closest structural homologue within the bilin binding proteins is the UnaG protein. Like *Te*CpcS, UnaG is composed of a 10-stranded beta barrel, but these two proteins only have 11% sequence identity (PDB id: 4I3B). UnaG protein from Japanese freshwater eel Unagi (*Anguilla japonica*) was first reported to be a fluorescent low molecular mass protein in small-diameter muscle fibers.[96] It belongs to the fatty-acid-binding protein family, however its main ligand is unconjugated (i.e. not bound to glucuronic acid) bilirubin (BR), a bilin.[97] As the crystallized structure of *Te*CpcS does not contain a ligand, the structure of UnaG including bilirubin was used by Kronfel et al. (2013) to align the two structures and this overlay showed only marginal clashes of the ligand with the *Te*CpcS side chains. Additionally the two unresolved loops of the *Te*CpcS structure (residues 77−83 and 108−117) could be modeled by using the UnaG structure as a template. It is hypotethized that these loops only become rigid upon ligand binding.[89]

Manual docking of the cognate ligand PCB to *Te*CpcS was performed by using the interaction of bilirubin and UnaG as a model. After the docking exercise by using XtalView and refinement to reduce steric clashes, the structure showed that ring D of PCB was buried inside the funnel and ring A, where the thioether-bond to the PBPs would be formed, was on top of the funnel and therefore more accessible. The major interactions between the ligand and the protein side chains are shown in Figure 14. The carbonyl group of ring D forms a hydrogen bond with Arg-151. The two propionate groups of ring B and ring C interact with the polar groups of Ser-155 and Trp-73, respectively.

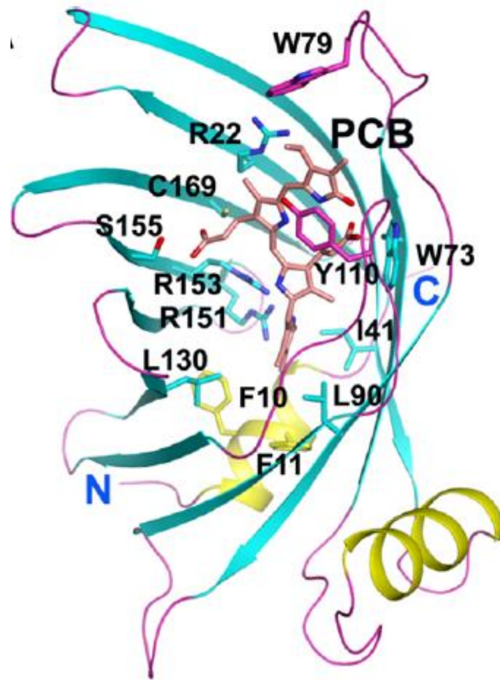


Figure 15 Structure of *Te*CpcS with PCB from docking experiments, shows the position of the ligand in the beta-barrel cavity of the protein. The ligand PCB and strictly conserved side chains are shown as balls and sticks. The C- and N-terminal positions are indicated. (adapted from Kronfel et al., 2013)

The second goal of this diploma thesis is to investigate the importance of the above mentioned residues Trp-73, Arg-151 and Ser-155 for the intrinsic bilin binding as well as their role in lyase activity.

Since cyanobacteria have one the simplest nutrient requirement in nature, because they thrive photoautotrophically on simple inorganic media, tolerate marginal agricultural environments and many of them do not need combined nitrogen in their medium, they offer a broad range of possible applications.[33] Cyanobacterial synthetic biology seems very promising for enhancing efforts to produce biofuels and other useful chemicals in photoautotrophic hosts.[98] They have been engineered to produce a wide range of products including fatty acids, alkanes, ethylene, polyhydroxybutyrate, 2,3-butanediol, ethanol, and hydrogen.[99] Cyanobacterial proteins are not optimal to feed to cattle but can be used as dietary supplements with various positive effects for humans and animals.[33] They have been identified as a rich source of biologically active compounds with antiviral, antibacterial, antifungal, immunosuppressant and anticancer activities.[100] Several strains of cyanobacteria can accumulate polyhydroxyalkanoates, which can be used as biodegradable plastic. Also, they can be found in big amounts at sites of oil-spills, providing the associated oil-degrading bacteria with the necessary oxygen, organics and fixed nitrogen. Wastewater treatment, food, fertilizers, production of secondary metabolites including exopolysaccharides, vitamins, toxins and enzymes are further possibilities. The cosmetic industry recently tries to exploit the cyanobacterial feature of overcoming the toxicity of ultraviolet radiation by means of UV-absorbing/screening compounds, such as mycosporine-like amino acids and scytonemin.[101] Phycobiliproteins conjugated to diverse proteins developed into fluorescent probes and applications are being found in histochemistry, fluorescence microscopy, flow cytometry, fluorescence-activated cell sorting and fluorescence immunoassays. Phycocyanin shows beneficial antioxidant reactivity in humans due to its association with phycocyanobilin.[102,103]

2. EXPERIMENTAL PROCEDURES

2.1. EXPRESSION VECTORS

Plasmids used for expression of phycobiliprotein subunits and phycobilin producing enzymes were previously described by Biswas et al. (2010), Kronfel et al. (2013) and Dammeyer et al. (2008). [60,72,89]

Table 1 Plasmids used for the experimental studies described in this study

Plasmid name	Recombinant proteins produced	Parental vector	Anti-biotic[a]	Reference
pCpcBA	*Synechocystis sp.* PCC 6803 HT-CpcB and CpcA	pCDF Duet	Sp	Biswas et al. 2010[72]
pApcAB	*Synechococcus sp*. PCC 7002 HT-ApcA and ApcB	pCDF Duet	Sp	Schluchter lab UNO, Shen et al. 2006[78]
pPcyA	*Synechocystis sp.* PCC 6803 HO1 and Synechococcus sp. PCC 7002 PcyA	pACYC	Cm	Biswas et al. 2010[72]
pPebS	*Myovirus* HO1 and PebS	pACYC	Cm	Dammeyer et al. 2008[60]
pTER13-30	*Thermosynechococcus elongatus* HT-CpcS	pET-30c	Km	Kronfel et al. 2013[89]
pTER13(W73G)	*Thermosynechococcus elongatus* HT-CpcS(W73G)	pTER13-30	Km	This thesis
pTER13(R151G)	*Thermosynechococcus elongatus* HT-CpcS (R151G)	pTER13-30	Km	This thesis
pTER13(S155G)	*Thermosynechococcus elongatus* HT-CpcS (S155G)	pTER13-30	Km	This thesis
pTER13(C2S)	*Thermosynechococcus elongatus* HT-CpcS (C2S)	pTER13-30	Km	This thesis
pTER13(C169S)	*Thermosynechococcus elongatus* HT-CpcS (C169S)	pTER13-30	Km	This thesis
pTER13(C2S/C169S)	*Thermosynechococcus elongatus* HT-CpcS (C2S/C169S)	pTER13-30 (C2S)	Km	This thesis
pTER13(W73G/R151G)	*Thermosynechococcus elongatus* HT-CpcS (W73G/R151G)	pTER13-30	Km	This thesis
pTER13(W73G/ R151G/S155G)	*Thermosynechococcus elongatus* HT-CpcS (W73G/R151G/S155G)	pTER13-30	Km	This thesis

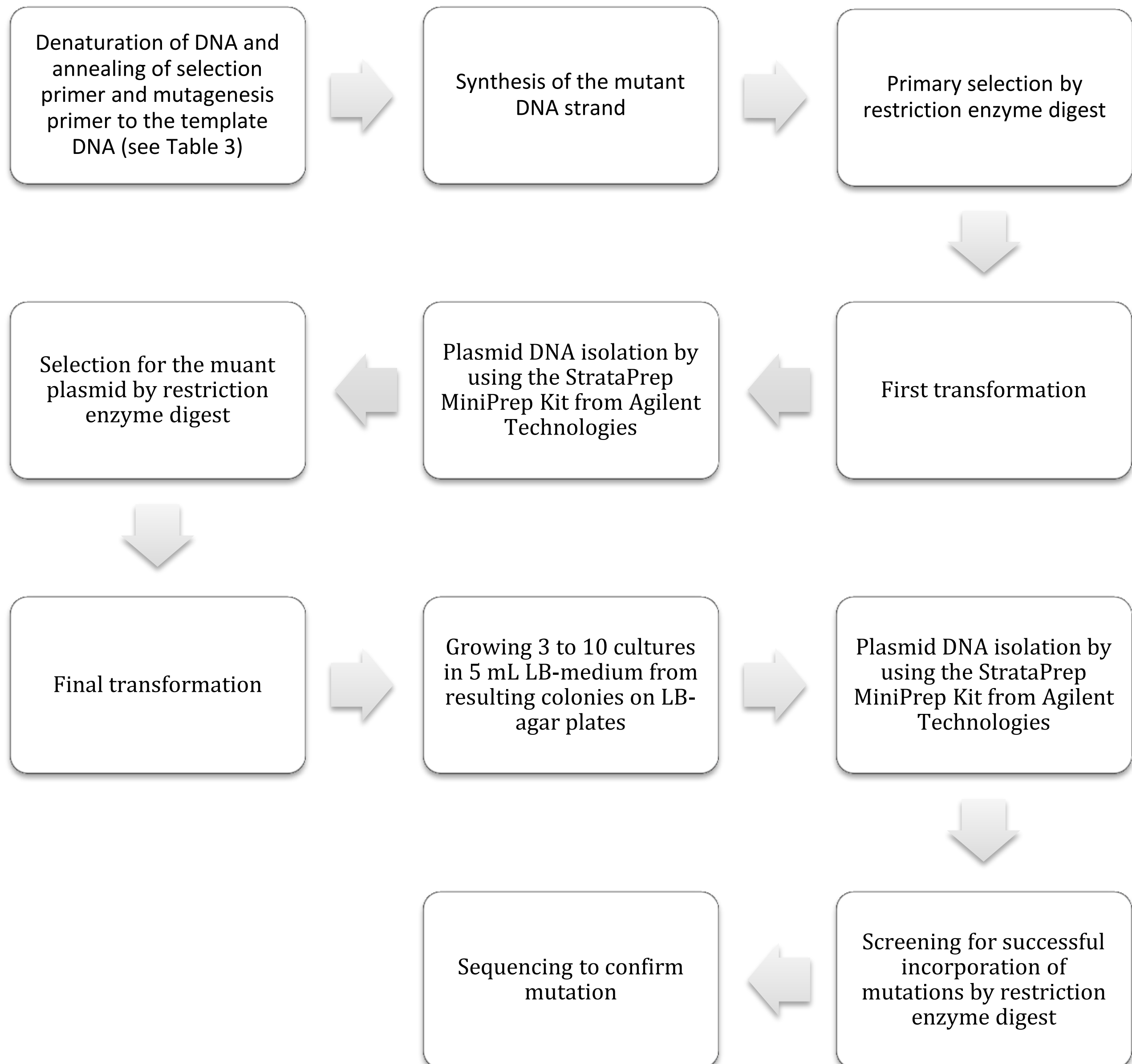
[a]Anitibiotic resistance used to select for the presence of the plasmid (Sp: spectinomycin, Cm: chloramphenicol, Km: kanamycin)

For expression of the α- and β-Phycocyanin subunits of *Synechocystis sp.* PCC 6803 the vector pCpcBA was used. In this vector derived from pCDF Duet, CpcB contains an N-terminal hexahistidine-tag. The vector pApcAB containing genes for the α- and β-subunits of allophycocyanin was produced in the Schluchter lab at University of New Orleans from a previous construct described by Shen et al. (2006).[74] The α-subunit of allophycocyanin is fused to a hexahistidine-tag at the N-terminal (HT-ApcA). The production of the phycobilins PCB or PEB from heme in *E.coli* was achieved by using the expression vectors pPcyA or pPebS, respectively. The plasmid pPcyA contains the genes for the enzymes Heme-Oxygenase1 (HO1) from *Synechocystis sp.* PCC 6803 and PcyA from *Synechococcus sp.* PCC 7002. The *myovirus* Heme-Oxygenase1 and Phycoerythrobilin Synthase (PebS) are incorporated in the vector pPebS. The plasmid containing the CpcS wildtype lyase from *Thermosynechococcus elongatus* (*Te*CpcS) was cloned into pET-30c from a different vector in a previous study by Kronfel et al. (2013).[89]

Table 2 List of proteins used in this study

Protein	Number of amino acids	Molecular Weight [Da]	Extinction coefficient $\varepsilon_{280\ nm}$ [$M^{-1}\cdot cm^{-1}$]
HT-TeCpcS	191	21,428.51	33710
CpcA	163	17,586.47	20525
HT-CpcB	186	19,739.02	9665
HT-ApcA	175	18,911.25	11920
ApcB	161	17,221.53	18005

Mutagenesis was performed by the Transformer™ Site-Directed Mutagenesis Kit from Clontech Laboratories, Inc. This procedure included the following steps:



All mutants except of *Te*CpcS C2S and *Te*CpcS C2S/C169S were created by the method above. For *Te*CpcS C2S and *Te*CpcS C2S/C169S, standard PCR and subsequent ligation was used to incorporate the mutation at position Cys-2 of *Te*CpcS wildtype or *Te*CpcS C169S, respectively. (see Table 3)

Table 3 List of primers used in the mutagenesis procedures. All primers were purchased from Eurofins MWG operon (Huntsville, AL)

Primer name	Sequence	Use
TeCpcS(W73G)	5'-[Phos]gcgggtcagtggggacggcaccat-3'	mutagenesis
TeCpcS(R151G)	5'-[Phos]ccccaatttaggtctgcgcacca-3'	mutagenesis
TeCpcS(S155G)	5'-[Phos]tctgcgcaccggtattctcaagc-3'	mutagenesis
TeCpcS(C169S)	5'-[Phos]ggcctccttctcctcggaaattcg-3'	mutagenesis
TeCpcS(C2S).F.Nde	5'-gatataCATATGtccataggtatggacatccgcg-3'	mutagenesis (PCR)
TeCpcS(C2S).R.Xho	5'-ggtgCTCGAGggagttggcgggttg-3'	mutagenesis (PCR)
TeCpcS(R151,S155G)	5'-[Phos]ccccaatttaggtctgcgcaccggtattctcaagc-3'	mutagenesis
pET-30c(XbaI)del	5'-[Phos]gtgagcggataacaattcccctctacaaataattttg-3'	mutagenesis selection primer
pET Upstream	5'-atgcgtccggcgtagagg-3'	sequencing
T7 Terminator	5'-gctagttattgctcagcgg-3'	sequencing

All resulting mutants were sequenced by the W. M. Keck Conservation and Molecular Genetics laboratory (University of New Orleans) to confirm the mutations that have been introduced.

TRANSFORMATIONS

Expression plasmids were co-transformed into *E.coli* BL21 DE3 cells purchased from Merck (Darmstadt, Germany) using 1 µL of plasmid DNA (concentration = 50 ng/µL) in 20-50 µL of competent cells. After incubation of 30 minutes on ice, the cells were heat shocked for 30 seconds in the water bath at 42°C and immediately cooled on ice for 2 minutes. After incubation of the cells in 150 µL of SOC medium in 37°C and shaking at 220 rpm, the cell suspension was streaked onto LB-agar plates containing the appropriate antibiotic combination for plasmid selection. For antibiotic concentrations see Table 4. The cells were grown at 37°C over night. Media: LB-medium (10g Bacto-Trytone/L, 5g Yeast extract/L, 10g NaCl/L), SOC (20g Bacto-Trytone/L, 5g Yeast extract/L, 0.5g NaCl/L, 360g glucose/L 2.5mM KCl, 100mM $MgCl_2$) LB-agar plates (15g Bacto-agar/L LB)

Table 4 Antibiotic concentrations in LB-agar plates and cultures for plasmid selection

Antibiotic	Symbol	Concentration [µg/mL]
Chloramphenicol	Cm	34
Spectinomycin	Sp	100
Kanamycin	Km	50

PROTEIN EXPRESSION

E.coli cells were picked from LB-agar plates and grown in 200 mL LB-medium containing appropriate antibiotics at 37°C and 220 rpm until optical density $OD_{600\ nm}$ = 0.6 (approximately 3.5 – 5 hours). Production of T7 RNA polymerase was induced by the

addition of 200 µL of 1M isopropyl β-D thiogalactoside (IPTG). For production of PCB (cells containing the plasmid pPcyA) cells were incubated at 30°C and 220 rpm for another 3 hours. For production of PEB (cells containing the plasmid pPebS) cells were incubated at 18°C for 16 hours over night. Subsequently the cells were collected by centrifugation for 15 minutes at 10000 x g. The supernatant was discarded and cell pellets were washed twice with ddH_2O. The cells were stored at -20°C until required.

PROTEIN PURIFICATION

The *E.coli* cells containing his-tagged recombinant protein were thawed and resuspended in 15 mL Buffer 0 (50mM Tris-HCl, 150 mM NaCl, pH 8.0) and homogenized in glass homogenizers. After addition of 150 µL of lysozyme (10mg/mL) and 150 µL of protease inhibitor (1 tablet in 1mL ddH_2O, cOmplete tablets, Roche, Germany) the cell suspension was incubated on ice for 25 minutes. The cells were then lysed by three passages through a French Press at 138 MPa. The lysed cell suspension was then centrifuged at 13,000 x g to remove inclusion bodies, cell debris and unbroken cells. In order to purify hexa-histidine tagged proteins, the protein was loaded on 4 mL of nickel-nitrilotriacetic acid Superflow-affinity column resin (Qiagen, Inc., Chatsworth, CA). The protein was washed with one column volume of each Buffer A1 (20mM Tris-HCl pH 8.0, 100mM Na/KCl, 20mM imidazole, 5% glycerol), Buffer B (20mM Tris-HCl pH 8.0, 0.5M Na/KCl), Buffer A2 (20mM Tris-HCl pH 8.0, 100mM Na/KCl, 30mM imidazole) and then eluted in 15 mL of Buffer C (20mM Tris HCl pH 8.0, 100mM Na/KCl, 200mM imidazole). Removal of imidazole in the buffer was achieved by dialysis at 4°C in buffer 0 containing 10mM 2-mercaptoethanol overnight. Purified proteins were used for analysis without concentration in case of presence of phycobiliproteins whereas samples only containing the lyase *Te*CpcS or derived mutants were concentrated 10X by centrifugal filter units with molecular weight limit 10,000 Da (Millipore, Darmstadt, Germany).

ABSORBANCE AND FLUORESCENCE EMISSION SPECTROMETRY

Absorbance spectra were acquired with a lambda 35, dual-beam UV-Vis spectrophotometer (Perkin Elmer, Waltham, MA). Fluorescence emission spectra were recorded with a LS55 fluorescence spectrophotometer (Perkin Elmer, Waltham, MA). Depending on the type of bilin present, the excitation wavelength was either set to 490 nm for PEB or 590 nm for PCB. The slit widths were set at 10 nm for excitation and emission. For the semiquantitative determination of the chromophorylation of CpcB by *Te*CpcS a dilution to an equal protein concentration was performed, based on the absorbance $A_{280\ nm}$ which corresponds to the abundance of Trp and Tyr residues in the protein.

PROTEIN ANALYSIS

Protein separation was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 13 minutes at 100 V and 1 hour at 200V in an electrophoresis cell containing Laemmli buffer (Tris 31g/L, Glycine 144g/L, SDS 10g/L). 15% acrylamide SDS-Gels (75mm x 50mm x 1 mm) with a 10% acrylamide stacking layer were prepared in the lab. To reveal covalent bilin attachment to the proteins, the gels were soaked in 10mM $ZnSO_4$ for 10 minutes and zinc-enhanced fluorescence of bilins was recorded using an FX imaging system (BioRad, Hercules, CA). For excitation at 532 nm an external laser light source was used. Visualization of total protein on the gel was achieved through staining with Coomassie Brilliant Blue (3.5g in 1L of MeOH/HOAc/H_2O; v/v = 4/1/5) over night and consecutive destaining the next day.

3. RESULTS

Previous studies of *Te*CpcS implied the importance of certain residues in its amino acid sequence for either covalent or non-covalent bilin binding.
Data from MS/MS spectra showed the occurrence of covalent bilin binding to *Te*CpcS at the two cysteine residues Cys-2 and Cys-169.[89] Manual docking experiments performed by Kronfel et al. (2013) of the bilin PCB to *Te*CpcS using the UnaG protein as a template, suggested strong non-covalent binding of the bilin to the residues Trp-73, Arg-151 and Ser-155 through hydrogen bonds.[89]

3.1. INTRINSIC BILIN BINDING

Site–directed mutants of *Te*CpcS were produced at positions Cys-2, Cys-169, Trp-73, Arg-151, Ser-155 as well as the double mutants Cys-2/Cys-169, Trp-73/Arg-151 and the triple mutant Trp-73/Arg-151/Ser-155. These mutants were then used in heterologous coexpressions of recombinant proteins in *E.coli* with genes that encode the enzymes for bilin chromophore production. The produced bilin was either phycocyanobilin (PCB) or phycoerythrobilin (PEB).

CYSTEINE-MUTANTS

His-tagged *Te*CpcS wildtype and the His-tagged mutants *Te*CpcS C2S, *Te*CpcS C169S, *Te*CpcS C2S/C169S were coexpressed with Phycocyanobilin Synthase (PcyA) and Heme Oxygenase1 (HO1) in *E.coli* in order to examine the PCB binding properties of this lyase. After concentration of the protein sample (10X) and purification by Nickel-NTA chromatography, absorbance and fluorescence emission spectra (excitation at 590 nm) were recorded as shown in Figure 16 (A). As the bilin binding is partially retained during Ni-NTA chromatography, the spectra represent a mixed state of covalent and non-covalent bilin binding to the lyase. All of the mutants show an absorbance peak at 605

nm and fluorescence emission at 633 nm, although the amounts seen are significantly lower than for phycobiliproteins. However, after SDS-PAGE and Zn-enhanced bilin fluorescence emission imaging, only the wild-type and the *Te*CpcS C169S mutant showed detectable bilin fluorescence (see Figure 16 (C)). Figure 16 (D) shows a slightly different colour of the *E.coli* cell pellets for *Te*CpcS C2S and the *Te*CpcS C2S/C169S compared to the samples without the mutation at the Cys-2 position. These results suggest, that covalent PCB binding at the lyase occurs predominantly at the Cys-2 position.

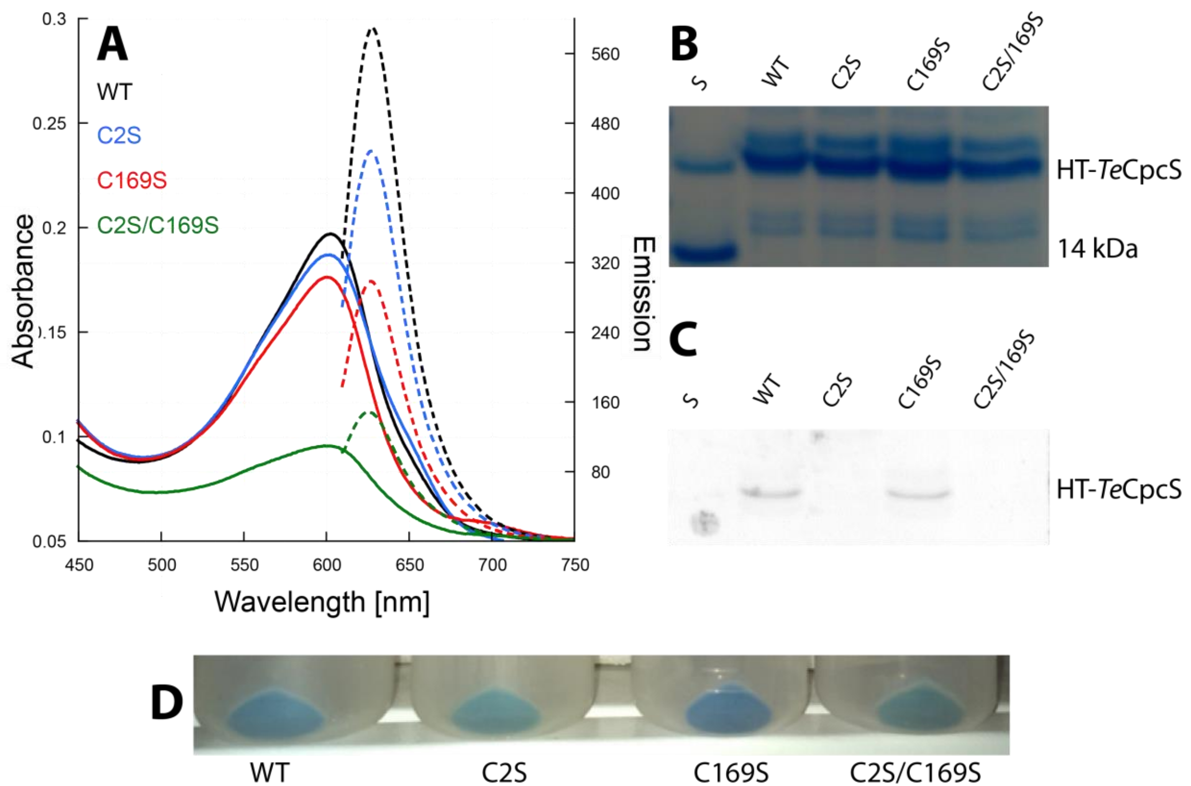


Figure 16 Intrinsic binding of PCB by *Te*CpcS wildtype and Cysteine-mutants. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-*Te*CpcS wildtype (black), HT-*Te*CpcS C2S (blue), HT-*Te*CpcS C169S (red) and HT-*Te*CpcS C2S/C169S (green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of *Te*CpcS wildtype, HT-*Te*CpcS C2S, HT-*Te*CpcS C169S, HT-*Te*CpcS C2S/C169S and molecular weight standards (lane S). (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB and HT-*Te*CpcS wildtype, HT-*Te*CpcS C2S, HT-*Te*CpcS C169S, HT-*Te*CpcS C2S/C169S.

The same expressions as above were also performed by substitution of PcyA by PebS, which leads to the production of PEB (red) instead of PCB (blue) in the cells. Figure 17 shows a very similar pattern of bilin binding for PEB as for PCB in Figure 16 above. Figure 17 (A) shows absorbance peaks for all samples at 560 nm and fluorescence emission peaks at 575 nm. A second peak at around 600 nm might be caused by the intermediate of PEB synthesis 15,16-dihydrobiliverdin.[65]

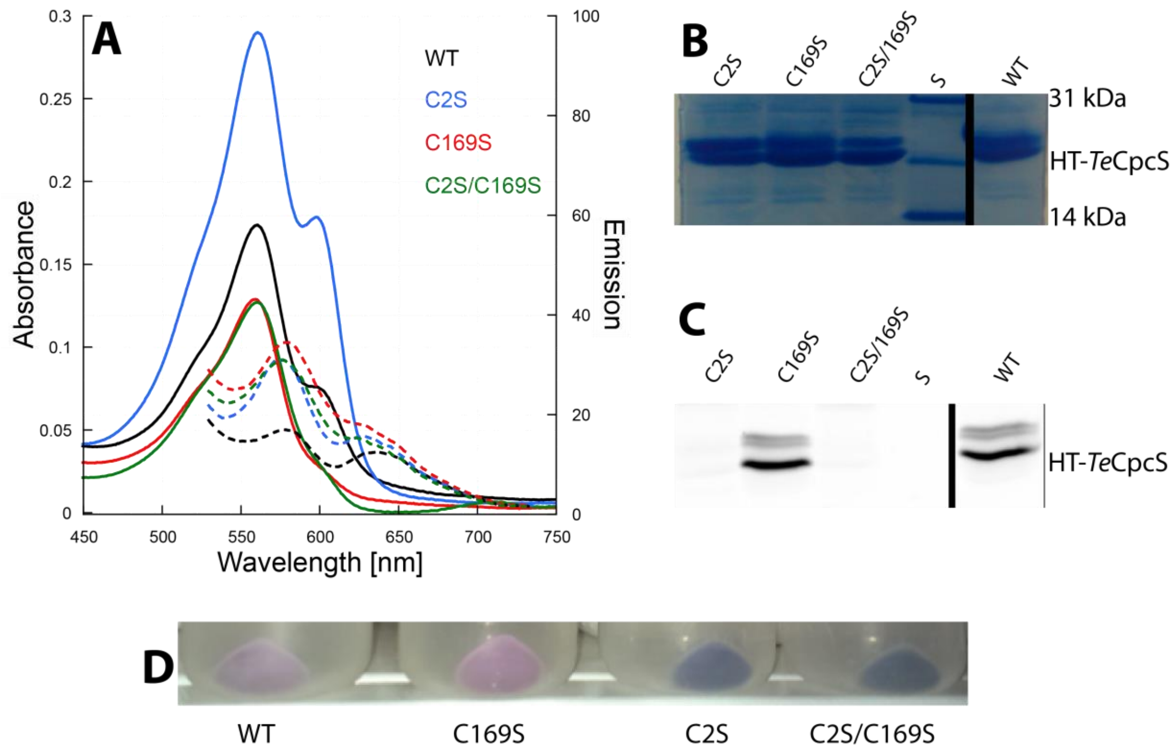


Figure 17 Intrinsic binding of PEB by HT-*Te*CpcS wild type and cysteine-mutants. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-*Te*CpcS wildtype (black), HT-*Te*CpcS C2S (blue), HT-*Te*CpcS C169S (red) and HT-*Te*CpcS C2S/C169S (green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of HT-*Te*CpcS C2S, HT-*Te*CpcS C169S, HT-*Te*CpcS C2S/C169S and molecular weight standards (lane S). (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB and HT-*Te*CpcS wildtype, HT-*Te*CpcS C2S, HT-*Te*CpcS C169S, HT-*Te*CpcS C2S/C169S.

The Zn-enhanced bilin fluorescence in Figure 17 (C) shows covalent attachment for the *Te*CpcS wildtype and the *Te*CpcS C169S variant, but not for the Cys-2 mutants. This is

supported by the *E.coli* cell pellet colour in Figure 17 (D) that shows similar colour for the Cys-2 mutants compared to the wildtype.

MUTANTS OF BILIN-HYDROGEN BONDING RESIDUES

The coexpression of PcyA and HO1 with His-tagged *Te*CpcS wildtype or HT-*Te*CpcS W73G, HT-*Te*CpcS R151G, HT-*Te*CpcS S155G showed a significant loss of the PCB binding ability for the mutants W73G and R151G, but only a partial decrease of bilin binding for the S155G mutant. This can be seen easily by looking at the *E.coli* cell pellets in Figure 18 (D) and is confirmed by the absorbance spectra, fluorescence emission spectra in Figure 18 (A) and zinc-enhanced bilin fluorescence emission of the SDS-PAGE gel in Figure 18 (C). The mobility of the *Te*CpcS W73G mutant was consistently lower in SDS-PAGE compared to the wildtype *Te*CpcS protein (see Figure 18 (B)). This may be due to post-translational modifications that consistently occur over all the experiments or the mutation affects the mobility in SDS-PAGE by changing the folding pattern of the protein or SDS adsorption to it. Repeated DNA-sequencing of the *Te*CpcS W73G mutant gene did not show any differences from the wildtype except for the intentional mutation. (see Appendix Figure 42)

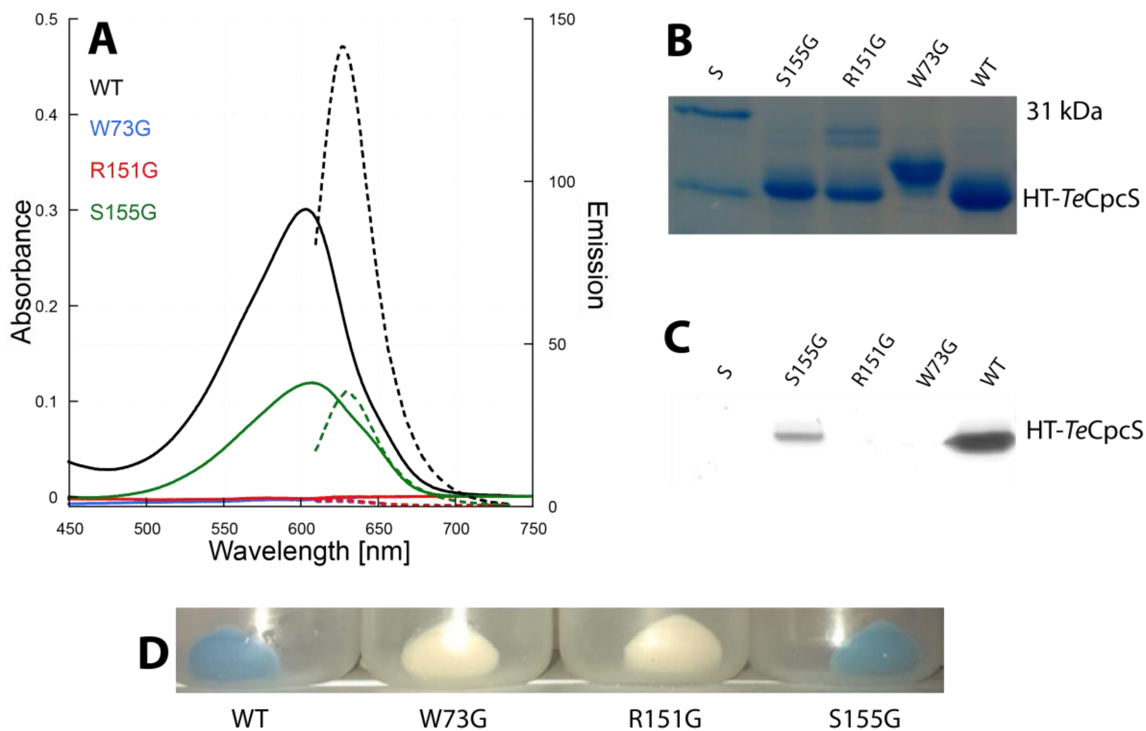


Figure 18 Intrinsic binding of PCB by HT-*Te*CpcS wild type and mutants of bilin-hydrogen bonding residues. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-*Te*CpcS wildtype (black), HT-*Te*CpcS W73G (blue), HT-*Te*CpcS R151G (red) and HT-*Te*CpcS S155G (green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of HT-*Te*CpcS wildtype, HT-*Te*CpcS W73G, HT-*Te*CpcS R151G, HT-*Te*CpcS S155G and molecular weight standards (lane S). (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB and HT-*Te*CpcS wildtype, HT-*Te*CpcS W73G, HT-*Te*CpcS R151G, HT-*Te*CpcS S155G.

The same expressions as for the bilin-hydrogen bonding mutants and PCB were also performed by substitution of PcyA by PebS, which leads to the production of PEB instead of PCB in the cells. Also for the bilin-hydrogen bonding mutants the results are very similar for binding of PEB and PCB. Figure 19 (A) shows a high absorption of the wildtype *Te*CpcS and *Te*CpcS S155G at 560 nm corresponding to the attached PEB and a secondary peak at 600 nm, which probably refers to the intermediate of PEB synthesis 15,16-dihydrobiliverdin. The absence of the chromophore in *Te*CpcS W73G and *Te*CpcS

R151G is also shown by the *E.coli* cell pellets in Figure 19 (D). Panel C in Figure 19 shows that covalent binding seems to occurs only at the wildtype *Te*CpcS and the *Te*CpcS S155G mutant.

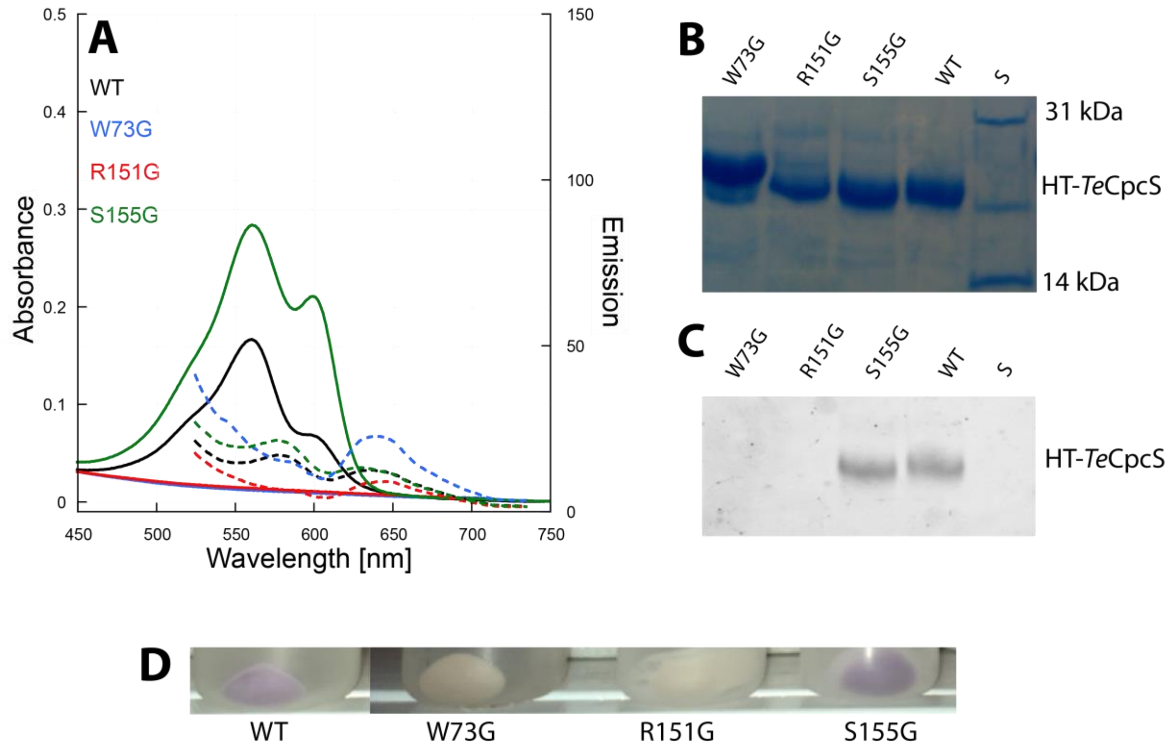


Figure 19 Intrinsic binding of PEB by HT-*Te*CpcS wildtype and mutants of bilin-hydrogen bonding residues. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of HT-*Te*CpcS wildtype (black), HT-*Te*CpcS W73G (blue), HT-*Te*CpcS R151G (red) and HT-*Te*CpcS S155G (green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of HT-*Te*CpcS wildtype (lane 4), HT-*Te*CpcS W73G, HT-*Te*CpcS R151G, HT-*Te*CpcS S155G and molecular weight standards (lane S). (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PEB and HT-*Te*CpcS wildtype, HT-*Te*CpcS W73G, HT-*Te*CpcS R151G, HT-*Te*CpcS S155G.

DOUBLE AND TRIPLE-MUTANTS OF BILIN-HYDROGEN BONDING RESIDUES

The double mutant HT-*Te*CpcS W73G/R151G and the triple mutant HT-*Te*CpcS W73G/R151G/S155G have strongly decreased solubility in buffer 0 and presumably also in *E.coli* and are therefore neither binding the bilins nor showing any catalytic activity. In the case of the coexpressions of these mutants with PCB there was no detectable amount of protein on the SDS-gel as showed in Figure 19(A). In the case of the coexpression with PEB, the mutants *Te*CpcS W73G/R151G and *Te*CpcS W73G/R151G/S155G could be detected, but the protein concentration was strongly decreased compared to the wildtype. (see Figure 20 (D)). This difference is probably due to the different expression conditions of the two bilins. PCB is produced by a 3 hour expression at 30°C whereas PEB is produced by a 16 hour expression at 18°C. The mobility of these mutants in the SDS-PAGE seemed to be similar to the *Te*CpcS W73G mutant. Figure 20 (B) and (E) show the absence of zinc-enhanced bilin fluorescence for the double and triple mutants. The absence of colour in *E.coli* pellets for both coexpressions with PCB and PEB is shown in Figure 20 (C) and (F), respectively. Samples taken from the *E.coli* pellet after cell lysis were analyzed by SDS-PAGE and showed the expression of *Te*CpcS double and triple mutants within insoluble inclusion body pellet (data not shown). This is an additional indication that the lack of purified protein is due to insolubility. The mutations could affect protein folding and/or stability in *E. coli.*

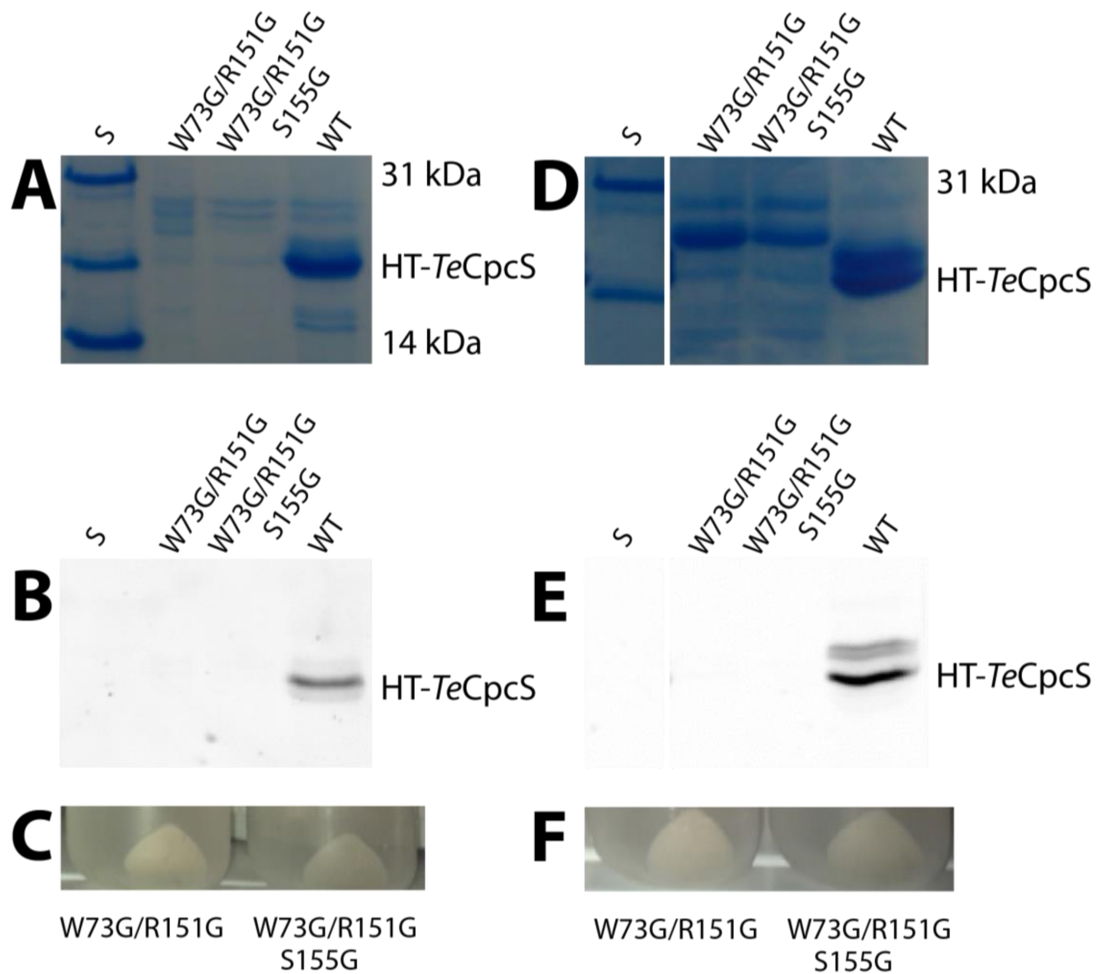


Figure 20 Intrinsic binding of PCB and PEB by HT-*Te*CpcS wildtype, double mutants and triple mutants. Coomassie-brilliant blue stained SDS-PAGE gel of HT-*Te*CpcS wildtype, HT-*Te*CpcS W73G/R151G, HT-*Te*CpcS W73G/R151G/S155G coexpressed with PcyA/HO1 in Panel A and PebS/HO1 in Panel D. Molecular weight standards were loaded in lane S. Panel B and E show zinc-enhanced bilin fluorescence of the gels (excitation at 532 nm) in panel A and D, respectively. *E.coli* cell pellets of coexpressed enzymes for production of PCB(C) or PEB(F) and HT-*Te*CpcS W73G/R151G and HT-*Te*CpcS W73G/R151G/S155G.

ATTACHMENT OF PCB TO CpcBA

Next, the activity of the *Te*CpcS lyase mutants was examined using a heterologous coexpression of HT-CpcB and CpcA from *Synechocystis sp.* PCC 6803 with HT-*Te*CpcS wild type and its cysteine-mutants as described above was performed. To include the production of the cognate chromophore PCB, the pPcyA plasmid was transformed into the *E.coli* cells. The samples were not further concentrated for absorbance and fluorescence spectroscopy. The native α-subunit of phycocyanin (CpcA) copurifies during Ni-NTA chromatography due to binding to the β-subunit (CpcB). Although previous studies showed only chromophorylation of CpcB and not CpcA, this construct containing both subunits was used due to the increase of solubility of CpcB.[89] The HT-*Te*CpcS wild type and all cysteine-mutants were able to chromophorylate HT-CpcB, but not CpcA. The absorbance maximum at 621 nm and the fluorescence emission peak at 644 nm, that are expected for the correct CpcB-PCB adduct at Cys-82, can be seen in Figure 21 (A). The efficiency of chromophorylation can only be determined semiquantitatively by this experiment. However, since all the coexpressions are performed under the exactly same conditions, an estimate insight into the activity of the lyase is shown by the height of the absorbance and fluorescence emission peaks and the intensity of the zinc-enhanced bilin fluorescence. Figure 21 (A) reveals that the mutants containing the mutation at Cys-2 seem to show higher ligation activity than the wild type. Although these mutants show lower protein concentrations on the SDS-gels in Figure 21 (B), the zinc-enhanced bilin fluorescence in Panel C shows efficient chromophorylation of CpcB in all cases.

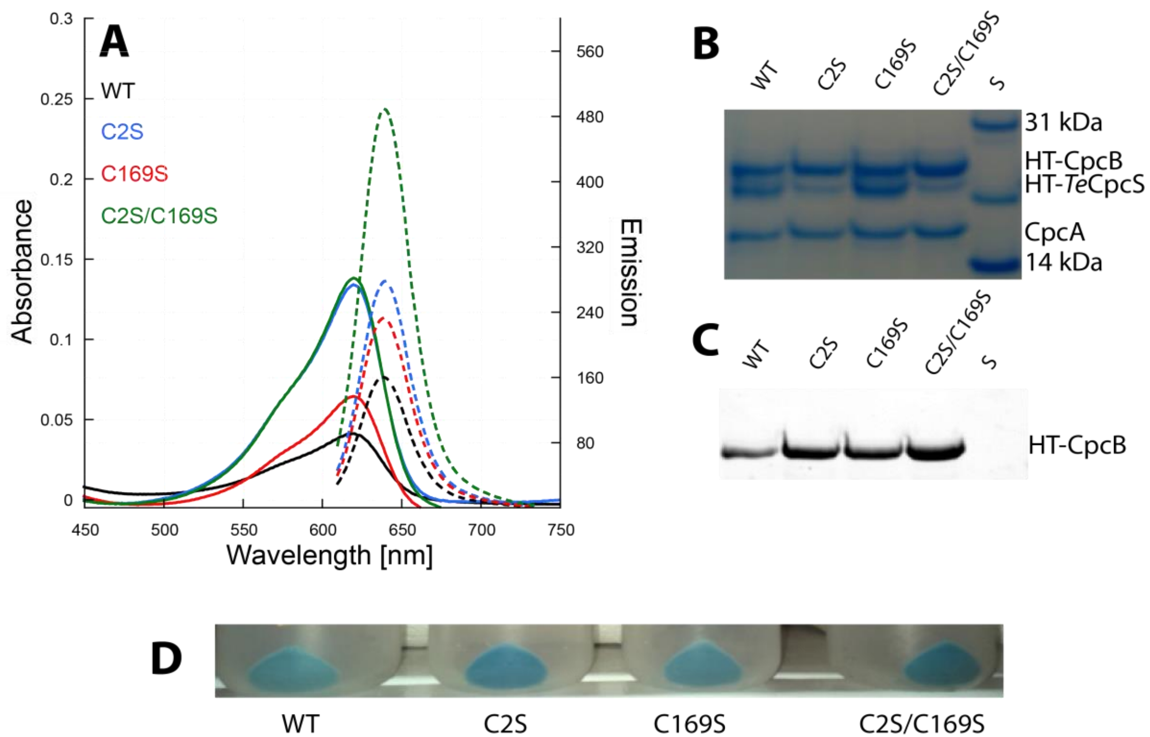


Figure 21 Attachment of PCB to HT-CpcB by HT-*Te*CpcS wildtype and cysteine mutants. (A) Absorbance (solid lines) and fluorescence emission (dashed lines) spectra of PCB, HT-CpcB, CpcA and one of HT-*Te*CpcS wildtype (black), HT-*Te*CpcS C2S (blue), HT-*Te*CpcS C169S (red) or HT-*Te*CpcS C2S/C169S (green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of HT-CpcB, CpcA and one of HT-*Te*CpcS wildtype, HT-*Te*CpcS C2S, HT-*Te*CpcS C169S or HT-*Te*CpcS C2S/C169S. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB, CpcB, CpcA and one of *Te*CpcS wildtype, *Te*CpcS C2S, *Te*CpcS C169S,*Te*CpcS-III C2S/C169S.

Like above, heterologous coexpressions were also performed for CpcBA and *Te*CpcS wildtype and its mutants of the bilin-hydrogen bonding sites. In this case the expressions were stopped after 1.5 hours instead of 3 hours to prevent a saturation effect of chromophorylation and therefore get a better estimate of lyase activities. Additionally the samples were diluted to an equal total protein concentration by adjusting the dilutions according to the absorbances at 280 nm to 0.25. Although all of the mutants seem to attach the bilin correctly to the CpcB subunit, Figure 22 (A) shows

the increased activity of *Te*CpcS W73G compared to the wildtype and the decreased activity of *Te*CpcS R151G. The lack of the *Te*CpcS W73G band in Figure 22 (B) is due to the overlapping with CpcB and the mutants *Te*CpcS R151G and *Te*CpcS S155G seem to have lower intensities than the phycobiliprotein subunits. This could partly be due to decreased solubility. Figure 22 (C) shows a slightly decreased chromophorylation of CpcB by the mutant *Te*CpcS R151G.

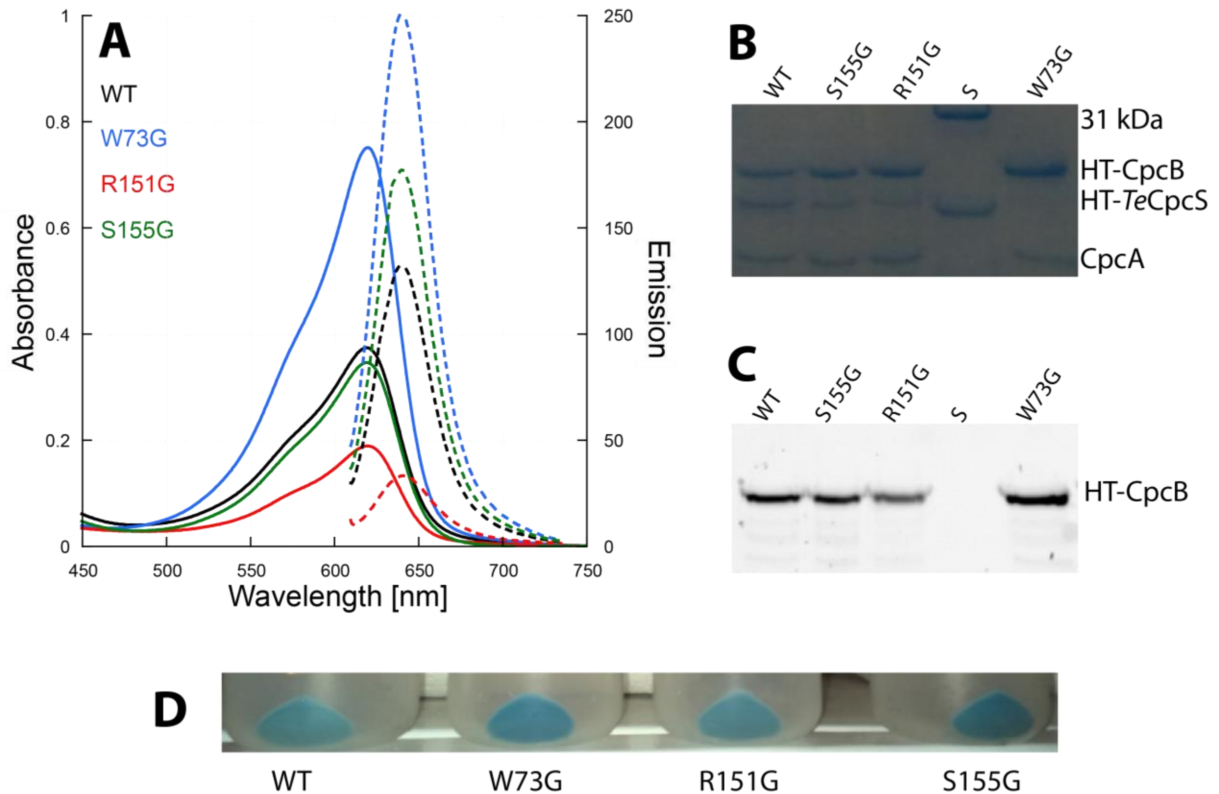


Figure 22 Attachment of PCB to CpcB by *Te*CpcS wildtype and mutants of bilin-hydrogen bonding residues. (A) Absorbance (solid lines) and fluorescence (dashed lines) spectra of PCB, CpcB, CpcA and one of *Te*CpcS wildtype (black), *Te*CpcS W73G (blue), *Te*CpcS R151G (red) or *Te*CpcS S155G(green). (B) Coomassie-brilliant blue stained SDS-PAGE gel of CpcB, CpcA and one of *Te*CpcS wildtype, *Te*CpcS W73G, *Te*CpcS R151G or *Te*CpcS S155G. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB, CpcB, CpcA and one of *Te*CpcS wildtype, *Te*CpcS W73G, *Te*CpcS R151G,*Te*CpcS S155G.

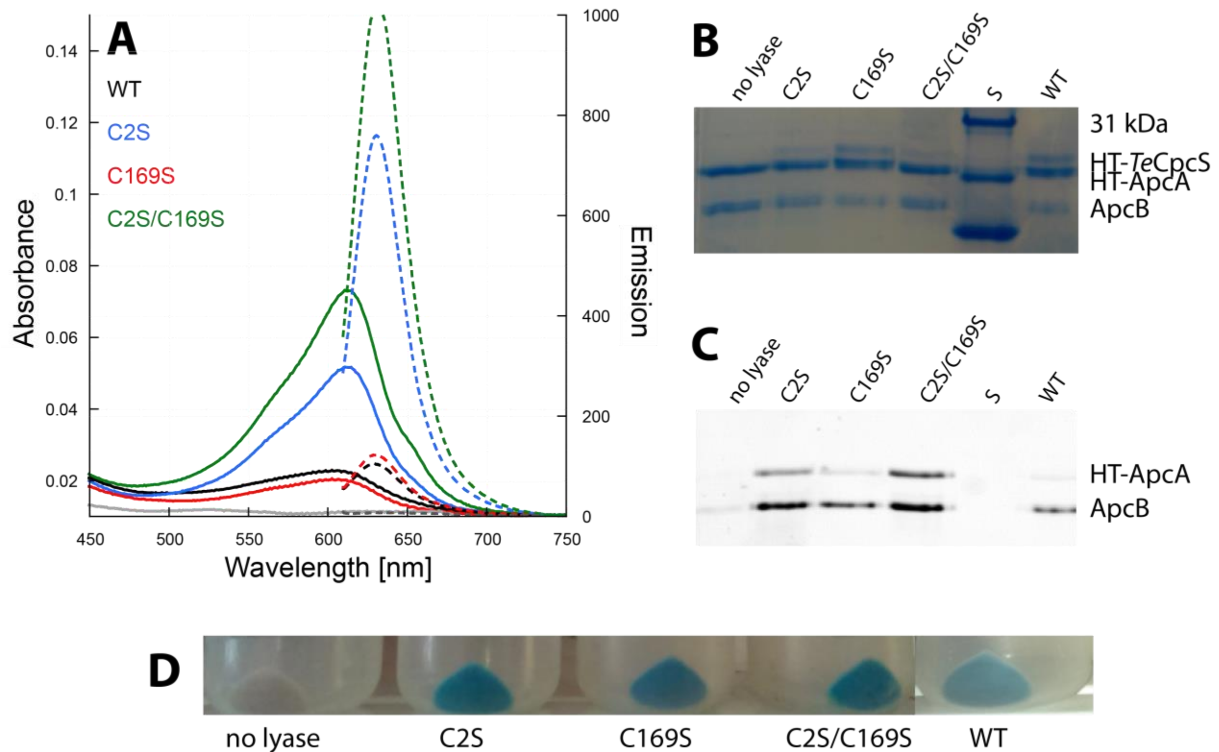


Figure 23 Attachment of PCB to ApcAB by *Te*CpcS wildtype and cysteine mutants. (A) Absorbance (solid lines) and fluorescence (dashed lines) spectra of PCB, ApcA, ApcB and one of *Te*CpcS wildtype (black), *Te*CpcS C2S (blue), *Te*CpcS C169S (red) or *Te*CpcS C2S/C169S (green).The grey lines correspond to the expression of PCB, ApcA and ApcB without lyase. (B) Coomassie-brilliant blue stained SDS-PAGE gel of ApcA, ApcB and one of *Te*CpcS wildtype, *Te*CpcS C2S, *Te*CpcS C169S or *Te*CpcS C2S/C169S. Lane 1 shows the PCB, ApcA and ApcB without lyase. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB, ApcA, ApcB and one of *Te*CpcS C2S, *Te*CpcS C169S,*Te*CpcS C2S/C169S.

In order to investigate the lyase activities of *Te*CpcS and the derived mutants with a different substrate, heterologous coexpressions of PCB producing enzymes PcyA and HO1, the two subunits of allophycocyanin (ApcA and ApcB) from *Synechococcus sp.* PCC 7002 and wildtype *Te*CpcS or the *Te*CpcS variants were carried out. All lyases in Figure

23 show correct attachment of PCB to ApcA and ApcB, indicated by absorbance peaks at 614 nm and fluorescence emission maxima at 636 nm.[81] However, considering the zinc-enhanced bilin fluorescence emission in Figure 23 (C) the chromophorylation of ApcB seems to be more efficient. This becomes even more evident by comparing the amount of ApcB to ApcA on the gel. This is probably due to the fact, that only ApcA is histidine-tagged and ApcB is copurifing with the former. Very similar to the attachment of PCB to CpcBA, the cysteine mutants lacking the Cys-2 show higher fluorescence emissions and absorbances than those of proteins produced with wildtype *Te*CpcS.

The results of the coexpression of PCB, ApcA, ApcB and *Te*CpcS or the mutants of hydrogen bonding residues in Figure 24 show increased absorbances for the mutants *Te*CpcS W73G and S155G, whereas only the latter one also shows an increase of fluorescence emission, as shown in Figure 24 (A). In Figure 24 (B) the SDS-gel shows an overlap of the bands HT-ApcA and HT-*Te*CpcS because these proteins are very similar in size (19 kDa and 21.4 kDa, respectively). Like in earlier experiments described above, the *Te*CpcS W73G shows an altered mobility on this SDS-gel. Figure 24 (C) reveals a lower chromophorylation of the α-subunit (ApcA) by the wildtype *Te*CpcS and *Te*CpcS R151G compared to *Te*CpcS W73G and *Te*CpcS S155G. In general, for ApcB higher intensities of zinc-enhanced bilin fluorescence can be seen compared to HT-ApcA. However, the colour of the *E.coli* cell pellets in Figure 24 (D) seems to be very similar for all of the samples.

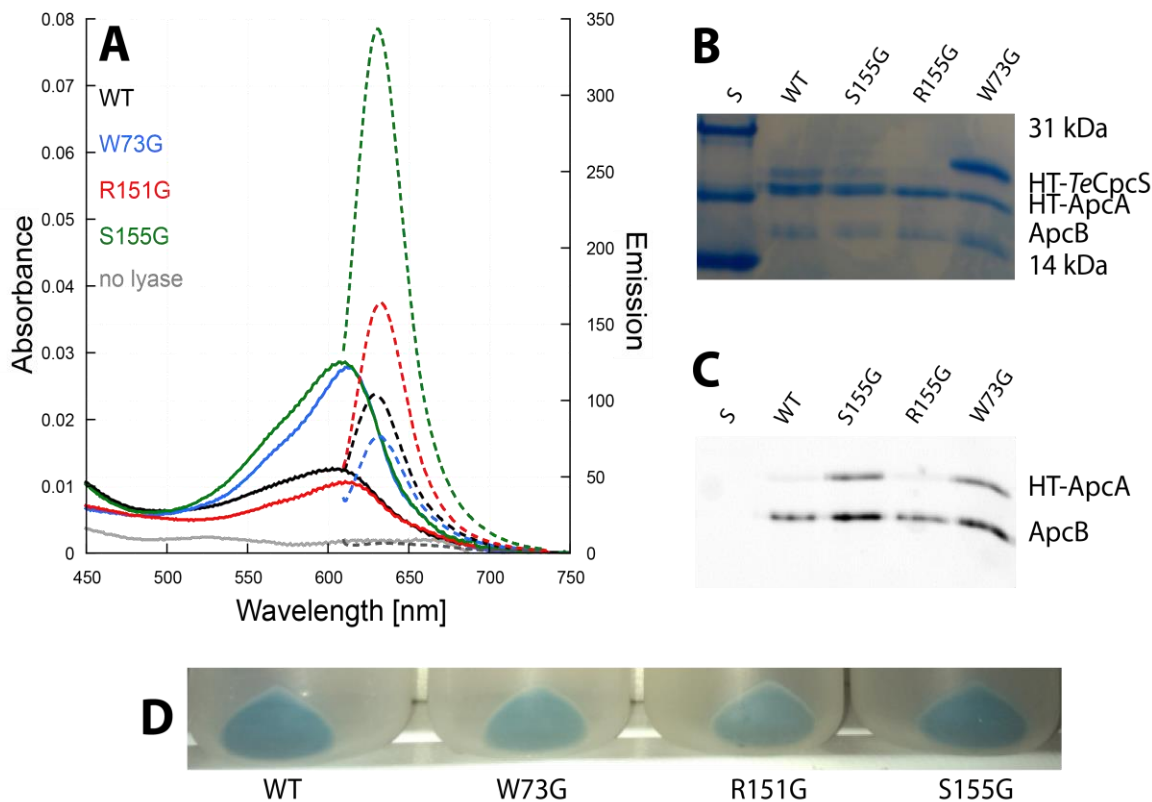


Figure 24 Attachment of PCB to ApcAB by *Te*CpcS wild type and mutants of bilin-hydrogen bonding residues. (A) Absorbance (solid lines) and fluorescence (dashed lines) spectra of PCB, ApcA, ApcB and one of *Te*CpcS wildtype (black), *Te*CpcS W73G (blue), *Te*CpcS R151G (red) or *Te*CpcS S155G (green).The grey lines correspond to the expression of PCB, ApcA and ApcB without lyase. (B) Coomassie-brilliant blue stained SDS-PAGE gel of ApcA, ApcB and one of *Te*CpcS wildtype, *Te*CpcS S155G, *Te*CpcS R151G or *Te*CpcS W73G. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PCB, ApcA, ApcB and one of HT-*Te*CpcS wildtype, *Te*CpcS W73G, *Te*CpcS R151G,*Te*CpcS S155G.

The heterologous coexpression of ApcA, ApcB and *Te*CpcS wildtype or the cysteine mutants with the non-cognate bilin PEB showed very similar results to the same expressions with PCB instead of PEB.

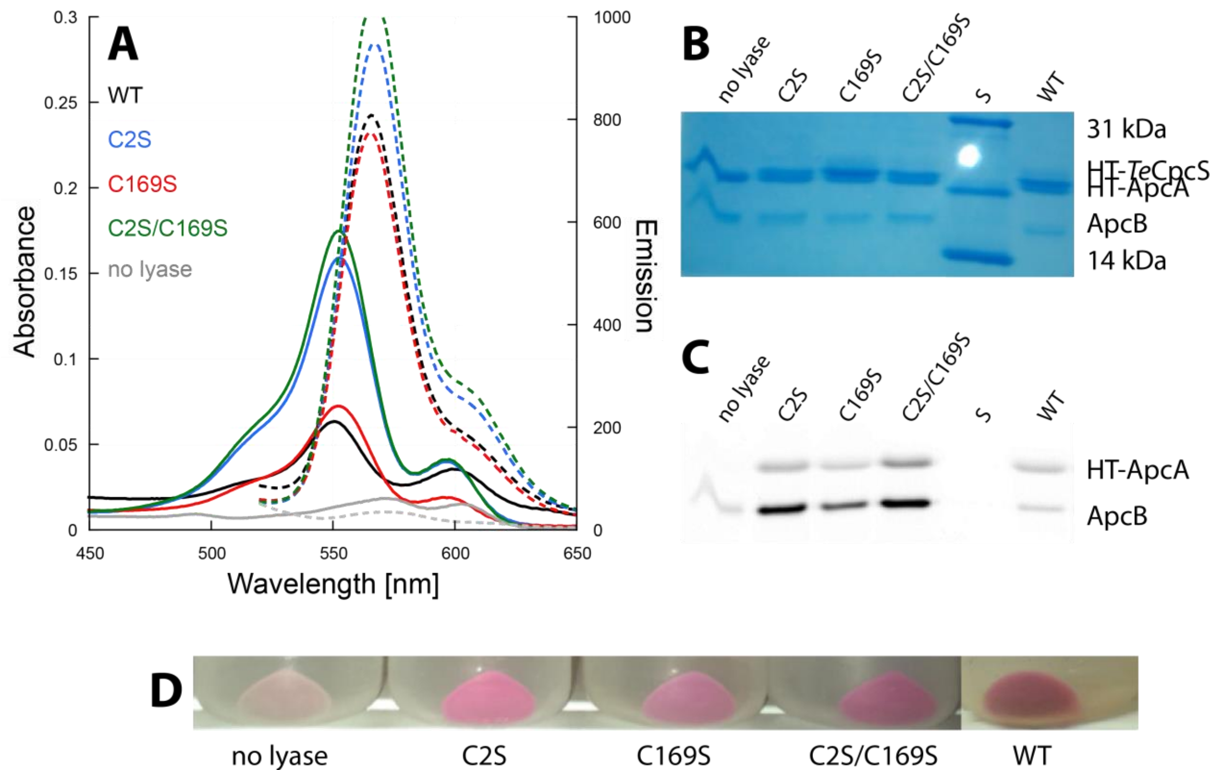


Figure 25 Attachment of PEB to ApcAB by *Te*CpcS wildtype and cysteine mutants. (A) Absorbance (solid lines) and fluorescence (dashed lines) spectra of PEB, ApcA, ApcB and one of *Te*CpcS wildtype (black), *Te*CpcS C2S (blue), *Te*CpcS C169S (red) or *Te*CpcS C2S/C169S (green).The grey lines correspond to the expression of PCB, ApcA and ApcB without lyase. (B) Coomassie-brilliant blue stained SDS-PAGE gel of ApcA, ApcB and one of *Te*CpcS wildtype, *Te*CpcS C2S, *Te*CpcS C169S or *Te*CpcS C2S/C169S. Lane 1 shows the PCB, ApcA and ApcB without lyase. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PEB, ApcA, ApcB and one of *Te*CpcS C2S, *Te*CpcS C169S, *Te*CpcS C2S/C169S.

The substitution of Cys-2 of the lyase leads to an increase in absorbance, fluorescence emission of the PBP in solution and after Zn-enhanced SDS-PAGE fluorescence imaging, shown in Figure 25 (A) and (C). As with PCB, the β-subunit of allophycocyanin showed relatively higher chromophorylation than the α-subunit. The autocatalytic attachment of the chromophore to ApcB in the absence of a lyase can be seen in Figure 25 (C). However, the corresponding intensity of the zinc-enhanced bilin fluorescence is very weak and the peaks in the absorbance and fluorescence emission spectra are shifted toward longer wavelengths, which indicates incorrect attachment.

Figure 26 (A) shows the absorbance and fluorescence emission spectra derived from purified protein expressions from *E.coli* that incorporated plasmids producing PebS, HO1, HT-ApcA, ApcB and either *Te*CpcS wildtype or one of the mutants of bilin-hydrogen bonding residues. In these coexpressions the decrease of biliprotein chromophorylation for the *Te*CpcS R151G mutant compared to the other lyases becomes even more evident than in earlier experiments. Although the peak of the TeCpcS R151G absorbance curve in Figure 26 (A) is at the same wavelength as for the other samples (550 nm), the peak is very low. Figure 26 (C) reveals that the chromophorylation occurs predominantly at ApcB for *Te*CpcS W73G and *Te*CpcS R151, whereas the other samples show similar zinc-enhanced bilin fluorescence for ApcA and ApcB. Panel D in Figure 26 shows a very faint pink colour of the *E.coli* cell pellet for TeCpcS R151G.

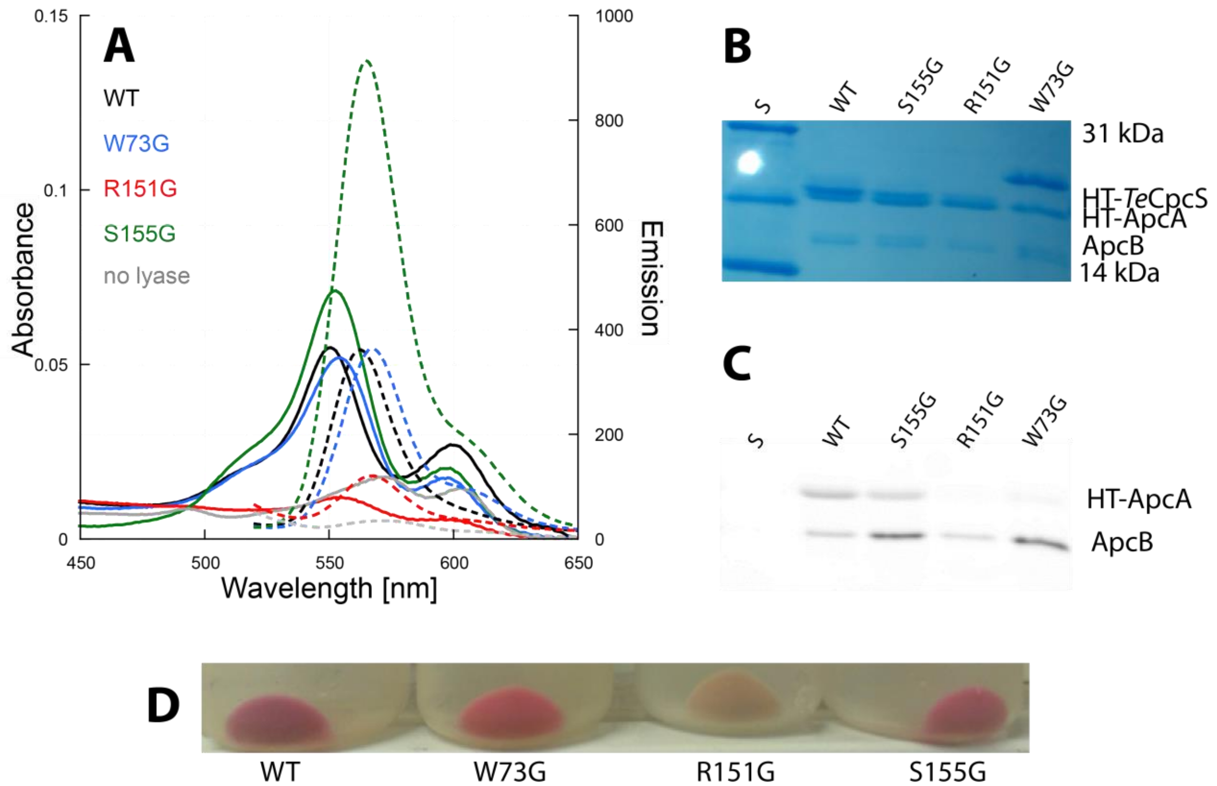


Figure 26 Attachment of PEB to ApcAB by *Te*CpcS wildtype and mutants of bilin-hydrogen bonding residues. (A) Absorbance (solid lines) and fluorescence (dashed lines) spectra of PEB, ApcA, ApcB and one of *Te*CpcS wildtype (black), *Te*CpcS W73G (blue), *Te*CpcS R151G (red) or *Te*CpcS S155G (green).The grey lines correspond to the expression of PCB, ApcA and ApcB without lyase. (B) Coomassie-brilliant blue stained SDS-PAGE gel of ApcA, ApcB and one of *Te*CpcS wildtype, *Te*CpcS S155G, *Te*CpcS R151G or *Te*CpcS W73G. Molecular weight standards were loaded in lane S. (C) Zn-enhanced bilin fluorescence of the gel (excitation at 532 nm) in panel B. (D) *E.coli* cell pellets of coexpressed PEB, ApcA, ApcB and one of *Te*CpcS wildtype, *Te*CpcS W73G, *Te*CpcS R151G, *Te*CpcS S155G.

Although the *Te*CpcS cysteine variants C2S, C169S and C2S/C169S did not show significant differences to the wildtype protein in bilin binding after Ni-NTA purification, SDS-PAGE analysis revealed a predominant occurrence of covalent binding of the chromophores PCB and PEB at Cys-2. Bilin attachment to various phycobiliprotein subunits is enhanced by the absence of the Cys-2 residue of the lyase *Te*CpcS.

The capability of bilin binding decreased drastically for the *Te*CpcS R151G mutant and for the *Te*CpcS W73G mutant. Only *Te*CpcS S155G of the tested mutants of bilin-hydrogen bonding residues retained its ability of bilin binding for PCB and PEB.

A decrease of the lyase activity could be shown for the *Te*CpcS R151G mutant for the attachment of PCB to CpcB and PEB to ApcAB.

The double mutant *Te*CpcS W73G/R151G and the triple mutant *Te*CpcS W73G/R151G/S155G were unable to test for chromophore binding, because the mutants´ solubility was decreased significantly.

4. DISCUSSION

The sites of covalent bilin attachment in *Te*CpcS, Cys-2 and Cys-169, do not seem to be involved in the catalytic process of bilin attachment to phycobiliproteins. First, a direct involvement of these residues is unlikely, due to their position in the crystal structure. Cys-2 is situated at the first α1-helix at the outside of the protein. Cys-169 is located at the center of the funnel-like beta barrel. Therefore they are far from the reactive ethylidene group of the bilin when the bilin is bound in the funnel-like structure. Second, these cysteine residues are not conserved in other S-type lyases of different

species.[89] Third, as the results of this thesis show, the efficiency of chromophorylation in the absence of the cysteine residues is increased, rather then decreased. This suggests that the observed covalent bonds between chromophores and *Te*CpcS occur adventitiously during the purification process. This could be due to the conditions in the performed experiments, as the long time of interaction between bilins and lyase might allow these side reactions. The presence of competing thiol-groups of the cysteine residues in the lyase even seems to have a detrimental effect on the lyase activity. However, investigations whether the cysteine groups could help with chromophore detachment from phycobiliproteins would be interesting for future work. Additionally, crystallization of *Te*CpcS mutants deficient of cysteine residues might be more successful without interfering attached bilins on the surface of the protein. The Cys-2 residue seems to be chromophorylated to a higher extent because it is situated on the surface of the protein and therefore more accessible for the bilin than the Cys-169, which is placed in the center of the beta-barrel funnel of *Te*CpcS. These Cys mutants will be used in obtaining a co-crystal structure with PCB allowing confirmation of the binding model with PCB.

The second set of mutants, here referred to as bilin-hydrogen bonding residues, show quite big discrepancies in terms of their importance. As expected, because of its strong interaction in the structure derived from docking, the evolutionarily conserved Arg-151 residue in the center of the lyase funnel seems to form the most important interaction with the bilin. The strong polar interaction of the positively charged guanidinium group and the negatively charged propionate that was formed in the manual docking experiment of PCB to *Te*CpcS, also seems to be irreplaceable for bilin binding *in vivo*. Furthermore, there has also been reported an interaction of this Arg group with a phosphate group in the crystal structure of *Te*CpcS in the absence of bilin. However, without the Arg-151 group, phycobiliprotein chromophorylation can occur, which indicates that also a very weak interaction between the bilin and the lyase can be sufficient for enzymatic activity within *E.coli*. These insights support the already postulated mechanism of chaperone-like activity of lyases in the chromophorylation

process. [84, 83] Also, the UnaG protein, that was used for the modeling of the interaction between *Te*CpcS and the bilin PCB, does not have a known catalytic function in the Japanese freshwater eel Unagi despite its very close structural similarity to *Te*CpcS.[97] Furthermore, *Te*CpcS neither shows a very high specificity for its ligands or substrates, nor is it easily affected by the substitution of some amino acids as shown here for the cysteine mutants, *Te*CpcS W73G and *Te*CpcS S155G. The exception to the substrate promiscuity of *Te*CpcS is CpcA, which is likely due to a difference in charge distribution along the CpcA surface.[89]

II. DIDACTIC SECTION

1. THEORETICAL BACKGROUND

1.1. INTRODUCTION

The way of teaching sciences changed significantly in the past century. Modern didactics include a lot more than just a teacher who is presenting material to his students, and students who are supposed to take up facts from teacher´s presentations.

Science education has constantly been and is still in the process of development. Many adults might still have experienced a very rigid way of teaching in their biology and chemistry classes in school. Modern teacher education in Austria includes many principles of progressive education to continue with the reformation of the school system.

In the following chapters a connection between the scientific part of this diploma thesis and the biology curriculum in secondary school will be provided as well as different concrete examples how to implement the scientific part into biology lessons. To ensure a reasonable integration of these applications in school, they will be built on pedagogical fundamentals of learning theory and didactic literature of teaching sciences.

The Project “Growing Cyanobacteria” that will be introduced in this thesis, has several connections to the Austrian curriculum of biology in secondary school. According to the official biology curriculum given by the Austrian government, the best suited year to incorporate this project would be 9th grade biology.

There are links to the topic of microorganisms ‘am Beispiel Mikroorganismen: An Hand ausgewählter Beispiele die Unterschiede zwischen Pro- und Eukaryoten erfassen; Mikroorganismen als Besiedler aller, auch extremer Lebensräume kennen lernen und ihre zentrale Bedeutung für die Natur verstehen’ [104] as this year´s curriculum includes photosynthesis ‘am Beispiel Pflanzen: An Hand ausgewählter Beispiele Wissen über Entwicklung, Keimung und Wachstum sowie mögliche Anpassungen an unterschiedliche Standorte erwerben und grundlegendes Verständnis für Stoffwechselvorgänge (Fotosynthese, Dissimilation) gewinnen’.[104]

Furthermore many ecological aspects concerning world sustenance and feeding the world can be found in the curriculum: ‘Verständnis für die Probleme der Welternährung, der Ressourcenverteilung und der verschiedenen Formen der Landwirtschaft (intensiv und extensiv) erwerben, Ursachen für den Nord-Süd-Konflikt erkennen und Zukunftsszenarien entwickeln’ [104]

In this research project students have the opportunity to investigate microorganisms that play a very important role for the world’s oxygen production and they will get important information about nutrient demands of different organisms as cyanobacteria also are able to perform nitrogen fixation from nitrogen gas. There is even the possibility to give an outlook on plant evolution as cyanobacteria are thought to be the evolutionary precursors of chloroplasts in plants and of course the general mechanism of photosynthesis is ubiquitous during the whole project.

The first step to become a competent science teacher is having a profound understanding of the way knowledge is acquired in the human brain. The latter half of the twentieth century produced an interest in understanding cognitive psychology and metacognition.[105] At the beginning of the 20th century behaviorism was still dominating the educational psychology. Teachers used positive and negative conditioning of their students by rewarding them for good behavior, i.e. repeating the teacher´s information as exactly as possible or negative enforcement by punishing behavior that was not leading to the desired result. There was very little space for children to have their own way of learning, and it was not a common belief that different ways could lead to the correct results. What is right and wrong was simply determined by the teacher and therefore discussions and opinions of the students haven´t been taken into account by the teacher.

Although the first known constructivist writing *De antiquissima Italorum sapientia* dates back until 1710 and has been written by Giambattista Vico, there are several 20th century constructivists who shaped the modern way of teaching through constructivist ideas.[105] There were a couple of teachers and scientists spread out over the world. John Dewey (1859-1952) was teaching at the University of Chicago and was one of the first who applied his ideas by granting his students an active intellectual learning environment. The Swiss scientist Jean Piaget (1896-1980) argued that knowledge is not out there somewhere, waiting to be discovered, but rather is acquired and constructed through a process of interaction with materials[106] Piaget´s work was especially for science teachers very important, because he was proposing many connections to hands-on experiences and to scientific models as well as conceptual changes in a child´s mind.

Applications of Piaget´s theory include:[105]

- Learning should be active and discovery-based
- Children should be given many opportunities to interact with their peers
- Instructional strategies should be adapted to fit the cognitive structures of children
- Conceptual change should be promoted by allowing children to test their presently held theories and become aware of inconsistencies in their thinking when compared with scientific models.

Recent research tries to implement the ideas of constructivism into the educational process, which means getting rid of the obsolete ideas of behavioristic funnel-like knowledge transmission.

According to Murphy (1997) we have to deal with the question how we perceive knowledge and the process of coming to know provides the basis for educational practice. If we believe that learners passively receive information then priority in instruction will be on knowledge transmission. If, on the other hand, we believe that learners actively construct knowledge in their attempts to make sense of their world, then learning will likely emphasize the development of meaning and understanding.[107]

Jonassen (1991) notes that many educators and cognitive psychologists have applied constructivism to the development of learning environments. From these applications, he has isolated a number of design principles:

- Create real-world environments that employ the context in which learning is relevant
- Focus on realistic approaches to solving real-world problems

- The instructor is a coach and analyzer of the strategies used to solve these problems
- Stress conceptual interrelatedness, providing multiple representations or perspectives on the content
- Instructional goals and objectives should be negotiated and not imposed
- Evaluation should serve as a self-analysis tool
- Provide tools and environments that help learners interpret the multiple perspectives of the world
- Learning should be internally controlled and mediated by the learner.[108]

Wilson and Cole (1991) provide a description of cognitive teaching models which "embody"constructivist concepts. From these descriptions, we can isolate some concepts central to constructivist design, teaching and learning:

- Embed learning in a rich authentic problem-solving environment
- Provide for authentic versus academic contexts for learning
- Provide for learner control
- Use errors as a mechanism to provide feedback on learners' understanding.[109]

These were just some examples for concepts that use constructivist ideas as a basis. To develop an education, that serves students with ideal circumstances to learn, must be the most important goal for a teacher. It is definitely helpful to consider points like the above mentioned while creating a curriculum or a project in school.

Inquiry-based Education is a well suited way for implementing constructivist concepts into the science classroom.

For the last 30 years, science educators have been concerned about probing and characterizing teachers’ understandings of the “Nature of Science“ (NOS) [110, 111]. This construct is in the United States of America a strongly debated topic in science education, because it includes many controversial points of view. Nevertheless understanding NOS facilitates the learning of science subject.matter.[112] First of all there are various definitions for the concept, but according to Lederman (1992) NOS typically refers to the epistemology of science, science as a way of knowing, or the values and beliefs inherent to scientific knowledge and its development .[111]

In practice this means that students should be able to see science as a way to see the world and there are some aspects the teacher should consider in order to provide students with a learning environment according to NOS:[112]

- Understand the crucial distinction between observation and inference. Observations are descriptive statements about natural phenomena that are “directly” accessible to the senses (or extensions of the senses) and about which several observers can reach consensus with relative ease (e.g., descriptions of the morphology of the remnants of a once living organism). Inferences, on the other hand, go beyond the senses.

- Distinction between scientific laws and theories. Individuals often hold a simplistic, hierarchical view of the relationship between theories and laws whereby theories become laws, depending on the availability of supporting evidence.

- Even though scientific knowledge is, at least partially, based on and/or derived from observations of the natural world (i.e., empirical), it nevertheless involves human imagination and creativity. Science, contrary to common belief, is not a totally lifeless, rational, and orderly activity. Science involves the invention of explanations, and this requires a great deal of creativity by scientists.

- Scientific knowledge is subjective and/or theory-laden. Scientists' theoretical commitments, beliefs, previous knowledge, training, experiences, and expectations actually influence their work.

- Science as a human enterprise is practiced in the context of a larger culture, and its practitioners (scientists) are the product of that culture. Science, it follows, affects and is affected by the various elements and intellectual spheres of the culture in which it is embedded. These elements include, but are not limited to, social fabric, power structures, politics, socioeconomic factors, philosophy, and religion. The practice of acupuncture, for example, was not accepted by western science until western science explanations for the success of acupuncture could be provided.

- Scientific knowledge is never absolute or certain. This knowledge, including "facts," theories, and laws, is tentative and subject to change.

These points show, that the "Nature of Science" deals more with the profound aspects of teaching and understanding the background of science. Considering this background information seems to be very important though, especially for teaching sciences connected to a constructivist basis. For example, it is just possible to allow children to invent their own theories if the teacher is aware, that all scientific theories have originally been "inventions" of scientists and even though the vast majority of theories are proven very profoundly, they are still subject to change in case new evidence came up.

The importance of teachers´ knowledge and understanding of NOS is shown in Table 5 from an empirical study by Bartholomew & Osborne (2004) that shows the most distinctive variables to perform a successful science education.[113] At the right hand side of the table the positive effects on the learning success are shown. All of these 5 Dimensions are absolutely capable of being integrated into conststructivist based education. For example the above mentioned criteria by Piaget (see chapter 1.3. From Behaviorism to Constructivismfit perfectly with the statements in Table 5, that the teachers´ use of discourse should be open and dialogic as well as the activities should be

owned by students. The role of the teacher as a facilitator or also frequently called "learning-coach" is evidently fostering a constructivist formation of thoughts.

Table 5 The Five Dimensions of Practice according to Bartholomew & Osborne (2004)[11]

	1. Teachers knowledge and understanding of the nature of science	
Teacher is anxious about their understanding	←——→	Confident that they have a sufficient understanding of NOS
	2. Teacher's conceptions of their own role	
Dispenser of knowledge	←——→	Facilitator of learning
	3. Teachers' use of discourse	
Closed and authoritative	←——→	Open and dialogic
	4. Teachers' conception of learning goals	
Limited to knowledge gains	←——→	Includes the development of reasoning skills
	5. The nature of classroom activities	
Student activities are contrived and inauthentic	←——→	Activities are owned by students and are authentic

There should be no confusion about the differences between NOS and scientific processes and inquiry. Although there is a big overlap between these subjects it is still necessary to distinguish. Both constructs are related and can be combined in teaching. However, the scientific process and inquiry are more connected to the activities in science and NOS is more about how knowledge is acquired. Furthermore none of these aspects should be seen as more important than the other one.[112]

Lederman (2006) states 'There is much evidence that NOS is best taught within a context of scientific inquiry or activities that are reasonable facsimiles of inquiry. That is, inquiry experiences provide students with foundational experiences upon which to reflect about aspects of NOS.' [112] Therefore the next chapter will deal with Inquiry-based Teaching in more detail.

In pedagogical literature “Inquiry-based Teaching“ very often seems to be defined by two primary points: The performance of an experiment and the knowledge about the experiment.

For example the definition that is given by the National Research Council is: ‘Inquiry teaching includes practices that promote the learning of scientific concepts and processes as well as “how scientists study the natural world”‘ (National Research Council, 1996).[114] Flick and Lederman (2006) define it as „inquiry is viewed as two different student outcomes, ability to do scientific processes and the knowledge about the processes“ [115]

Douglas Llewellyn (2007) points out a systematic “inquiry circle” that shows common phases in an inquiry process. These phases are: [105]

1. Inquisition – stating the question to be investigated
2. Acquisition – Brainstorming possible solutions
3. Supposition – Selecting a statement to test
4. Implementation – Designing and carrying out a plan
5. Summation – Collecting evidence and drawing conclusions
6. Exhibition – Sharing and communicating results

These points give a general outlook on the structure a research project plan should underlie. It is helpful in practice to overthink a project plan by making a checklist, if all these phases are integrated into the didactical method.

Although these points already present a well-defined model, there are some refinements that should be mentioned about it. First, there is the initial part of inquisition, which is usually observation.[116] There should be something that catches the eye of the students and arouses their interest. This could for example be a cover story or initial presentation, ideally though a personal observation that is connected to the

actual life of the student. Second, there might be the possibility to perform literature research in the acquisition phase. This can be beneficial for students who experienced inquiry-based methods before and want to bring their research on a higher level. Third, the very important aspect of reflection isn´t mentioned in the inquiry circle.

One of the most important parameters about any kind of pedagogical methods is of course the learning output that children have experienced when they have been taught by a certain method. Inquiry-based learning has been openly criticized not to be as effective as it seems and that this way of teaching is just intuitively appealing to many teachers who would like to be modern thinking and want to use modern ways of teaching.[117] This is definitely a problem that should be taken into consideration. The future of children´s education should not easily be risked by trusting popular ideas, all the more empirical studies should be taken into account.

However, Inquiry-based teaching is not invariant. According to Banchi & Bell (2008) there are different levels of inquiry. The increments reach from Confirmational Inquiry, where the teacher is providing the whole procedure from the question until the results and the students just have to verify the experiments, until open inquiry where the teacher doesn´t provide anything and let the student do everything on their own.[118] It might seem obvious that the more previous knowledge the students have the more they might be able to make their own decisions and it might not be beneficial for the learning output if novices try to investigate nature completely on their own. The form of "Guided Inquiry" presents a way where the teacher provides a general question and accompanies the student progress by acting as a coach and by guiding the students into the right direction. This seems to provide both the possibility for students to make their own decisions and the necessary structure given by the teacher that keeps students from losing track. According to a collection of empirical studies about successful tuition by Meyer (2004) a clear structure ("klare Strukturierung") is the most important factor for a high learning output.[119]

2. PROJECT: GROWING CYANOBACTERIA

The project “Growing Cyanobacteria”, which will be introduced in this section of the thesis, is definitely a very demanding venture for both students and teacher. The methods in this project might partly seem very difficult to conduct in a 9th grade biology class, but this also introduces new opportunities for the students to show and acquire skills, that are not always demanded in science classrooms. These skills include creativity, decision making, long-term planning, individual working and many more.

2.1. PROJECT PLAN

The project plan below shows the general overview of the time schedule, the contents of the different steps, the equipment that is required as well as a short description of the goals of the single steps in terms of learning output.

This Project plan can easily be adapted to different projects using inquiry-based methods. The use of a cover story to gain the students interest is optional and generally not required for a complete research project, but it is a good opportunity to sensibilize students for general topics as in this case the growth of the world population.

Table 6 Project plan for “Growing Cyanobacteria”

Nr.	Time	Title	Method	Content	Tools/ Equipment	Goals
1	10 min	Introduction	Cover story	Fossile fuels - Alternative Energies	Internet Ressources (Presentation if no Internet availale)	Gain Interest
2	10 min	Problem Evaluation	Open Discussion	What is the reason and what are possible solutions?	Board	Sensibilization
3	10 min	Introducing Cyanobacteria and Biotechnology (Inquisition)	Presentation	A possible solution! Providing the research question: How to grow cyanobacteria best?	Power Point Presentation	Introduction to the research project
4	20 min	Concept Building (Acquisition, Supposition, Implemen-tation)	Mind map	What are the main factors for growth? How do we test it?	Board & Clipboard	Planning
5	10 min	Introduction into Using Science Notebooks	2-Person Partnerwork	Oral Description By Teacher	Science Notebook Example	Introduction in new way of taking notes
6	2 h	Microscopy	Observation and Description	Sample Preparation, Correct Use of Microscope Investigate Cyanobacteria/ Microorganisms	Science Notebooks, Microscopes, Samples	Apply new research technique
7	5 min	Group formation	Raffle	Forming groups (ideally 4 to 5 students per group) and distribute topics	Raffle Topics for Presentations	Organisation

8	8 wks	Research phase (Summation)	Independent research	Students take care of their tasks, observe changes, take notes	Variable – according to research tasks	Training to responsibility
9	1 h	Presenting Results (Exhibition)	Student Presentation	Students create posters and present results	Posters, colours, etc.	Combining Information

The following section will provide a more detailed description of the steps within the table above and gives background information for the teacher to use this project plan.

2.1.1. Introduction

As mentioned above the use of a ”cover story“ as introduction is optional and can be skipped to save time. However, on the other hand a “cover story“ provides the great opportunity to connect the topic of research to the life of the students and to bring up new ideas. This way of introduction can even use fictional or fantasy-based stories – although the children of course would not believe the story in this case, they might identify with the project and can even practice their skills in role playing.

In this project the “cover story“ is the depletion of ressources like fossil energies and the problems arising from them. This will also be connected to the problem of overpopulation on planet earth.

As the number of people on our world more than doubled in the last 50 years, many problems arise with this overpopulation. Pollution of our environment, starvation in developing countries, energy shortages, diseases and many more are problems in modern societies that are connected to the use of fossil energy.

There are many easily available internet resources to show in class. A very impressive one is a counter, where you can see the number of people that actually live on earth –

and it´s changing every second. This can be found at www.worldometers.info/world-population.

Further resources that can be helpful are graphs that show the recent history of population growth and the global production of fossil fuels: These graphs are easily available online.

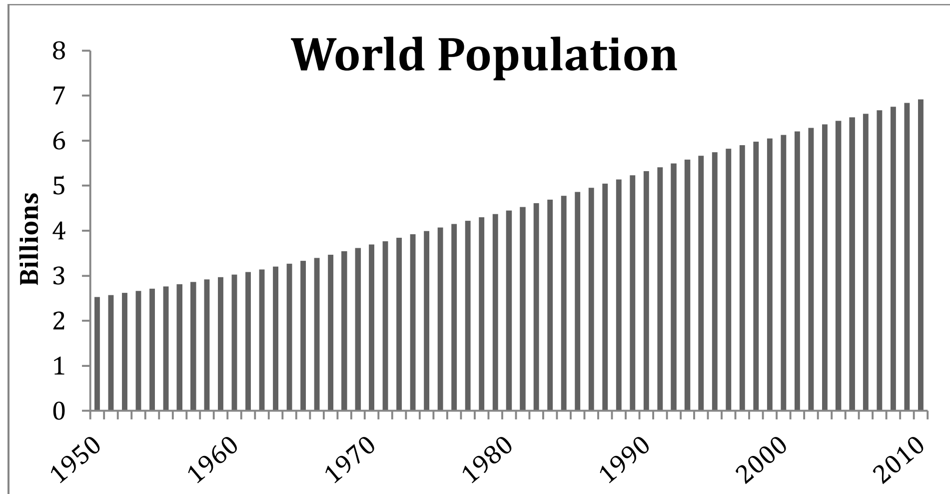


Figure 27 World Population Growth (data from United Nations Organization)[120]

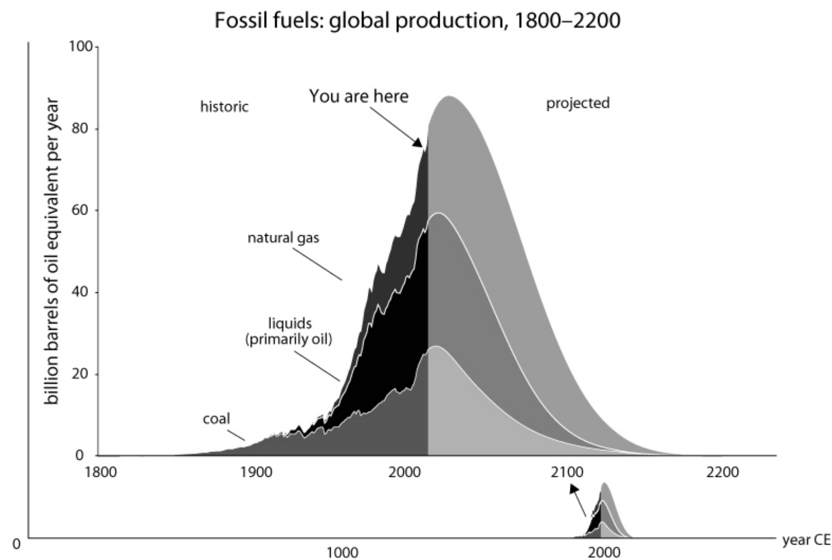


Figure 28 Fossil fuels: global production (Rocky Mountain Institute © 2011, Published by Chelsea Green in Reinventing Fire)[121]

The massive growth of population in combination with the depletion of fossil energy might lead to big social problems in the future. The growth of wealth in western societies during the past century was strongly supported by the exploitation of easily available fossil energy, whereas future development will be dependent on new ideas and technologies.

Additionally, a connection to starvation in developing countries due to the difficult circumstances in agriculture can be made. Students can be sensibilized by showing them a picture of children that suffer from Protein Energy Malnutrition (Kwashiorkor).

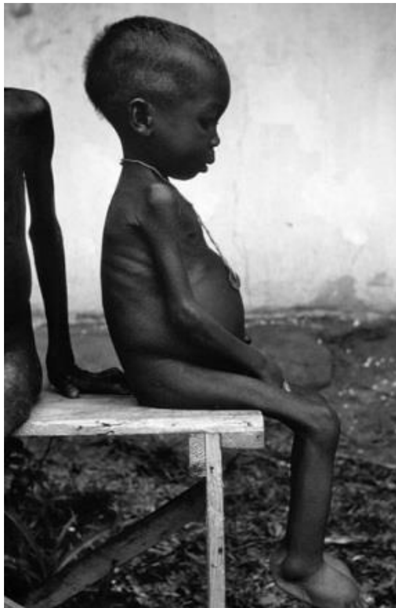


Figure 29 Typical symptoms of Kwashiorkor [122]

The typical symptom of Kwashiorkor is a bloated abdomen. This is due to a very low level of Albumin in the blood serum, which leads to an insufficient oncotic pressure and subsequently to an insufficient transport of fluid from the abdomen tissue.[123]

2.1.2. Open Discussion

As students should always have the opportunity to contribute to the course of events in the classroom, a discussion of these very critical points is an appropriate method to develop a broad overview of the topics connected to World Population.

It would be beneficial to collect the ideas of the students by using the board and drawing a common mind map.

On the one hand, students should be challenged to think about problems that are connected to fossil fuels or overpopulation and on the other hand the possible solutions should be discussed openly.

In general it might be useful to take a picture of the mind map before erasing it – this can be used as a cover page in the end of the project report.

2.1.3. Introducing Cyanobacteria and Biotechnology

As the second part of the „cover story“ and introduction into a solution of some problems cyanobacteria can be introduced as an organism that is probably new to most of the students. As cyanobacteria have so many special characteristics that make them feasible for many applications, these organisms are very interesting to investigate. Biotechnology is a very fast growing sector in economy and might be one way to overcome future problems in energy shortage, malnutrition, health care and many more.

Cyanobacteria can… convert energy of light into chemical energy (photosynthesis)
grow under difficult circumstances and in extreme environments
produce biofuels / hydrogen
produce protein / fertilizer

However, the first step to use cyanobacteria in biotechnology is to know how to grow them. So in this project the main task is to test the different parameters to successfully grow cyanobacteria and to investigate their growth and appearance.

It should be mentioned that cyanobacteria do not only have useful applications, but can also be very problematic. Either they can be a simple unpleasant guest in an aquarium, or they can proliferate incontrollable in nature and lead to a phenomenon that is commonly known as an algal bloom. Furthermore certain species of cyanobacteria are able to produce toxic substances and harm their environment.

2.1.4. Concept Building

This is one of the most important parts of the project. First, because the students have to bring up their own ideas, what the main factors of cyanobacterial growth could be. Second, because the whole success of the project depends on the planning. Third, this is the most crucial part for the teacher. On the one hand he or she is able to influence the project to be successful, but on the other hand it is also beneficial to give the students their freedom to choose what their research is going to be about.

To begin the investigation, the students should come up with a mind map or a general list about factors, which could influence the growth of cyanobacteria.

These factors could be:

- Temperature
- Light intensity
- Colour of Light (different wavelengths)
- Amount of Water
- Flowing water/silent water/stirring
- Salt concentration (different minerals, trace elements)
- Nutrient concentration (Sugar, fertilizer)
- Carbon dioxide concentration
- Oxygen concentration
- pH value

In this section of the project a large variety of factors can be defined by the students and subsequently also a large variety of ways to investigate these factors.

There are factors that are very easy to influence like temperature (samples in the refrigerator or on the heating element or water bath) or light intensity (covering samples, put samples next to the windows) and others that demand more equipment to investigate like the oxygen concentration.

Algal and Cyanobacterial Cultures can be purchased for teaching purposes at different suppliers. One of them is: Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG)

http://www.uni-goettingen.de/en/184982.html

In general students tend to be very disappointed when their cultures won´t grow at all. As this is very likely for most of the cultures, students should be prepared to that by emphasizing the rare possibility to find the perfect conditions at the first time. Even if the cultures don´t grow very efficiently it will be very important for the students to observe the changes in the culture when the culture dies.

2.1.5. Introduction into Using Science Notebooks

Science notebooks are a combination of science journals and science logs as they combine both data collections and reflections of the students´ thoughts. Elements of a Science Notebook are usually notes, lists, technical drawings, diagrams with labels, charts, tables, graphs and written observations. Important organizational elements are date, time and headings (question, topic, etc.).

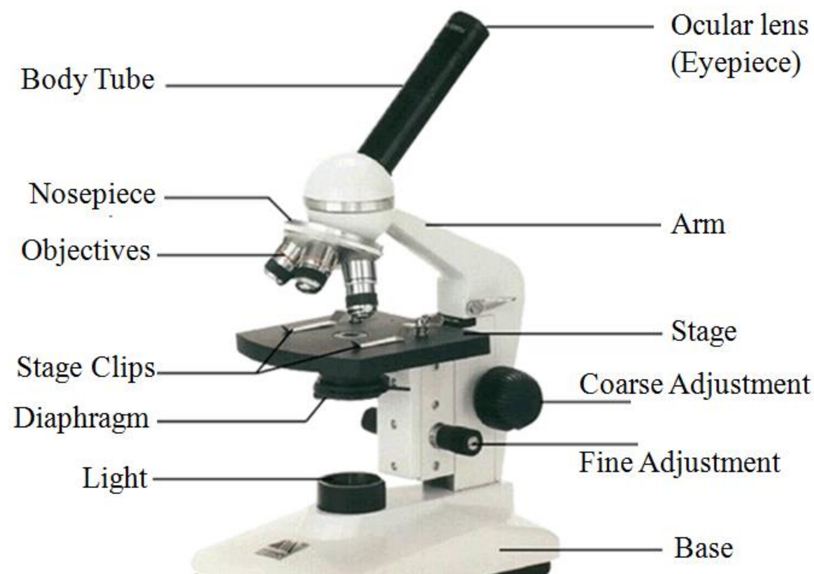
In this short oral introduction by the teacher, it would not be necessary to give a detailed description about the use of science notebooks. However, it seems appropriate to give the students a general idea, what science notebooks are, what kind of information they are supposed to write down and how the structure of a science book should generally look like.

Hence the students should make sure to include the above mentioned organizational elements in every single entry. Further they should be encouraged to use different elements to describe their observations and also to include personal thoughts in the notebooks.

This project is an ideal opportunity to implement microscopy into the curriculum and the use of science notebooks can be practiced easily within this method.

Depending on the previous knowledge of the students about microscopy, it will be more or less time consuming to give an introduction into this observation technique. As most of the schools are equipped with a small number of microscopes and these microscopes are very expensive compared to many schools´ budgets, it is very important to keep students from damaging these.

Therefore the handout below gives a short description of the first steps to use a microscope.



1. Carry the microscope very carefully with both hands! Do not touch the Objectives!
2. Turn the nosepiece to the lowest magnitude objective (this is also the shortest one)
3. Place the slide with the specimen on the stage and fix it with the stage clips
4. Move the stage up as far as it will go by turning the coarse adjustment.
5. Look through the ocular lens (eyepiece) and adjust the light source and the diaphragm to the highest light intensity.
6. Focus the image by moving the stage away from the objective (turn the coarse adjustment)and refine the focus by turning the fine adjustment.
7. Move the slide with the sample, so that the specimen is in the center of the image.
8. Increase the magnification by turning the nosepiece. Adjust the focus by using the fine adjustment. Do not let the objective touch the slide or cover glass!
9. Observe the specimen. Vary the position of diaphragm and the light intensity to get the best image.
10. When finished, lower the stage, remove the specimen, turn off the light source and cover the microscope to prevent it from getting in contact with dust.

Further online learning resources can be found under the following links:

http://www.wisc-online.com/Objects/ViewObject.aspx?ID=BIO905

http://tap-into-science.wikispaces.com/Bugs+%26+Microscopes

To practice the use of science notebooks and microscopy, samples have to be prepared. To gain as much interest for microscopy as possible, living samples are usually a good choice. For example rotifers and tardigrades are very interesting to look at. Ideally these samples are collected in the surroundings of the school and directly prepared with the students in the classroom.

An appropriate task for the students would be to describe all the different organisms in their sample and to estimate the abundance in their sample. They can make tables in their notebooks to list the numbers of different species they are able to distinguish as well as trying to make a technical drawing of individuals.

At the end of the microscopy tasks it might be beneficial for the teacher to collect the notebooks of the students, take a look at them and give them feedback about it.

Additionally the teacher should prepare samples of cyanobacteria to show the different species to the students. As there are big variations in the phenotypes of cyanobacteria it would be interesting to show the students species from filamentous genera like *Nostoc* as well as unicellular cyanobacteria like *Synechococcus*.

2.1.7. Group formation

Depending on how many different factors of cyanobacterial growth the students want to investigate, the group sizes can be varied. Ideally the groups should be made up by three to four students. To break up the common peer groups in a classroom a raffle can be used to form groups.

Each group is supposed to investigate at least one factor of growth. The factors can by chosen by the "first come, first-served" principle.

Each group member is responsible for his or her own science notebook, although the notes can be taken together by the group.

2.1.8. Research Phase

This might be seen as the main part of the research project, although it might not create a lot of work for the students. Once the work of preparation is done, the only task of the students is to observe the changes in the cultures and take simple notes about it.

The research phase might be very different from one group to another as the conditions for the cyanobacterial growth might diverge strongly dependent on the factors they are investigating.

One of the main problems when dealing with microorganisms is the contamination with other organisms in the samples. As most of the schools do not have appropriate equipment to sterilize the appliances, the students must be told to be very careful not to get in contact with unsterile surfaces and the flasks that are used to grow the cyanobacteria should be purchased recently.

As standard medium that serves as a control for the students there are different commercially available media that have been proven to provide cyanobacteria with sufficient nutrients and minerals. These Media are blue-green medium BG-11, B-HEPES (BG-11 with 4.6 mM HEPES-KOH and 18 mg/L ferric ammonium citrate) or SN-Medium. [124, 125,] Media compositions are available online at cyanosite from Purdue University. http://www-cyanosite.bio.purdue.edu/media/table/media.html

Alternatively these media can also be mixed by the students. However, this requires a basic knowledge of laboratory practice and might be too time consuming.

The students might have difficulties to see the difference between living and dead cyanobacteria – and indeed it is not as easy to see as for moving animals. The easiest way to find out if cyanobacteria are still alive is by shaking the cultures and observing if the whole medium is still coloured green or if the medium is faint yellow with green flakes of cyanobacterial cultures. The first one would be the case for live cultures and the latter one for dead ones.

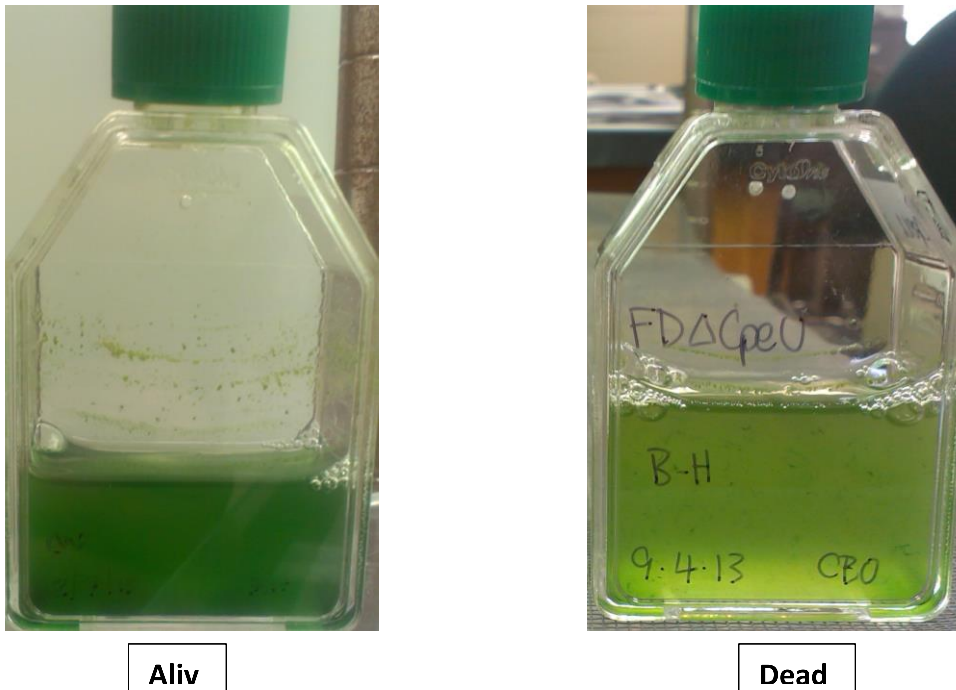


Figure 30 Comparison of Alive and Dead cyanobacterial cultures

This might be a difficulty on the one hand, but on the other hand this task gives students space for their own interpretations and thoughts. Ideally a microscope should be available for the students to look at the organisms.

2.1.9. Presentation of results and Reflection Research

For the collection of all the results a poster is very useful. Each group is supposed to design one sheet of paper, including one or two statements about their results and one graph or table that corresponds to their results. Of course also other elements like pictures are allowed as long as they have an illustrative effect.

The groups have to present their results within a short presentation that includes their strategy of investigation, their tools, their measurements and of course the final result.

The independent sheets produced by each group, can be in different colours and then they should be combined to one big poster.

Furthermore the students have to write down a reflection of their thoughts during the research phase. Students should make connections to their notes in their science notebooks by citing their own statements.

2.2. ADDITIONAL PRESENTATIONS WITHIN THE PROJECT

As this project covers a long time period, there is plenty of room for additional work while the research phase is being performed. This offers a perfect opportunity to have students presenting topics that are connected to the project.

Possible topics for their presentations would be for example: Biotechnology, photosynthesis, cyanobacterial toxins, algal blooms, algae (Differences between Red/Green/Blue-Green), differences between prokaryotes and eukaryotes, etc.

2.3. POSSIBLE VARIATIONS OF THE PROJECT

In place of cyanobacteria also green algae can be used. Although in this case the students would not have the chance to work with a prokaryote as a different type of cell, which is a very interesting aspect of cyanobacteria.

Ideally sample collection could be performed in a local water body. However, this is of course under the condition, that there is a local water body that contains sufficient populations of cyanobacteria or algae, and it would be recommendable for the teacher to investigate the abundant species before starting the project.

3. ADDITIONAL TEACHING MATERIAL - COLOURS

The following chapter is not connected to the project “growing cyanobacteria” but should provide an idea how to implement the topic “colours” in the science classroom. The basic understanding of colour absorption and perception is not only a fundamental concept that can be useful to students in everyday life, but is also inherently very interesting to students. The following outline for a 50 minute lesson on colours should provide teachers with a general idea of how to give a short introduction into the topic colours and their interaction with organic molecules.

Table 7 Teaching plan for "colours"

Time	Method	Contents	Material	Goal
20 min	Teacher-student question based method	colours, spectrum of visible light, idea: why does the sky appear red on sunset and blue during the day? → diffusion of light → concept of different wavelengths	Image files(circle of colours, addition of colours, spectrum), board	Recalling of fundamentals of light from physics classes
5 min	experiment	Base reaction of phenolphthalein (colour change from colourless to pink)	Glass flask, phenolphthalein, Sodium hydroxide	Gain interest and bring up questions
5 min	Question based teaching	Introduction into organic molecules with Pi-conjugated systems: Why do some absorb light and others don´t?	Image: deprotonation of phenolphthalein	Forming connection between Pi-conjugated systems and colours
19 min	Group work	Working on texts about plant and animal chromophores (chlorophyll, heme, bilirubin)	texts	Independent working of students, increasing reading skills of scientific texts
1 min	Giving homework assignment	Homework: Preparing a short 2 min presentation about the text	---	

The colour circle in Figure 31 **Complementary Colours** [126] shows complementary colours and the idea of contrast between them.

Figure 31 Complementary Colours [126]

In order to help the students getting a deeper understanding of the addition of colours, the additive colour scheme in Figure 32 can be projected to the board in the classroom and the students should answer questions from the teacher like: Which colour is percepted when all three primary colours are mixed? Which colour is seen when only two of them are abundant? What kind of light is sunlight? Why do we perceive red things as red?

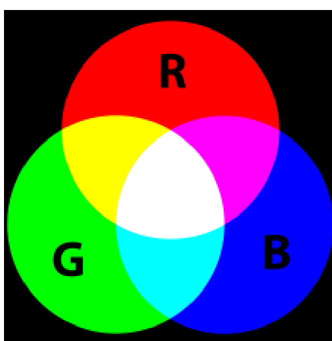


Figure 32 Additive Colour Perception [127]

To gain more of the interest of the students, it is usually helpful to bring up an example of the students life. The phenomenon of diffusion of light during sunset that leads to the well known red sky is a very interesting example. However, to explain this phenomenon, the students have to recall the principles of wavelengths and the compostion of light. Therefore the spectrum of visible light in Figure 33 should be discussed. Also the

relation between energy content of different wavelenghts or frequencies should be mentioned. (E = hf)

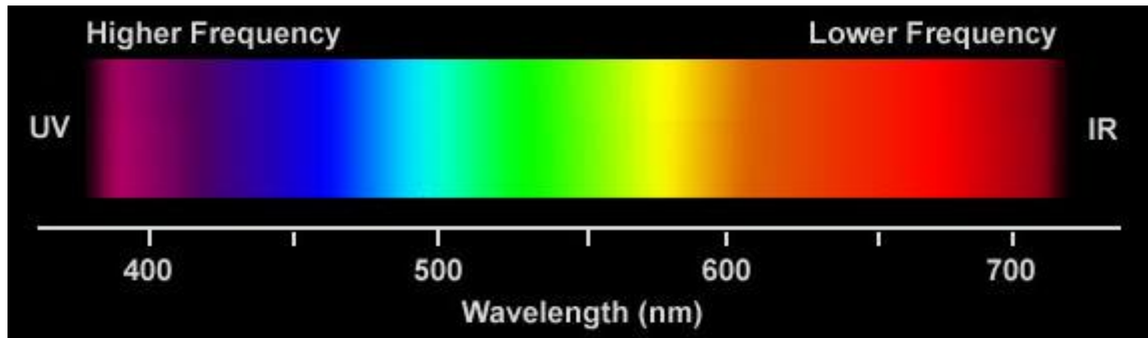


Figure 33 Spectrum of Light [128]

With the understanding of different wavelengths in their mind, students should be able to understand that different wavelenghts of light are more or less scattered by the particles in our atmosphere. As red light with longer wavelength is less likely to be scattered, it can penetrate the atmosphere deeper and is therefore also visible when the sunlight has to go through a long way in the atmosphere, like it is the case during sunset. This can be demonstrated by a drawing on the board that looks similar to Figure 34.[129]

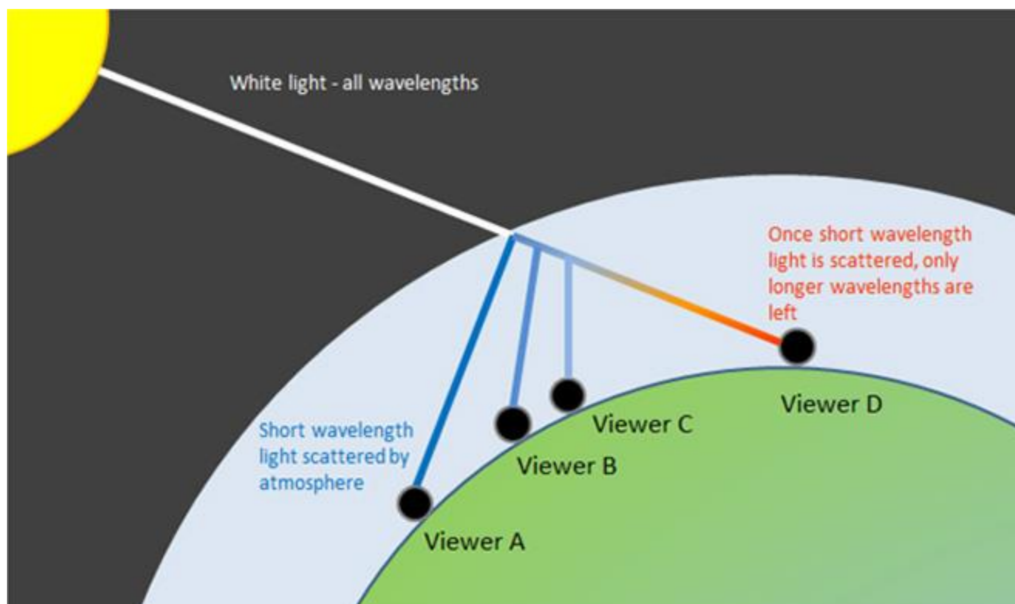


Figure 34 Colours of the sky [129]

Another example is the perception of light under water that scuba divers might experience when they are diving in high depths. As red light is not able to penetrate the water column very deeply, the vision of the diver is shifted to green and blue colours.

For the basic understanding of light absorption in biological molecules, the students should be provided with the infromation, that electrons are the main absorbers of light. Electrons can take up energy of photons or light waves and are lifted onto a higher energy level, which basically means that they just move faster. This relation shows the conversion of energy from light to thermal energy.

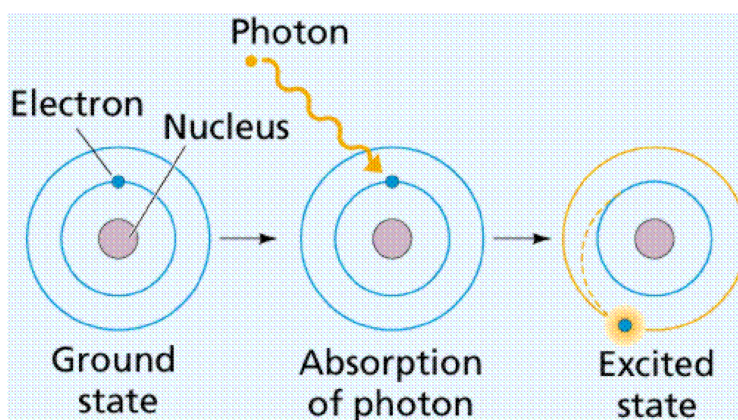


Figure 35 Electronic absorption of light [130]

Dependent on how much energy these electrons can absorb, they are absorbing the corresponding wavelength of light and therefore the corresponding colour. The important thing to teach the students is the relationship of absorption of one wavelength and the transmission of the complementary colour.

To bring a hands-on experience for the students in context with their theoretical knowledge a widely used pH-indicator can be used to show the connection between Pi-conjugation and colour absorption. The addition of sodium hydroxide or any other base will lead to a deprotonation of the colourless phenolphthalein and the resulting Pi-conjugated system in the molecule will lead to light absorption and the pink-coloued liquid.

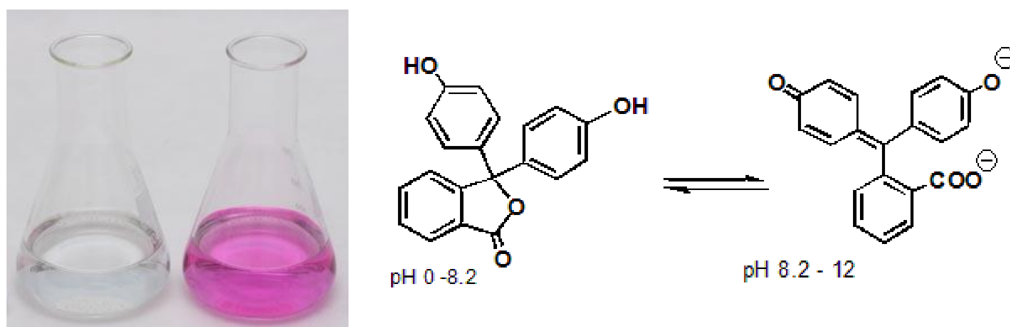


Figure 36 Deprotonation of Phenolphthalein [131,132]

The vivid explanation for the students is, that a Pi-conjugation in a molecule allows the electrons to move all over the molecule and therefor makes the electrons more „flexible“ and more accessible for light absorption.

Further examples of colourful biomolecules can be found in the texts in the Appendix. These are the texts that the students in this lesson should work on in their group activity and present in a 2 minute presentation in the next classroom session. The main task for the students on working with these texts could be: knowing the meaning of all the words that come up in the texts, showing the longest Pi-conjugation in the molecules and understanding the main biological function of the pigment molecule in the organism of origin. As these texts contain many difficult words and concepts, students should use internet research to look up words they do not understand. The texts include different biological pigments like Heme[133,134], Chlorophyll[135], Retinal/Opsin[135], Melanin[136,137], Anthocyanins[138] and Firefly Luciferin/Luciferase.[139] These texts should challenge the reading skills of the students and their ability of working on an unknown subject in small groups. The recent results of the OECD Programme for International Student Assessment (PISA) in the category reading revealed a weakness in the Austrian education system.[140] Therefore students should be confronted with scientific literature more frequently.

After the presentation of all these topics in the next lesson, a conclusion by the teacher should include the general similarity of all pigment molecules to insure the understanding of the interaction of photons with organic molecules.

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APPENDIX

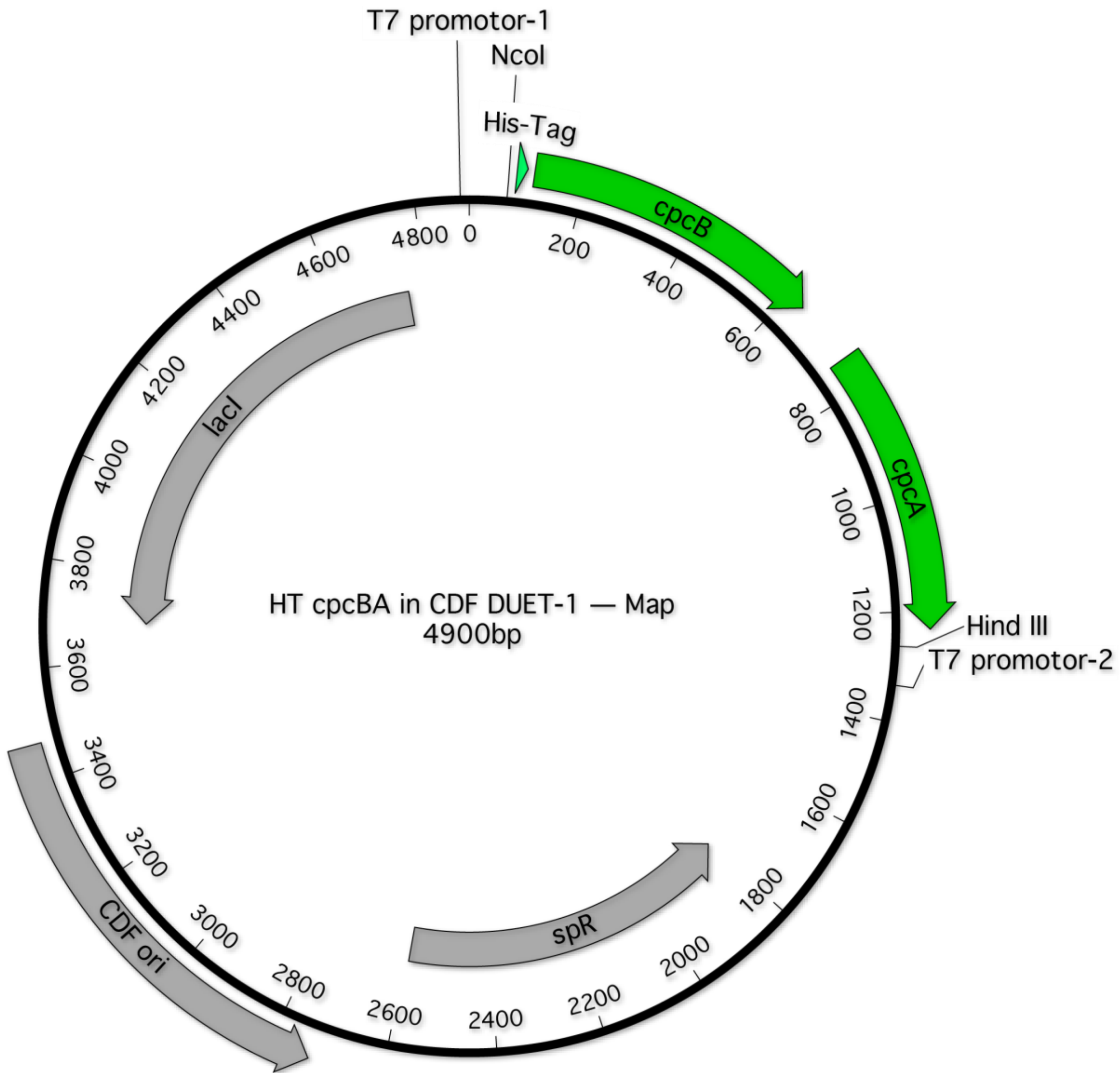


Figure 37 Vector map of pCpcBA. The plasmid pCDF Duet-1 including the genes for the α- and hexa-histidine tagged β-subunit of phycocyanin from *Synechocystis sp.* PCC 6803**.**

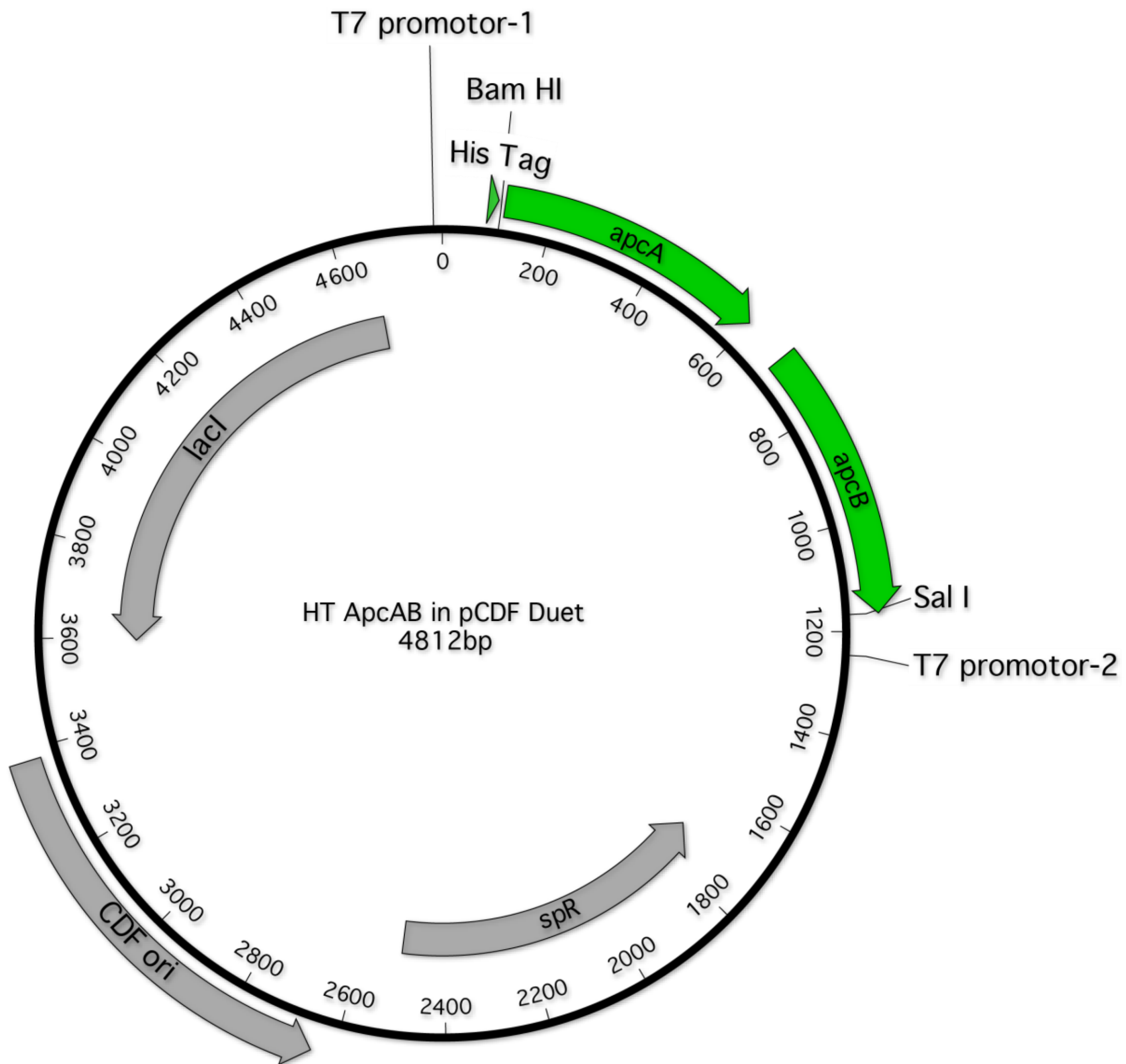


Figure 38 Vector map of pApcAB The plasmid pCDF Duet-1 including the genes for the hexa-histidine tagged α- and β-subunit of allophycocyanin from *Synechochoccus sp.* PCC 7002.

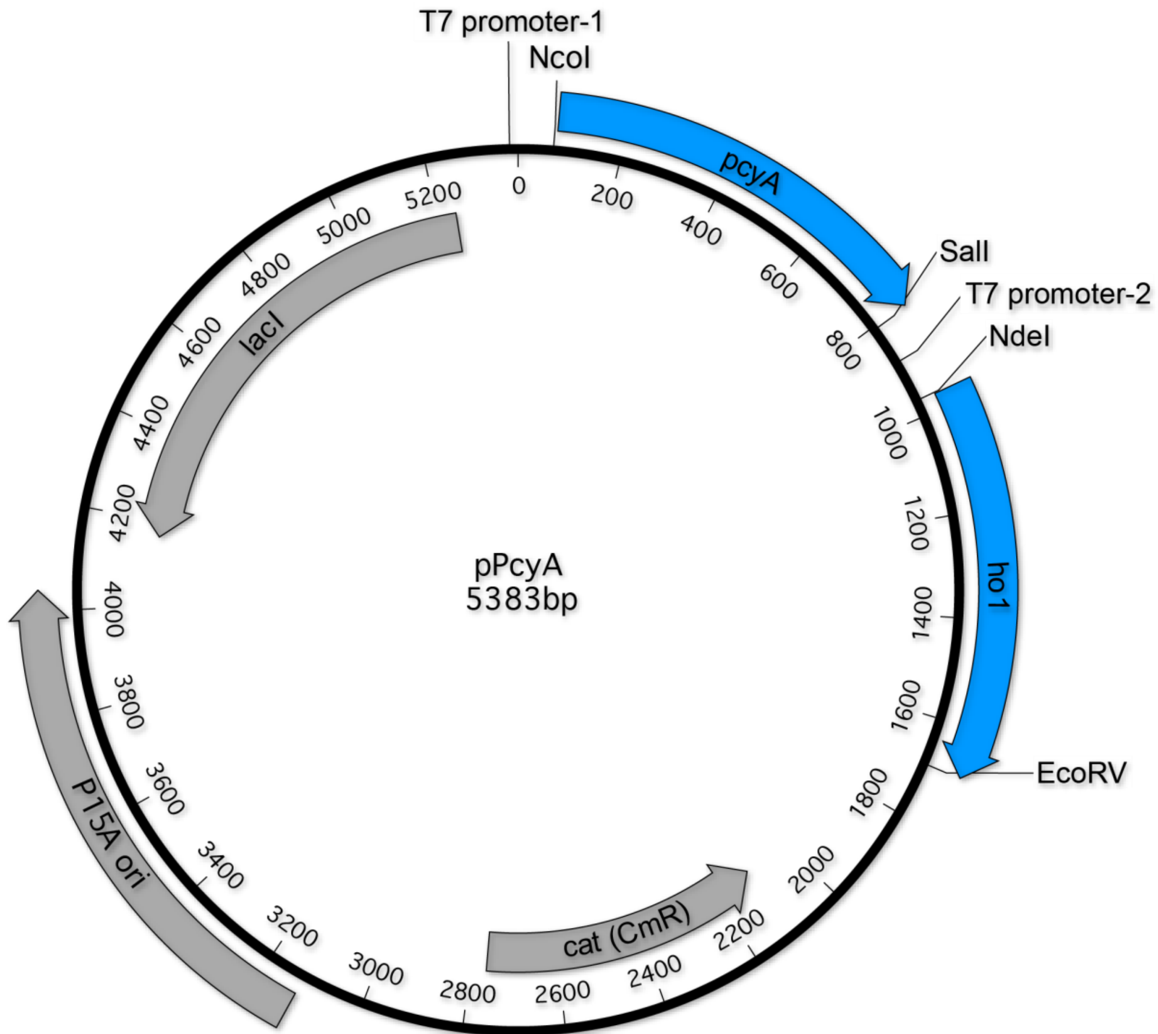


Figure 39 Vector map of pPcyA The plasmid pACYC including the genes for heme oxygenase 1 (HO1) from *Synechocystis sp.* PCC 6803 and phycocyanobilin:ferredoxin oxidoreductase (PcyA) from *Synechochoccus sp.* PCC 7002.

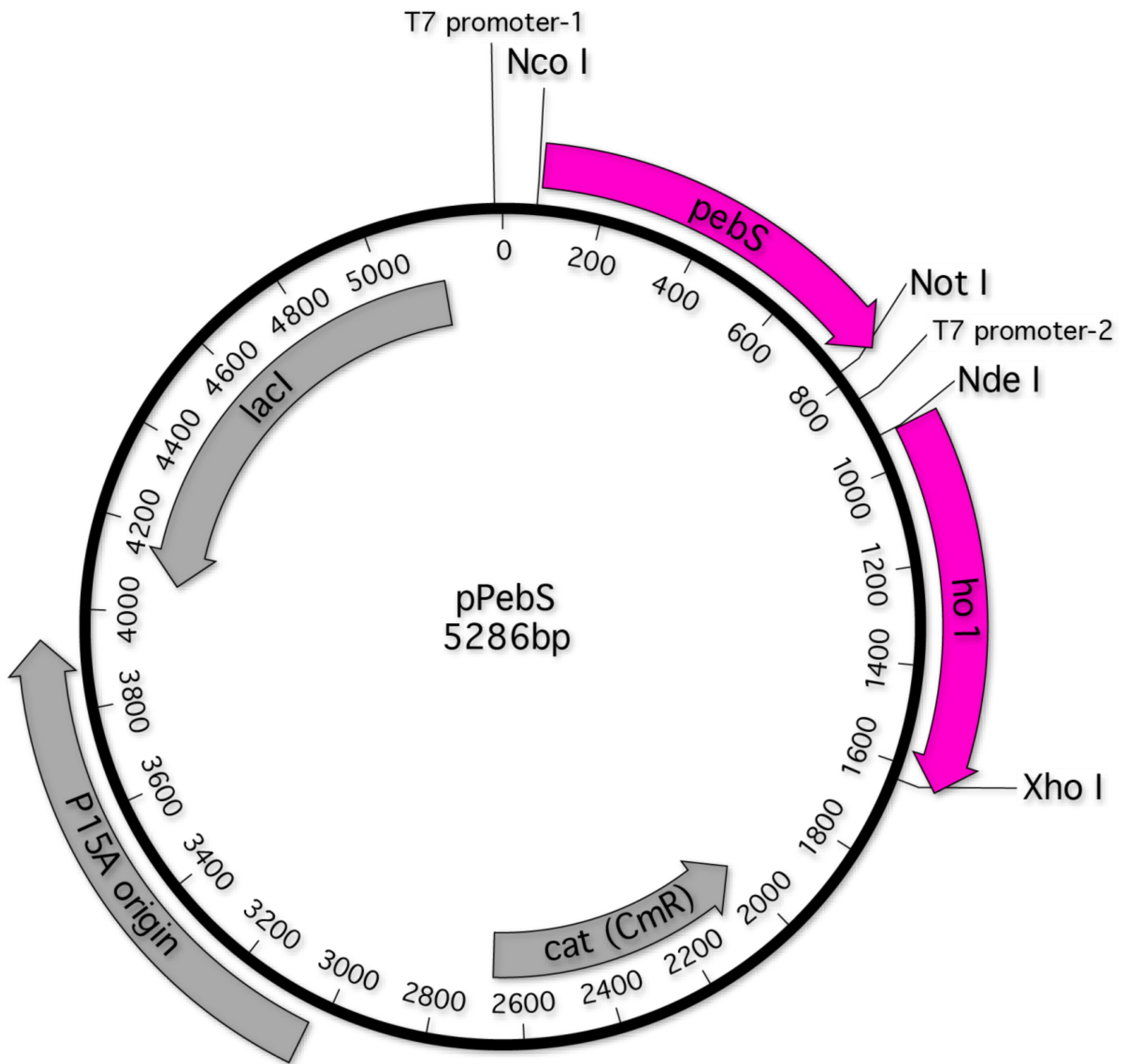


Figure 40 Vector map of pPebS Plasmid pACYC including the genes for heme oxygenase1 (HO1) and phycoerythrobilin synthase (PebS) from *myovirus*.[60]

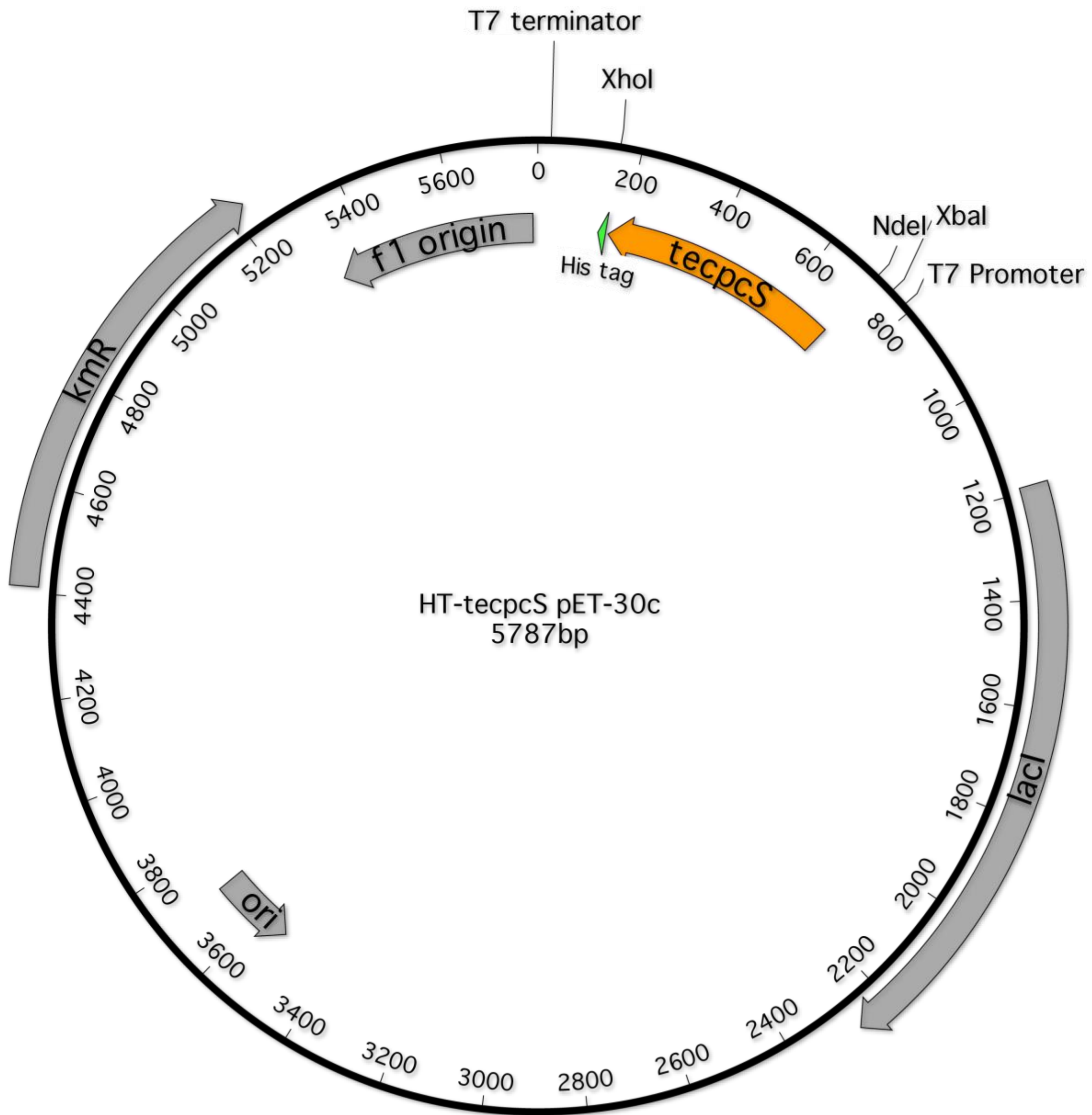


Figure 41 Vector map of pTER13-30. The plasmid pET-30c including the gene for hexa-histidine tagged CpcS lyase from *Thermosynechococcus elongates* or CpcS lyase mutants, respectively.

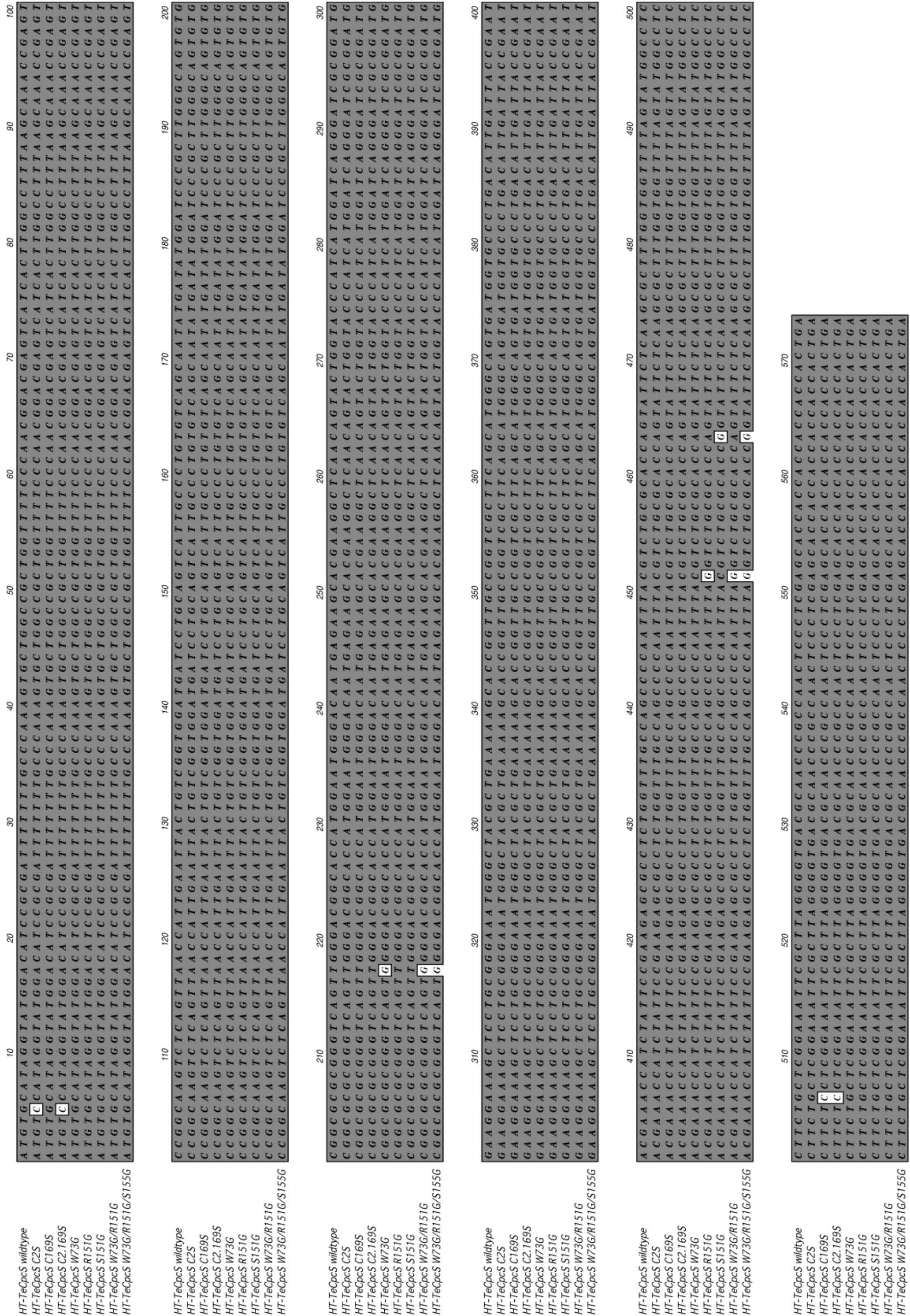
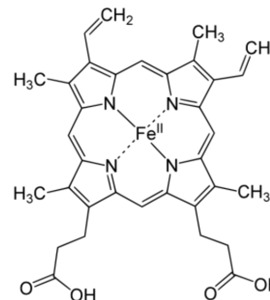


Figure 42 Alignment of nucleotide sequence of HT-*Te*CpcS wildtype and all variants derived from it in this thesis.

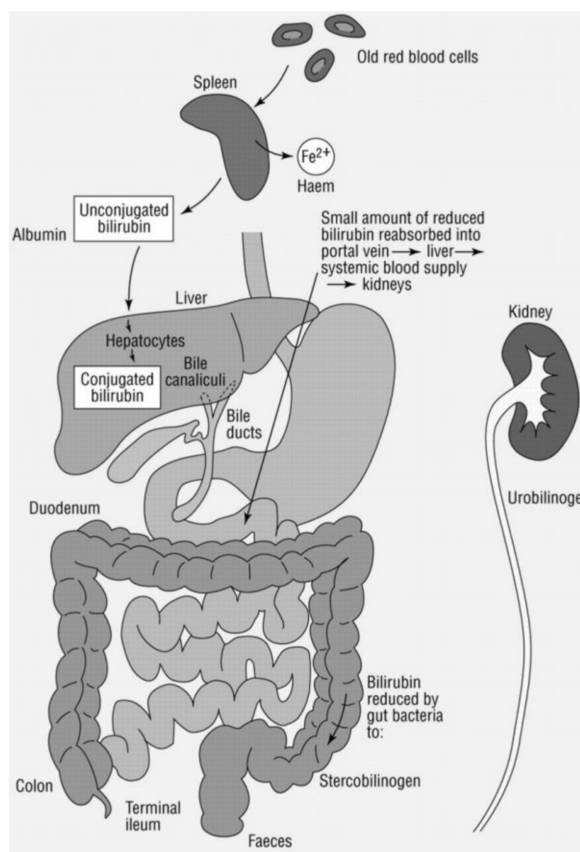
Heme – the red in our blood



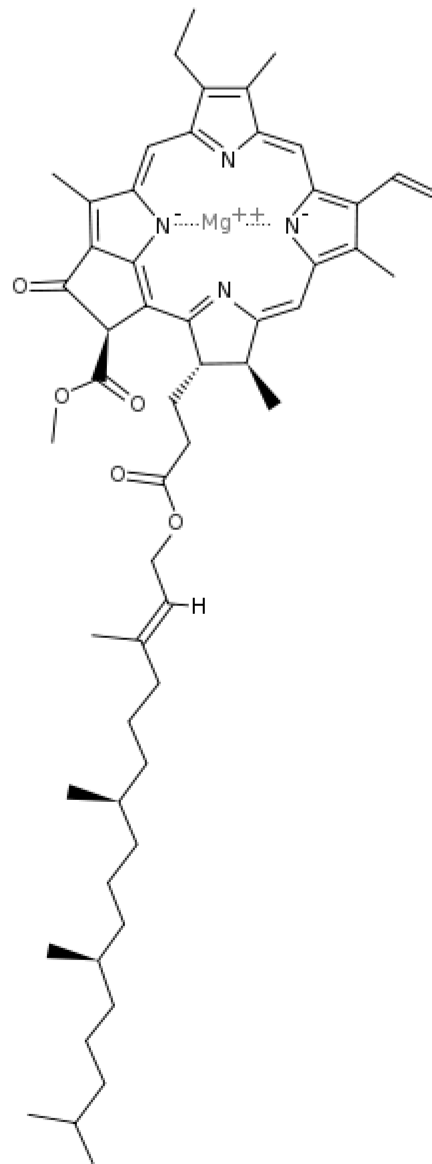
Heme is a chemical compound consisting of a Fe^{2+} ion contained in the centre of a heterocyclic organic ring called a porphyrin. Not all porphyrins contain iron, but many porphyrin-containing metalloproteins have heme. These are known as hemoproteins. Hemes are most commonly known as components of hemoglobin, **the red pigment in blood**, but they are also components of a number of other hemo-proteins such as myoglobin, cytochrome, catalase, etc.

Hemoproteins have diverse biological functions including the transportation of gases like oxygen, chemical catalysis and electron transfer. In the transportation of gases, the gas binds to the heme iron. Not only oxygen, but also carbon monoxide can bind to the heme group in red blood cells. As carbon monoxide is binding stronger to heme than oxygen, oxygen transport is inhibited by CO. This can lead to internal suffocation. Cigarette smoke and especially car exhausts contain high concentrations of CO. The ability of hemoglobin to effectively deliver oxygen to tissues is due to specific amino acid residues located near the heme molecule. Hemoglobin binds oxygen in the pulmonary system, where the pH is high and the concentration of CO_2 is low, and releases it in the tissues, where the situation is reversed. This phenomenon is known as the Bohr-effect.

Degradation of heme begins inside macrophages of the spleen, which remove old and damaged erythrocytes from the circulation. In the first step, heme is converted to biliverdin. In the second reaction, biliverdin is converted to bilirubin. Bilirubin is transported into the liver, where it is conjugated with glucuronic acid to become more water soluble. This form of bilirubin is excreted from the liver in bile. The intestinal bacteria deconjugate bilirubin diglucuronide and convert bilirubin to urobilinogens. Some urobilinogen is absorbed by intestinal cells and transported into the kidneys. The excretion of urobilin, which is a derivative of urobilinogen, is responsible for the **yellow colour of urine**. The remainder is converted to stercobilinogen by gut bacteria and transported down the intestine, where it is oxidized to stercobilin, which is excreted and is responsible for **the colour of feces**.



Chlorophyll – the green in plants



Chlorophyll (from Greek "chloros" = green and "phyllon" = leaf) is a chemical compound that consists of a magnesium ion in the center of a chlorin ring. In the most common forms of chlorophyll (chlorophyll a and b) a long phythol side chain is attached to the chlorin ring.

The green pigment is found in cyanobacteria and chloroplasts of algae and plants. Chlorophyll is an extremely important biomolecule for photosynthesis, which allows plants to convert energy from light to chemical energy. Chlorophyll absorbs light most strongly in the blue and the red portion of the electromagnetic spectrum. However, it absorbs green and near-green light only poorly. Therefore the colour of chlorophyll-containing tissues is green.

Chlorophyll can be found in and around photosystems. These photosystems are embedded in the membranes of chloroplasts, known as thylakoid membranes. In the light-harvesting complexes, chlorophyll has two primary functions. First, the function of the majority of chlorophyll is to absorb light and transfer that light energy by resonance energy transfer to the reaction center of the photosystems (These chlorophylls can be abundant in very high amounts up to several hundred molecules per photosystem). Second, the specific chlorophyll pair in the reaction center of the photosystems is photooxidized and starts the electron transport chain that is necessary for photosynthesis.

The two photosystems are Photosystem II and Photosystem I, which have distinct reaction centers, named P680 and P700, respectively. The names correspond to their wavelength in nanometers of their red-peak absorption maximum.

The different chlorophyll pigments and associated carotinoids can easily be extracted from the protein into a solvent, such as acetone or methanol and subsequently separated in a simple paper chromatography experiment. The pigment components will chemically separate on the paper based on the difference in number of polar groups between them.

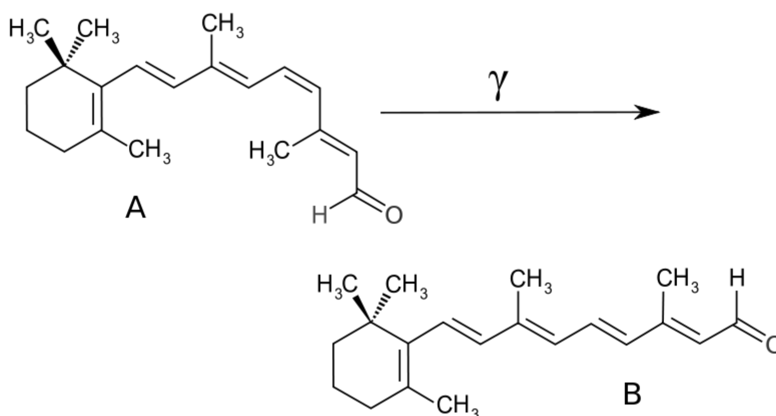
Retinal – the light receptor in our eyes

Retinal, also called vitamin A aldehyde, is one of the many forms of vitamin A. Retinal, a polyene chromophore, is bound to proteins called opsins and is the chemical basis of animal vision.

Opsins, the retinal binding proteins are found in the photoreceptor cells in the retinas of eyes. Different variations of opsins can be found in rod cells and in cone cells of the retina. Rod cells are more sensitive to light but do not distinguish colours. They are therefore responsible for night vision. Cone cells are responsible for colour vision. Opsins are typical G protein-coupled receptors. These receptors are important in signal transduction in various cell types and lead to specific cellular responses. In the case of vision the cellular response leads to a signal in the optic nerve.

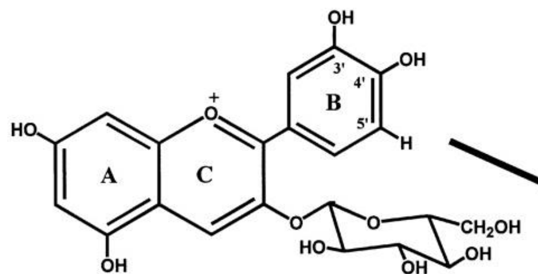
Vertebrate animals (this includes humans) ingest retinal directly from meat, or produce retinal from carotenes (α-carotene, β-carotene) or β-cryptoxanthin, a type of xanthophyll. These precursers must be synthesized by plants or other photosynthetic organisms. Some carnivores cannot convert any carotenoids to Vitamin A and are therefore dependent on direct intake of it. The other main form of vitamin A, retinol, may be produced from retinal.

The process of vision begins with the photoisomerization of retinal. When 11-cis-retinal (molecule A in image below) absorbs a photon, it isomerizes to all-trans-retinal(molecule B in image below). The absorbance spectrum of the chromophore depends on its interactions with the opsin protein to which it is bound. Different opsins lead to different absorbance spectra and are therefore responsible for vision of different colours.



Anthocyanins – colours of berries and flowers

Anthocyanins (from Greek: *anthos* = flower and *kyanos* = blue) are water-soluble pigments that may appear in different colours from red, purple or blue. They belong to a parent class of molecules called flavonoids. They are usually odorless and nearly flavorless. Anthocyanins can be found in various tissues of higher plants, including leaves, stems, roots, flowers, and fruits.



Flowers use anthocyanins for attracting pollinators. In fruits, the colorful skins attract the attention of animals, which may eat the fruits and disperse the seeds. In photosynthetic tissues, anthocyanins have been shown to act as a "sunscreen", protecting cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photoinhibition, or high-light stress.

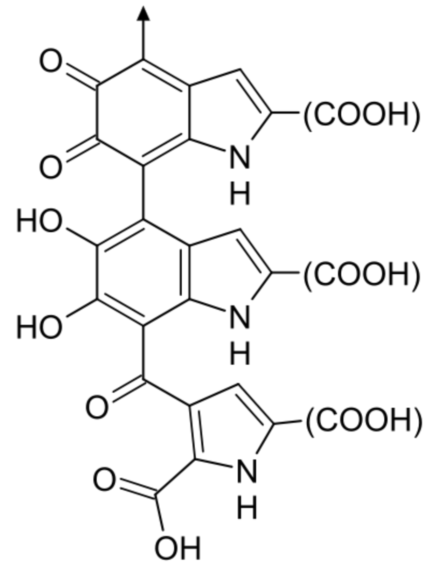
Plants rich in anthocyanins are blueberry, cranberry, and bilberry, black raspberry, red raspberry, blackberry, blackcurrant, cherry, eggplant peel, black rice, grapes, and red cabbage. Also red-fleshed peaches are rich in anthocyanins.

In addition to their role as light-attenuators, anthocyanins also act as powerful antioxidants. Based upon many studies, it has been suggested that anthocyanins possess anti-inflammatory and anti-carcinogenic activity, cardiovascular disease prevention, obesity control, and diabetes alleviation properties. All of these seem to be associated with their potent antioxidant property.

Anthocyanins can be used as pH indicators because their color changes with pH. They are pink in acidic solutions (pH < 7), purple in neutral solutions (pH ~ 7), greenish-yellow in alkaline solutions (pH > 7), and colourless in very alkaline solutions, where the pigment is completely reduced. Red cabbage extract is a well known pH indicator (shown in the image at the right).

Melanin – the colour of our skin

Melanin (from Greek: melas = "black, dark") is a broad term for a group of dark coloured natural pigments found in many organisms. Melanin is a derivative of the amino acid tyrosine. However, it is not produced from amino acids and is not a protein. The pigment is produced in a specialized group of cells known as melanocytes.



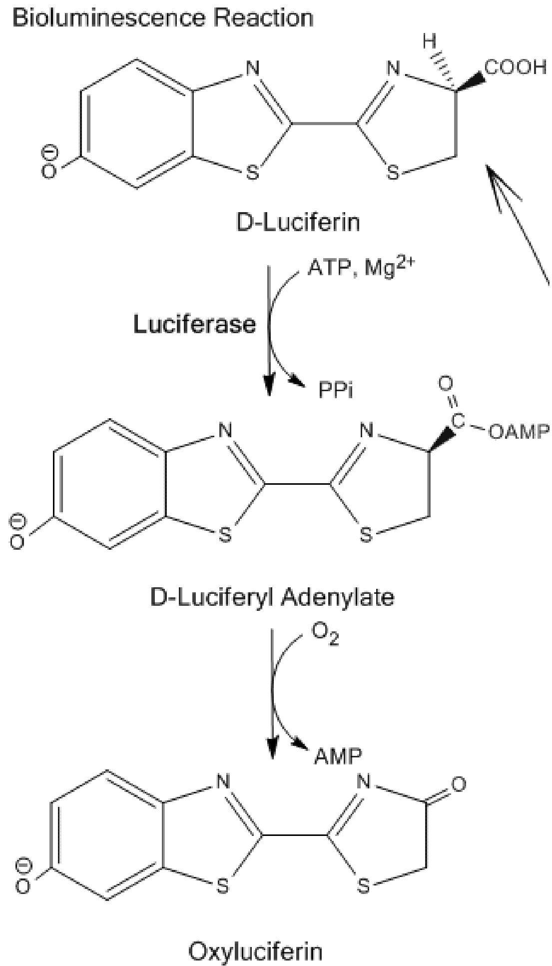
There are three basic types of melanin: eumelanin, pheomelanin, and neuromelanin. The most common type is eumelanin, and appears either black or brown. Pheomelanin is a red-brown polymer and is largely responsible for red hair and freckles. Neuromelanin is found in the brain, though its function is unknown.

In the skin, melanogenesis, the production of melanin occurs after exposure to UV radiation. The production of melanin leads to the appearance of tanned skin. Melanin is an effective absorber of light. The pigment is able to dissipate the majority of the absorbed UV radiation. Therefore melanin is thought to protect skin cells from UV radiation damage and therefore might reduce the risk of skin cancer.

In humans, melanin is not only the primary determinant of skin color, it is also found in hair, the pigmented tissue underlying the iris of the eye, and in the inner ear. In the brain, tissues with melanin include the medulla and pigment-bearing neurons within areas of the brainstem, such as the locus coeruleus and the substantia nigra. It also occurs in the zona reticularis of the adrenal gland.

Some individual animals and humans have a strongly decreased or no melanin synthesis. This condition is known as albinism. Eumelanin is the form that is most likely to be deficient in albinism.

Luciferin – the light of fireflies



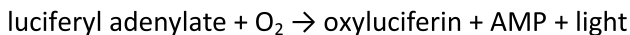
Firefly luciferin (Latin: “Lucifer” = Light-bringer) is the light-emitting compound, found in many firefly species. It is the substrate of luciferase, which is responsible for the characteristic yellow light emission from the abdomen of the fireflies. As with all other luciferins, oxygen is required to emit light. However, it has also been found that ATP and magnesium are required for light emission.

Lampyridae is a family of insects in the beetle order Coleoptera. They are winged beetles, and commonly called fireflies or lightning bugs for their ability of using bioluminescence to attract mates or prey.

The typical emission for luciferase is in the yellow-green region (550–570 nm), with a peak at 562 nm at basic media (pH ~ 7.5–7.8) However, luciferase is a pH-sensitive enzyme, and acid media (pH ~ 5–6) can shift the emission to red (maximum at 620 nm), as well as higher temperatures and heavy metal cations. It is believed that conformational changes, which influence the active site microenvironment, are responsible for the different color emission Different species of fireflies all use the same luciferin, however the color of the light emitted can differ greatly.

The chemical reaction catalyzed by firefly luciferase takes place in two steps:

luciferin + ATP → luciferyl adenylate + PP_i


luciferyl adenylate + O_2 → oxyluciferin + AMP + light

Light is emitted because the reaction forms oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state.

The luciferin reaction can also be used for scientific applications. In general there are two areas of interest. In molecular biology studies and bioimaging the luciferin gene is used as a reporter and the light reaction can be used for quantification of analytes connected to ATP or other participants.