PURIFICATION AND KINETIC CHARACTERIZATION OF
PROTOCHLOROPHYLLIDe a DIVINYl REDUCTASE IN GREEN SULFUR
BACTERIUM CHLOROBIUM TEPIDUM

A Thesis in
Plant Physiology
by
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(Bacterio)chlorophylls are magnesium tetrapyrroles involved in the light harvesting and photochemistry. In this thesis study, the *bciA* gene, encoding protochlorophyllide a divinyl reductase from *Chlorobium tepidum*, was overexpressed in *Escherichia coli* and purified by metal affinity chromatography.

As estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) analysis, purified BciA was greater than 95% pure. Peptide mass fingerprinting of the overproduced BciA excised from a SDS-PAGE gel slice matched the CT1063 protein with a high score (202) and high coverage (70%). Non-denaturing PAGE showed that BciA mostly exist as monomers and that about 10% of the protein exists as dimers.

Divinyl reductase assays using purified BciA confirmed that BciA was able to convert divinyl protochlorophyllide (DV-PChlide) into monovinyl protochlorophyllide (MV-PChlide) with NADPH serving as the reductant. NADH was not able to substitute for NADPH. Initial velocity studies suggest that BciA reacts via a ternary complex mechanism. Product inhibition studies indicated the binding sequence of the reactants. NADPH binds first and NADP⁺ is released last. The $K_m$ value for the NADPH was calculated to be $20.1 \pm 7.8 \mu M$. The $K_m$ value for the DV-PChlide was estimated to be $1.7 \pm 0.5 \mu M$. These $K_m$ values are comparable to those of other enzymes involved in the (B)Chl biosynthesis pathways. The low $K_m$ values for tetrapyrroles are consistent with the fact that the tetrapyrroles are very toxic to the cells, and therefore the cellular concentrations of these compounds are most likely to be very low.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALA</td>
<td>5-aminolevulinic acid</td>
</tr>
<tr>
<td>(B)Chl</td>
<td>(bacterio)chlorophyll</td>
</tr>
<tr>
<td>BCHl</td>
<td>bacteriochlorophyll</td>
</tr>
<tr>
<td>C.</td>
<td><em>Chlorobium</em></td>
</tr>
<tr>
<td>Chlide</td>
<td>chlorophyllide</td>
</tr>
<tr>
<td>DV</td>
<td>3,8-divinyl</td>
</tr>
<tr>
<td>FAP</td>
<td>filamentous anoxygenic prokaryotes</td>
</tr>
<tr>
<td>FMO protein</td>
<td>Fenna-Matthews-Olson protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>GSB</td>
<td>green sulfur bacteria</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-morpholino]ethane sulfonic acid</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
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<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>MP-IX</td>
<td>magnesium protoporphyrin IX</td>
</tr>
<tr>
<td>MPE</td>
<td>magnesium protoporphyrin monomethyl ester</td>
</tr>
<tr>
<td>MV</td>
<td>3-monovinyl</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced form of nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced form of nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PChlide</td>
<td>protochlorophyllide</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
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<td>protoporphyrin IX</td>
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<tr>
<td>R.</td>
<td>Rhodobacter</td>
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<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl methionine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)amino-methane</td>
</tr>
<tr>
<td>× g</td>
<td>gravitational constant</td>
</tr>
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CHAPTER 1

Introduction

1.1 Green Sulfur Bacteria

Photosynthesis, the reduction of carbon dioxide into biomass using energy derived from light, is considered to be the most important biological process on Earth. Two mechanisms exist for the collection and conversion of light energy into chemical energy (15). The first mechanism uses rhodopsins, which are retinal-binding proteins that act as light-driven proton or chloride pumps and light sensors (23). The second mechanism uses reaction centers that contain chlorophyll ((B)Chl) (23). Green sulfur bacteria (GSB, Chlorobi) is one of the only six eubacterial phyla (Cyanobacteria, Proteobacteria, Chlorobi, Chloroflexi, Firmicutes and Acidobacteria) employing this mechanism (14, 15). GSB strains have simple nutritional requirements and metabolic capabilities (30). Not surprisingly, GSB genomes are quite small, ranging from 2 to 3 Mb (27) and thus potentially encode 2000 to 3000 proteins (30).

1.1.1 Ecology

GSB are obligately anaerobic photolithoautotrophs. The growth of GSB requires the absence of molecular oxygen and the presence of sulfide and/or thiosulfate. GSB have been isolated from below the chemocline of stratified lakes and from the anoxic layers of
aquatic sediments (89). They can also be found in sulfidic springs and microbial mats (85, 90). GSB possess a highly specialized light-harvesting antenna, the chlorosome, which is the largest and one of the most efficient antenna structures known. The chlorosomes allow GSB to photosynthesize at extremely low light intensities, which would not normally be suitable for photosynthesis. For example, they have been found growing at a depth of 100 m below the surface of the Black Sea, where the light intensity is only 0.0026 µmol photons m\(^{-2}\)s\(^{-1}\) and at 2200 m below the surface of the Pacific Ocean close to a hydrothermal vent (30, 56, 69). The in situ temperatures for GSB range from 2 °C around the deep-sea hydrothermal vent to 68 °C in hot spring mats (7, 28). GSB have been found in acidic hydrothermal waters with pH values as low as 2.5, although GSB tend to grow best at nearly neutral pH (6.8-7.0) (26, 68).

1.1.2 Phylogeny

Molecular phylogenies using 16S rDNA (70, 71) and RecA protein sequences (36), as well as phylogenies based on whole-genome analysis (T. Li, personal communication), show that GSB are the sister group of the Cytophaga-Flexibacteria-Bacteroides (CFB) clade, and are only distantly related to all other phototrophic bacterial species (Figure 1).

Within the GSB clade, there exist five phylogenetically distinct groups (Figure 1.2). These are a marine cluster with average G+C values between 52.2 and 53.5 mol%; a second group of low-salt requiring strains with higher mol% G+C values; a third group of freshwater strains; and a fourth group comprised of *Chlorobaculum tepidum* (*C. tepidum*)
and some strains of *C. vibrioforme* and *C. limicola* (29). The fifth group has only one isolated member, *Chloroherpeton thalassium*, and is the most divergent genus (1).

1.1.3 Physiology

All known GSB fix carbon via the reductive tricarboxylic acid (TCA) cycle (21). The pathway of reductive TCA cycle is shown in Figure 1.3. Using reduced sulfur compounds or hydrogen as electron donors, two molecules of carbon dioxide are assimilated via reductive TCA cycle into one molecule of acetyl-CoA and a third carbon dioxide molecule is fixed to make pyruvate by pyruvate ferredoxin oxidoreductase (PFOR). The reductive TCA cycle has also been found in the Aquificales (42) and the δ- (76) and ε- proteobacteria (20). ATP-dependent citrate lyase, which converts citrate into acetyl CoA and oxaloacetate, is the diagnostic enzyme for the reductive TCA cycle in most GSB except Aquificales (3, 4, 45). It has been found that carbon originally reduced by GSB has a different isotopic signature than carbon reduced by oxygenic photosynthesis due to different carbon fixation pathways (75, 78). Investigation of these isotopic signatures and identification of GSB-specific biomarker isorenieratane as well as its derivatives have led to the conclusion of the existence of anoxic photic zones, at least temporarily, in certain environments (46).

As anoxygenic photoautotrophs, GSB do not use water as an electron donor but use diverse sulfur sources and hydrogen to provide electrons. All species of GSB, except *C. ferrooxidans* which uses ferrous iron as an electron donor, can grow by oxidizing hydrogen sulfide to elemental sulfur and sulfate. Some species have the ability to utilize thiosulfate for growth in the absence of other reduced sulfur sources (13).
GSB are able to fix nitrogen but can also use ammonia as a nitrogen source. The nitrogenase activity in *Chlorobium* species can be activated by the depletion of ammonia and inactivated by the addition of ammonia. The completed genome of *C. tepidum* revealed that all the nitrogen fixation genes are in a single region of the genome (27). It is interesting that the closest orthologs of the *C. tepidum nif* genes are identified in archaea with conserved gene organization (27).

### 1.1.4 Photosynthetic apparatus of green sulfur bacteria

The photosynthetic apparatus of GSB includes three parts: the light-harvesting antenna, the reaction center (RC) and the electron transfer pathway. Antenna complexes are responsible for harvesting light energy. Light is absorbed by the pigments and the energy is initially stored in the form of an excited electronic state. The excitation energy is transferred via the baseplate containing BChl $a$ to the FMO-BChl $a$ complex. FMO further transfers the excitation energy to the RC, where a pair of BChl $a$ serves as the primary electron donor and Chl $a$ is the primary acceptor $A_0$ (39). The excitation energy is used by the RC to produce proton gradient for ATP synthesis and/or reducing power (27).

### 1.1.5 Chlorosome

GSB have a highly specialized light-harvesting antenna, the chlorosome, which allows them to photosynthesize at very low light intensities. Besides GSB, chlorosomes have also been found in some filamentous anoxygenic phototrophs (FAP) of the phylum *Chloroflexi* and the newly discovered aerobic phototroph *Candidatus*
Chloroacidobacterium thermophilum (31). Chlorosomes are large, flattened, sac-like structures with arrays of bacteriochlorophyll (BChl) $c$, $d$ or $e$ that absorb in the near-infrared between 720 and 750 nm. Chlorosomes in GSB are connected to reaction centers through the Bchl $a$-containing Fenna-Matthews-Olson (FMO) protein (30). Chlorosomes are usually 100 to 200 nm long, 30 to 70 nm wide and 10 to 12 nm thick, with variations among species and according to different light intensities and other growth conditions (9). Chlorosomes in FAP are smaller than those in GSB and FAP, lack the FMO-BChl $a$ complex, while those in *Candidatus* Chloroacidobacterium thermophilum are similar in size and have an FMO-BChl $a$ complex (14). A typical chlorosome from *C. tepidum* has been estimated to contain as many as $215,000 \pm 80,000$ BChl $c$ molecules (63). Each reaction center receives excitation energy from roughly 5000-8000 BChl $c$ (30). This is the largest antenna pigment to RC ratio known among photosynthetic organisms. Antenna sizes for most other photosynthetic organisms are in the range of 50 to 300 pigment molecule per RC (24). Unlike other light-harvesting antenna structures in which chromophores are rigidly bound to and oriented by proteins, the antenna BChls in chlorosomes do not require a protein scaffold for assembly. The protein-free organization is supported by the following lines of evidence: firstly, BChl $c$ can form aggregates in non-polar solvents with an absorption spectrum resembling those of chlorosomes; secondly, proteins can be extracted from chlorosomes without affecting the spectral properties of the BChl $c$; lastly, proteins have been shown to be located only in the envelope but not the hydrophobic interior of the chlorosomes (9). As a result, the protein-to-chlorophyll ratio of chlorosomes is very low, and it greatly reduces the cellular energy costs to build such large arrays of pigments. The protein: pigment ratio of
chlorosomes is approximately two amino acids per BChl, while the ratios are about 15 amino acids per Chl in plant antenna protein complexes and 60-160 amino acids per bilin in cyanobacterial phycobiliproteins (30, 88). Given the large antenna pigment numbers, a protein-based antenna system would be a huge energy burden for GSB. The self-assembly of Bchl c, d or e molecules are facilitated by intermolecular interactions among the Bchl molecules. The BChls have a unique hydroxyl group at the C3\(^1\) position. The hydroxyl group of one BChl bridges to the magnesium ion of another BChl with the C-13\(^1\) keto groups of a third BChl, C=O \ldots HO (R) \ldots Mg (41). Long-range organization of Bchl layers in chlorosomes from wide-type \textit{C. tepidum} and from a mutant that produces a single homolog of Bchl d (8-ethyl, 12-methyl, Bchl d) have been examined by cryo-electron microscopy (67). End-on views revealed that chlorosomes are composed of several multi-layer tubules of variable diameter (20-30 nm) with some locally undulating non-tubular lamellae in between (67). The mutant synthesizing only [8-ethyl, 12-methyl]-Bchl d has a more regular and larger multi-layered tubular structure than the wide-type chlorosomes (67). The self-aggregated BChls are enveloped in a galactolipid monolayer with several chlorosome proteins. The chlorosome envelope in \textit{Chlorobium tepidum} has 10 identified proteins (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX, among which CsmA is the most abundant and the only one required for viability (16, 31). Cross-linking studies showed that CsmA is located in the baseplate and that CsmA directly interacts with FMO protein (53). CsmA, CsmC, CsmD, CsmH, CsmI, CsmJ and CsmX were shown to exist as homomultimers in the chlorosome envelope (53).

The molar ratio of carotenoids to BChl in chlorosomes is about 1:10 in \textit{Chlorobium} species, varying according to different species and light conditions (9).
Carotenoids in chlorosomes have been suggested to function in light harvesting (60, 64, 73), structural stabilization of the BChl aggregates (5, 32) and photoprotection (60). Recent studies found that the photoprotective role of carotenoids in chlorosomes is much less significant than in other parts of photosynthetic apparatus (48). Alternatively, BChl aggregates are naturally protected due to the formation of triplet excitons whose energy may fall below that of singlet oxygen and triplet carotenoids, preventing energy transfer from triplet BChl (48).

1.1.6 Reaction center

Photosynthetic RCs are protein-pigment complexes embedded in the photosynthetic membrane. Light energy is trapped by the RCs by a photochemical reaction to drive electron transport reactions leading to the production of proton gradient for ATP synthesis and/or reducing power. Reaction centers consist of a mixture of protein, chlorophyll and other redox-active cofactors (40). Mainly based on the identity of the terminal electron acceptor, two types of RCs are classified (7). Type I RCs use [4Fe-4S] clusters as their terminal electron acceptors and produce weak oxidants and strong reductants (reduced ferredoxin), whereas type II complexes uses (bacterio)pheophytins and quinones as terminal electron acceptors and produce strong oxidants and weak reductants (hydroquinone) (30, 39). GSB and heliobacteria contain type I RCs. Purple photosynthetic bacteria have type II RCs. Oxygenic photosynthetic organisms (cyanobacteria, algae and plants) have both type I and type II RCs, named separately as Photosystem I and Photosystem II (7).
The structures of type II RC from two species of purple bacteria, *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* were determined by X-ray crystallography in mid-1980s (2, 25). The three-dimensional structures of Photosystem I and Photosystem II from cyanobacterium *Synechococcus* (*Thermosynechococcus* *elongatus*) were also determined at high resolution more recently (Figure 1.4 a,b) (47, 55). The structure of the type I RC in GSB has not been resolved by crystallography due to their sensitivity to oxygen. Type I RC in GSB contains 4 subunits: PscA, PscB, PscC and PscD (40). RC in GSB and heliobacteria differ from PSI in that it contains a homodimeric core of two identical polypeptides termed PscA instead of the PsaA-PsaB heterodimer (19). This is explained by the presence of a single gene for the core polypeptides of the RC in GSB and heliobacteria, rather than the two genes seen in cyanobacteria, algae and higher plants (18, 54). PscA from *C. tepidum* is an 82 kDa protein with 731 amino acids that binds the primary electron donor P840 (a special pair of BChl *a*, the primary electron acceptor *A*0 (a monomeric Chl *a*-derivative) and the [4Fe-4S] cluster Fx (38). In addition to the PscA homodimer, type I RC in GSB also have the extrinsic proteins PscB and PscD, resembling the PSI PsaC and PsaD (Figure 1.4 c) (39). PscC is a monoheme cytochrome *c* molecule with three transmembrane helices at its N-terminus and electron transfer kinetics showed that two molecules of PscC are symmetrically arranged around the reaction center core (51, 66). PscD is a 15 kDa protein resides in the vicinity of PscB, which may function in the stabilization of PscB and/or the interaction with ferredoxin (37, 77). PscB is a membrane extrinsic protein that resembles the PsaC subunit in PSI (40).

Although the PscA homodimer is of the same size as PsaA and PsaB, the PscA homodimer binds many fewer chlorophyll molecules in comparison to the PsaA/PsaB
heterodimer. In contrast to the 96 chlorins bound by the PsaA and PsaB polypeptides, each RC core in GSB contains only 4 Chl $a$ and ~16 BChl $a$ (39). Two of the BChl $a$ molecules are C13$^2$ epimers that served as the primary donor termed P840 in GSB (49). There are also only two carotenoids per RC in comparison to the 22 carotenoids in each PSI RC, which may reflecting a reduced need for photoprotection in the strictly anaerobic phototrophic organisms (39).

1.1.7 Energy transfer pathway

In GSB, the excitation energy harvested by BChl $c$ is transferred to pigments with progressively red-shifted absorption and fluorescence spectra: BChl $c$ (chlorosome)-> BChl $a$ (baseplate) ->BChl $a$ (cytoplasmic membrane or FMO) -> BChl $a$ (reaction center) (9). The main absorption peaks of BChl molecules are between 720 to 750 nm, the baseplate BChl $a$ has an absorption maximum at 795 nm, the FMO-BChl $a$ complex has a absorption maximum at 808 nm and the special pair of BChl $a$ in reaction center serving as primary electron donor has an absorption maximum at 840 nm (61). The energy gradient provides a ‘funneling’ of excitation energy into the reaction center (9).

The energy transfer in GSB appears to be regulated by redox potential (9). Under reducing conditions, which is the natural growth state of the GSB, the chlorosomes are highly fluorescent while under oxidizing conditions, this fluorescence is quenched (87, 92). Energy transfer efficiency can be reduced from nearly 100% to less than 10% by adding oxidizing reagents, and the addition of reducing reagents can fully reverse the quenching (8, 92). Inhibition of excitation energy to the RC under oxic conditions might be a survival response by the GSB, because low-potential reductants produced by
photoactivation of the RC of GSB react readily with molecular oxygen to form activated oxygen species (34). The environmental niches of GSB, such as just below the chemocline in stratified lakes, may allow occasional exposure to the moderate oxygen levels and a mechanism that provides protection from oxidative damage would be of adaptive advantage (9). Quinones have been proposed to play a significant role in the inhibition of energy transfer under oxic conditions. (34). Chlorosomes from *C. tepidum* contain three isoprenoid quinone species (chlorobiumquinone, menaquinone-7, and an unidentified quinone that might be a chlorobiumquinone derivative) and the molar ratio of total quinone to BChl *c* in the chlorosomes is approximately 1:10 (34). These quinones have been shown to act as quenchers of BChl *c* fluorescence (34). Isoprenoid quinones are very hydrophobic and are presumed to be located in the interior of the chlorosomes rather than in the chlorosome envelope (9).

### 1.1.8 *Chlorobium tepidum*

The GSB *C. tepidum* is a moderate thermophile originally isolated from mildly acidic, high-sulfide hot springs in New Zealand (90). *C. tepidum* cells are Gram-negative, nonmotile rods with variable lengths (90). The optimal temperature for cultured *C. tepidum* is 47-48 °C and optimal pH is 6.8-7.0. The upper temperature for its growth is about 52 °C (52, 90). The doubling time of *C. tepidum* cultured under photoautotrophic conditions with thiosulfate as the major electron donor is around two hours, faster than that of any other anoxygenic phototroph (91). This fast growth rate and the availability of an efficient natural transformation system make *C. tepidum* a model organism for studies of photosynthesis and autotrophy in GSB. The 2.15-Mb genome of *C. tepidum* has been
sequenced, annotated and published in 2002 (27). *C. tepidum* cells contains about 200-250 chlorosomes. The major pigments of *C. tepidum* are BChl c₇ and chlorobactene (30, 33).

1.2 Pigment content and biosynthesis

All chlorophototrophs make two types of pigments: 1. carotenoids, which primarily function in photoprotection but are also involved in light harvesting 2. bacteriochlorophylls (BChls) and/or chlorophylls (Chls) which function in light harvesting and photochemistry (23). Hundreds of carotenoids have been identified while twelve types of Chls (Chl $a$, $b$, $d$; divinyl-Chl $a$ and $b$; 8'-hydroxy-Chl $a$) and BChls (BChl $a$, $b$, $c$, $d$, $e$, and $g$) have been identified so far (23).

1.2.1 Carotenoids in GSB

Most of carotenoids in GSB are located in the chlorosome and the major carotenoids of GSB contain aromatic rings at one or both ends (28, 74). The major carotenoid of the green-colored GSB species is monocyclic chlorobactene while that of the brown-colored GSB is dicyclic isorenieratene (44). *C. tepidum* also contains $\gamma$-carotene, the 1’,2’-dihydro derivatives of both chlorobactene, $\gamma$-carotene and in lesser amounts, OH-chlorobactene, OH-$\gamma$-carotene, and the glucoside laurate ester of both OH-chlorobactene and OH-$\gamma$-carotene (33, 82). The carotenoid species in RC are mostly the glucoside laurate esters (81). The biosynthesis pathway of chlorobactene is shown in Figure 1.5. The mutation in *crtB*, which encodes phytoene synthase, completely eliminates carotenoid biosynthesis. This mutant is viable but exhibits the most severely
impaired growth rate (30). Knock-out mutants of any of the first four genes in the
carotenoid biosynthesis show significant inhibition of growth under all light conditions
tested (32). Inhibition of carotenoid biosynthesis at any step after isomerization of poly-
cis-lycopene has little effect on growth rate, although the inhibition of the synthesis of
glycoside esters does reduce the growth rate to 90% at low light intensities (32, 58).

1.2.2 Structures of (bacterio)chlorophylls

(B)Chls are mostly cyclic Mg-tetrapyrroles with four pyrrole rings (designated A, B, C and D; clockwise, starting in the top-left corner) and a cyclopentanone ring (E) adjacent to ring C (Figure 1.7). Carbons of the tetrapyrrole are numbered from 1 to 20 clockwise beginning with the carbon next to the nitrogen in ring A (Figure 1.7). Chl \(a\) (Figure 1.7) is the main pigment of cyanobacteria with only a few exceptions. One exception is the \textit{Prochlorococcus} spp. which synthesizes divinyl-Chl \(a\) and divinyl-Chl \(b\) in addition to Chl \(a\) and \(b\) (Figure 1.6) (72). The other exception is \textit{Acaryochloris marina} which uses Chl \(d\) (Figure 1.6) as its principal light-harvesting pigment (50). BCHls \(a\), \(b\) and \(g\) are bacteriochlorins (7,8,17,18-tetraphydro-porphyrins) with reduced B and D rings. BCHl \(a\) is the most common pigment in purple bacteria and FAPs that do not produce chlorosomes and is the second most abundant pigment in GSB and green-colored FAPs (21). BCHl \(a\) has an acetyl group at the C3 position, and its C17 propionyl group is esterified with phytol (Figure 1.8). BCHl \(b\) is mostly identical to BCHl \(a\) except for an ethylidene group at C8 (Figure 1.6 E). BCHl \(g\) (Figure 1.6 D) is the major pigment in the reaction center complex of heliobacteria (62). It differs from Chl \(a\) by having a reduced B-ring and an ethylidene at C8 position. \(8^1\)-hydroxy-Chl \(a\_F\) (Figure 1.6 F) is a minor
pigment of the reaction centers in heliobacteria (62). GSB synthesize one of the three so-called *Chlorobium* chlorophylls, BChl *c*, *d*, or *e*, as antenna chlorophylls in the chlorosomes (23). BChl *c*, *d*, and *e* are chlorins (17, 18-dihydroporphyrin) rather than bacteriochlorins as their names might have suggested (Figure 1.6 G,H,F). These BChls have a hydroxyl substituent at C31 position and lack the bulky carboxymethyl group at C132 found in all other Chls and BChls (26). The hydroxyl group can chelate the magnesium of a neighboring BChl molecule as mentioned in 1.1.5. The carboxymethyl group would interfere with the hydrogen bonding network between the keto group of one BChl molecule and the C31 hydroxyl of a neighboring molecule in self-aggregation (86).

BChl *c*, *d*, and *e* differ in their C7 and C20 positions. BChl *c* is methylated at both C7 and C20 positions. BChl *d* is methylated at the C7 position. BChl *e* is methylated at C20 and formylated at the C20 position. BChls *c* and *d* are the main antenna pigments of green-colored GSB and FAPs while BChl *e* is the main antenna pigment of brown-colored GSB (35).

1.2.3 Proposed model for (B)Chl biosynthesis in GSB

It has been postulated that all Chls and BChls are synthesized from Chlide *a* with the exceptions of DV-Chl *a* and DV-Chl *b* which must be synthesized from 3,8-DV-Chlide *a* (21). The core pathway of all (B)Chls starts from protoporphyrin IX (Proto-IX), which is synthesized from eight molecules of 5-aminolevulinic acid (ALA) (Figure 1.10) (23). Proto-IX is also the precursor of heme and heme derivatives and is the last metal-free intermediate in (B)Chl biosynthesis (6). The insertion of Mg2+ into proto-IX is the first committed step in (B)Chl biosynthesis and is catalyzed by a multi-subunit Mg-
chelatase encoded by three genes, \textit{bchH}, \textit{bchD} and \textit{bchI} in photosynthetic bacteria (11). Methylation of Mg-ProtoIX (MP-IX) is then catalyzed by MP-IX methyltransferase to form Mg-protoIX-monomethyl ester (MPE) (11). The C13 propionic acid chain of MPE is oxidized and cyclized to form the isocyclic fifth ring (or E ring) of the (B)Chl molecule by the enzyme MPE oxidative cyclase. The cyclization forms 3, 8-divinyl-protochlorophyllide (DV-PChlide) whose C-8 vinyl group is reduced by 8-vinyl PChlide reductase (Figure 1.12). This reaction is further discussed in 1.2.5. The next step is the reduction of the C17/C18 double bond of the D ring of PChlide \textit{a} catalyzed by protochlorophyllide oxidoreductase (POR). Two types of POR exist: light-independent (dark-operative) POR (DPOR) and light-dependent POR. They are structurally unrelated and do not share any significant homology (11). The product of this step is Chlide \textit{a} (80).

Chlide \textit{a} can either be esterified by chlorophyll synthase to form Chl \textit{a} or be further modified to produce various other pigments (Figure 1.11) (80). Chlide \textit{a} can be converted to Chlide \textit{b} by Chlide \textit{a} oxygenase (CAO) (83). Chlide \textit{a} is reduced by Chlide \textit{a} reductase (COR) at the chlorin macrocycle to form a bacteriochlorin (17). A $C_{3}$ hydroxyl group is introduced and then oxidized to a keto group (11, 12). The resulting BChlide \textit{a} is esterified at $C_{17}^{2}$ position by BchG with a geranylgeranyl-pyrophosphate tail, which is later saturated to phytol by BchP (84). The end product is BChl \textit{a}. The steps from the hub intermediate Chlide \textit{a} to other pigments are shown in Figure 1.8.

\textbf{1.2.4 Proposed model for BChl \textit{c} biosynthesis in \textit{Chlorobium tepidum}}

\textit{C. tepidum} synthesizes three types of (B)Chl: BChl \textit{ap}, Chl \textit{apD}, and BChl \textit{cF}. 
BChl $c_F$ is the major antenna pigment of *C. tepidum*, making up about 97% of total (B)Chl content in *C. tepidum*. The BChl $c$ biosynthesis pathway in *C. tepidum* has been proposed according to the mutational and biochemical characterization of the genes identified by genomic analysis (Figure 1.10). The branching point of the biosynthesis pathway of BChl $c$ from those of Chl $a$ and BChl $a$ in *C. tepidum* was identified to be after the formation of Chlide $a$ (23). The only step yet to be defined in the synthesis of BChl $c$, $d$ and $e$ in GSB and FAPs is the removal of the C13\(^{1}\) methyl-carboxyl moiety of Chlide $a$ (23). Very recently, the knock-out mutant of *CT1077* was found not to be able to make BChl $c$ and was likely to be involved in this step (Z. Liu, personal communication).

After the demethylation and decarboxylation of Chlide $a$, two radical-SAM (S-adenosyl-$L$-methionine) oxidative methyltransferases, BchQ and BchR, add the methyl groups to the C8\(^{2}\) and C12\(^{1}\) positions of the 3-vinyl-BChlide $d$ (21, 23). The C3 chains are hydrated by the hydratases BchF and BchV (21). The C20 methyl group, which is the difference between BChl $c$ and BChl $d$, is added by BchU (23). The final step in the biosynthesis of BChl $c$ is the addition of the farnesol tail to the BChlide $c$ by BChl $c$ synthase, BchK (23).

### 1.2.5 Reduction of the C-8 vinyl group

Most BChls and Chls in bacteria have an ethyl group at the C8 position with the exception of marine *Prochlorococcus* spp which produces divinyl-Chl $a$ and divinyl-Chl $b$. The absorbance maximum of the divinyl chlorophylls in the Soret region red shifts by ~10 nm compared with monovinyl chlorophylls, enabling *Prochlorococcus* to have more efficient absorption of blue light that is enriched in deep water layers (65). GSB also
produce BChls with C8 ethyl group whose C8 carbon in the antenna BChls can be methylated to different extents (10). In 1995, a mutant of *Rhodobacter capsulatus* with an inactive copy of *bchJ* was found to accumulate and secret large amounts of DV-PChlide (72). It was therefore proposed that *bchJ* encoded the enzyme responsible for the reduction of C8 vinyl group of the DV-PChlide to an ethyl group (79). The *C. tepidum* genome contains an ortholog (*CT2014*) of *bchJ* and its gene product was presumed to perform the divinyl reductase (DVR) function (22). However, *bchJ* homologs were not detectable in higher plants and cyanobacteria that produce monovinyl (MV)-Chl a. In 2005, a gene named *AT5G18660* was found to encode the C-8 vinyl reductase in *Arabidopsis thaliana* (65). Interestingly, an ortholog of *AT5G18660*, *CT1063* was also found in the *C. tepidum* genome. To determine which of these two genes encodes DVR in *C. tepidum*, the *CT1063* and *bchJ* (*CT2014*) genes were insertionally inactivated and the resulting mutants were biochemically characterized (22). HPLC and HPLC-MS analyses revealed that the *bchJ* mutant produced detectable amounts of Chl *a*PD, BChl *a*F and BChl *c*F with reduced C8 groups (22). In contrast, all three (B)Chls synthesized by the *CT1063* mutant had C-8 vinyl groups. Whole-cell extracts from *Escherichia coli* (*E. coli*) cells expressing *CT1063* were mixed with DV-PChlide and NADPH and the absorbance of the Soret peak of the product was found to be blue-shifted around 5 nm with respect to DV-PChlide (22). These results suggested that *CT1063* (renamed as *bciA*) and not *bchJ* encodes the C-8 vinyl reductase in *C. tepidum*.

1.3 The objectives of this thesis study
The main objectives of this thesis project were to obtain purified BciA protein from *C. tepidum* after overexpression of the gene in *E. coli*, and to characterize its reductase activity. Kinetic studies were to be performed to obtain critical enzyme parameters and determine its catalytic mechanism. This information could be useful when studying other enzymes involved in the (B)Chl synthesis pathway and could facilitate the production of substrates for other (B)Chl biosynthesis enzymes.
1.4 References


Figure 1.1 Phylogeny of the major eubacterial taxa based on RecA sequences. Asterisks indicate lineages with photosynthetic members (Adapted from Maresca 2007 (57))
Figure 1.2 Phylogeny of green sulfur Bacteria. Phylogenetic tree of 16S rDNA from different strains of green sulfur bacteria (Adapted from Alexander et al. (1)).
Figure 1.3 The reductive tricarboxylic acid cycle for autotrophic CO$_2$ fixation (Adapted from Hugler et al., 2007 (43))
Figure 1.4 Structural organization of the photosystem I reaction center from *Synechococcus elongatus*, and a representation of the equivalent complex from *Chlorobium limicola* (39). (a) The electron transfer cofactors of the *S. elongatus* photosystem I complex. The cofactors are arranged around an axis of pseudo-twofold symmetry that runs from the P700 dimer to the FX iron–sulfur center. (b) The protein component of the *S. elongatus* photosystem I complex. The PsaA and PsaB polypeptides are shown in maroon and green, respectively. The PsaC subunit (cyan ribbons at the top of the structure) at the stromal surface of the complex binds the FA and FB iron sulfur centres. The binding and function of PsaC is assisted by two other small subunits (PsaD and PsaE) located on the stromal surface of the complex (orange and red ribbons, respectively, at the top of the structure). (c) Schematic of the type I reaction centre from *C. limicola*. This complex consists of a homodimer of two copies of the PscA gene (contrasted in green and maroon). The complex also has equivalents of the PsaC and PsaD subunits of the cyanobacterial photosystem I (cyan and orange ribbons, respectively, at the top of the structure).
Figure 1.5 Biosynthetic pathway of carotenoids in \textit{C. tepidum} (Adapted from Maresca \textit{et al}., 2008 (59))
Figure 1.6 Structure of bacterial chlorophylls. A) DV-Chl $a_p$, B) Chl $b_p$, C) Chl $d_p$, D) BChl $g_f$, E) BChl $b_f$, F) 81-hydroxy-Chl $a_f$, G) BChl $c_f$, H) BChl $d_f$, and I) BChl $e_f$. 
Figure 1.7 Structure of Chl $a_{\infty}$. 
Figure 1.8 Structure of BChl a.
Figure 1.9 Structure of BChl $c_t$. 

$R_e = \text{ethyl}$

$n$-propyl

iso-butyl

neo-pentyl

$R_{12} = \text{methyl}$

ethyl

farnesyl
Figure 1.10 Proposed pathway for BChl c biosynthesis in *C. tepidum* (Adapted from Gomez Maqueo Chew & Bryant, 2007 (23)).
Figure 1.11 Model for biosynthesis of bacterial (B)Chls from a central Chlide \(a\) intermediate (Adapted from Gomez Maqueo Chew & Bryant, 2007 (23)). Known and proposed pathways for the biosynthesis of Chls \(a\), \(b\), \(d\), and 8\(\alpha\)-hydroxy-Chl \(a\) and for BCHls \(a\), \(b\), \(c\), \(d\), \(e\), and \(g\) from a proposed Chlide \(a\) intermediate. Steps that remain to be elucidated are indicated with question marks. The (B)Chl synthase (ChlG, BchG) and the geranylgeranyl reductase reactions (catalyzed by ChlP or BchP) have been combined into one reaction step for convenience.
Figure 12 Reduction of DV-PChlide to MV-PChlide by a divinyl protochlorophyllide reductase (DVR) in *C. tepidum*, this reaction is catalyzed by the enzyme BciA (Adapted from Gomez Maqueo Chew & Bryant, 2007 (22)).
Chapter 2

Purification and kinetic characterization of BciA

2.1 Introduction

As described in 1.2.4, previous genetic and biochemical experiments showed that BciA acts as a DVR. In this thesis study, BciA was overproduced in *E. coli* and purified by immobilized metal affinity chromatography. The divinyl reductase properties of purified BciA were characterized kinetically.

Enzyme kinetics is the section of enzymology that includes studies of the rate of reactions catalyzed by enzymes and the factors which affect these rates. Substrate and product concentration, enzyme concentration, pH, temperature, ionic strength of the solvent, and concentration of activators and inhibitors, all affect the rate of enzyme-catalyzed reactions (6). The reaction velocities usually decrease over time. The reasons can be one or a combination of the following causes: 1. the enzymes become unstable during the course of the reaction; 2. the degree of saturation of the enzyme by substrate decreases as substrate is depleted; 3. the reverse reaction becomes more predominant as product accumulates; and 4. the products of the reaction inhibit the enzyme (22). Therefore, initial velocities are typically used as a measure of reaction rates. During the early stages of an enzyme-catalyzed reaction, the enzyme can be considered to be stable, conversion of substrate to product is small and substrate concentration can be considered to be constant and equal to the initial concentration. In the meanwhile, little product has accumulated and thus reverse reactions and product inhibition can be considered as
negligible (22). The initial velocity is operationally taken as the velocity measured before more than \( \sim 10\% \) of the substrate has been converted to the product (35).

One of the most important factors influencing reaction velocities is the concentration(s) of substrate(s) and/or product(s). When other factors are kept constant, the affects of substrate on the initial velocity can be monitored and the results give clues of possible kinetic mechanism, that is, the order by which substrate(s) add and product(s) leave the active site of the enzyme (29, 34).

An enzymatic reaction is usually modeled as a two steps: substrate (S) binding by enzyme (E) and formation of an enzyme-substrate (ES) complex, followed by irreversible breakdown of the enzyme and product (P) (22).

\[
E + S \xrightleftharpoons[k_1]{k_i} ES \xrightarrow{k_2} E + P
\]

The constant \( k_2 \) (or \( k_p \) or \( k_{\text{cat}} \)) is called the turnover number (or molecular activity or catalytic rate constant) and represents the maximum velocity per mole of enzyme (or per mole of active site if the enzyme has more than one active sites).

The first general rate equation for reactions involving enzymes was derived in 1903 by Henri (13). Ten years later, Michaelis and Menten confirmed Henri’s experimental work and derived the familiar Michaelis-Menten equation (eq. 2.1), which has been suggested by some people to be referred to as the Henri-Michaelis-Menten equation (25).

\[
v = \frac{V_{\text{max}}[S]}{K_s + [S]} \quad \text{(eq. 2.1)}
\]

\[
\frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_i} = K_s \quad \text{(eq. 2.2)}
\]
\( v = \text{initial velocity}; \ [S] = \text{substrate concentration}; \ V_{\text{max}} = \text{maximum velocity}; \ K_s = \text{the dissociation constant of the ES complex} \)

This equation was derived based on rapid equilibrium model. The model assumptions are: The substrate-binding step and formation of the ES complex are fast relative to the breakdown rate. This leads to the approximation that the substrate binding reaction is at equilibrium. 2. The substrate concentration is very much higher than the enzyme concentration ([S] \gg [E_T]), so that the formation of an ES complex does not alter substrate concentration. 3. The conversion of product back to substrate is negligible (22, 29).

In 1925, Briggs and Haldane provided a steady-state model which can obtain the rate equation for an enzyme-catalyzed reaction without the restriction of rapid equilibrium condition (1). The main assumption of the steady-state model is that the concentration of enzyme-substrate complex remains constant. Therefore, the differential equation that describes changes in the concentration of the ES complex in time equals zero (22):

\[
\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \quad (\text{eq. 2.3})
\]

Eq. 2.3 can be rearranged to eq 2.4:

\[
\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \quad (\text{eq. 2.4})
\]

The rate-limiting step of an enzyme-catalyzed reaction is the breakdown of the ES complex. The velocity of an enzyme-catalyzed reaction can thus be expressed as

\[
v = k_2[ES] = \frac{k_2[E][S]}{K_m} \quad (\text{eq. 2.5})
\]
After normalization of equation 2.5 with total enzyme concentration \([E_T] = [E+ES]\) and substituting \(V_{max}\) for \(k_2 [E_T]\), rearrangement of 2.5 can yield the familiar expression for the velocity of an enzyme-catalyzed reaction:

\[
v = \frac{V_{max} [S]}{K_m + [S]} \quad \text{(eq. 2.6)}
\]

Equation 2.6 is similar to the equation 2.1, except for \(K_s\) is replaced with \(K_m\). In most cases, substrate binding occurs much faster than the breakdown of the ES complex, that is, \(k_2\) is very small compared to \(k_1\), \(K_m \approx k_{-1}/k_1 = K_s\). Then the two models are equivalent (22).

\(K_m\) is known as the Michaelis constant. The numerical value of \(K_m\) is equivalent to the substrate concentration that yields half-maximal velocity as can be seen from equation 2.6. The numerical value of \(K_m\) is of interest for several reasons as explained by Segel (29). Firstly, the \(K_m\) represents an approximate value for the intracellular level of the substrate. The catalytic potential would be underutilized if \([S]_{\text{intracell}} \ll K_m\). It also makes no physiological sense to maintain a substrate concentration much higher than \(K_m\) since \(v\) cannot go beyond \(V_{max}\) and \(v\) becomes insensitive to small changes in \([S]\) at \([S] \gg K_m\). Secondly, as \(K_m\) is a constant for a particular enzyme, its numerical value can be used to compare enzymes from different organisms, or from different tissues of the same organism, or from the same tissue at different stages of development. In this way, one may determine whether enzyme A and enzyme B are identical or if they are different proteins that have the same function. Thirdly, activators and inhibitors for an enzyme can be identified by monitoring their effects on the apparent \(K_m\). The best substrate can also be determined because \(K_m\) is equal to \(K_s\) which is the dissociation constant of the ES
complex when \( k_2 \) is much smaller than \( k_1 \). \( K_m \) is therefore also a measure of the affinity of the enzyme for its substrate (35).

The equation 2.6 can be converted to a double reciprocal form which leads to equation 2.7. By measuring the initial velocities at various substrate concentrations, the values of \( K_m \) and \( V_{\text{max}} \) can be obtained.

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} \left( \frac{K_m}{[S]} \right) + \frac{1}{V_{\text{max}}} \tag{eq. 2.7}
\]

In the nomenclature system introduced by Cleland, the number of reactants and products are specified by the terms Uni (one), Bi (two), Ter (three), and Quad (four) (35). Up to this point, only a unireactant model has been considered in which only one substrate is involved. In enzyme kinetics, a cofactor is usually considered as a substrate and therefore the reaction catalyzed by DVR is considered as a bisubstrate reaction since both DV-PChlide and a pyridine nucleotide are substrates in a kinetic sense (34). Reactions that require two substrates and yield two products are named Bi-Bi reactions. Bi-Bi reactions can be classified as Ping-Pong or sequential. In Ping-Pong reactions, one or more products are released before all substrates have been added. In sequential reactions, all substrates must bind to the enzyme before a reaction can occur and products can be released. Sequential mechanisms can be further differentiated as ordered or random. In ordered sequential mechanisms, substrates react with enzyme, and products are then released in a compulsory order; in random sequential mechanisms, the substrate addition and product release do not have an order (35).

The velocity equations for the above-mentioned bisubstrate mechanisms in the absence of products are given below in double-reciprocal forms (21):

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} \left( \frac{K_m}{[S]} \right) + \frac{1}{V_{\text{max}}} \tag{eq. 2.7}
\]
a. Ordered sequential Bi-Bi

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K^A_M}{V_{\text{max}}[A]} + \frac{K^B_M}{V_{\text{max}}[B]} + \frac{K^A_S K^B_M}{V_{\text{max}}[A][B]} \quad \text{(eq. 2.8)}
\]

b. Random sequential Bi-Bi

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K^A_S K^B_M}{V_{\text{max}}[A]} + \frac{K^B_M}{V_{\text{max}}[B]} + \frac{K^A_M K^B_S}{V_{\text{max}}[A][B]} \quad \text{(eq. 2.9)}
\]

c. Ping-Pong Bi-Bi

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K^A_M}{V_{\text{max}}[A]} + \frac{K^B_M}{V_{\text{max}}[B]} \quad \text{(eq. 2.10)}
\]

\(v\) = initial velocity; \([A]\) and \([B]\) = substrate concentrations; \(K^A_M\) and \(K^B_M\) = respective concentrations of \(A\) and \(B\) needed to achieve 1/2 \(V_{\text{max}}\) in the presence of a saturating concentration of the other; \(K^A_S\) and \(K^B_S\) = dissociation constant of \(A\) and \(B\) from the enzyme, respectively

Ping-Pong and sequential mechanisms can be differentiated using their distinctive properties in double reciprocal plots (Lineweaver-Burk plot). From equation 2.10 representing the Ping-Pong mechanism, it can be seen that a plot of \(1/v\) versus \(1/[A]\) at constant \([B]\) should yield straight lines with the slope \(K^A_M/V_{\text{max}}\) and intercepts on the \(1/v\) axis equal to \(1/V_{\text{max}} + K^B_M/(V_{\text{max}}[B])\). Since the slope is independent of \([B]\), such plots for different levels of \([B]\) should yield a family of parallel lines. It is also the same case when \([B]\) is varied and \([A]\) is kept constant. Therefore, a Ping-Pong mechanism is characterized by a set of parallel lines in the double reciprocal plots (5). The rate equations of both random and ordered sequential mechanisms (equations 2.8 and 2.9) can be rearranged to show that the reciprocal plot of \(1/v\) versus \(1/[A]\) yields straight lines with slopes inversely proportional to \([B]\). Therefore, the diagnostic feature of the
reciprocal plots indicating a sequential mechanism is that lines of different levels of \([B]\) intersect to the left of the \(1/v\) axis (35).

Product inhibition patterns further distinguish the ordered sequential from the random sequential mechanism. It also gives information of the order of substrate addition and product release (29). Product inhibition is a kind of enzyme inhibition which can be competitive, uncompetitive, or linear mixed type. Competitive inhibitors bind reversibly to the free enzyme in a manner similar to the substrate but the EI (the enzyme-inhibitor complex) is catalytically inactive. The rate equation for competitive inhibition is shown in equation 2.11 (22). The result is an increase in the apparent \(K_m\) value but the \(V_{max}\) is not affected. The characteristic feature of the double-reciprocal plot for a competitive inhibitor is that lines of various \([I]\) intersect at \(1/V_{max}\) on the \(1/v\) axis (21).

\[
v = \frac{V_{max} [A]}{K_m (1 + [I]/K_i) + [A]}
\]

(eq. 2.11)

where \(K_i = \frac{[ES][I]}{[ESI]}\)

In uncompetitive inhibition, the inhibitor interacts with the enzyme-substrate complex at a site other than the active site (22). The rate equation for uncompetitive inhibition is shown as equation 2.12 (35):

\[
\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{\alpha}{V_{max}}
\]

(eq. 2.12)

where \(\alpha = 1 + \frac{[I]}{K_i} K_i = \frac{[ES][I]}{[ESI]}\)
Equation 2.12 shows that the a series of double reciprocal plots at various uncompetitive inhibitor concentrations consists of parallel lines with slope $K_m/V_{\text{max}}$ and with $1/v$ and $1/[S]$ intercepts of $\alpha/V_{\text{max}}$ and $-\alpha/K_m$, respectively (35).

In mixed inhibition, a compound can interact with both the free enzyme and the enzyme-substrate complex. The Lineweaver-Burk equation for mixed inhibition is shown as equation 2.13 (29).

$$\frac{1}{v} = \left(\frac{\alpha K_m}{V_{\text{max}}} \right) \frac{1}{[S]} + \frac{\alpha'}{V_{\text{max}}} \tag{eq. 2.13}$$

where $\alpha = 1 + \frac{[I]}{K_i}$ $\alpha' = 1 + \frac{[I]}{K_i'}$ $K_i = \frac{[E][I]}{[EI]}$ $K_i' = \frac{[ES][I]}{[ESI]}$

It can be seen from equation 2.13 that the plot of this equation consists of a family of lines intersect to the left of the $1/v$ axis. For a special case when $k_i = k_i'$, the intersection is on the $1/[S]$ axis. This situation is sometimes called noncompetitive inhibition (22).

Graphical analysis is a quick and useful way to visualize enzyme kinetic data, but for precise estimate of kinetic constants, computerized statistical analysis is essential (21). Most linear-transformations have inherent statistical bias. For example, in the most widely used double reciprocal plots (Lineweaver-Burk plot), the weight of experimental values obtained at low concentrations of substrate is overemphasized, and those are the values which normally have the largest experimental error (21). Computerized statistical analysis can also discriminate different models by non-linear fitting of the initial velocity data to different model equations iteratively, and the best-fitting equation is picked according to certain statistical criteria such as R square, F value, Akaike’s AIC (an
information criterion), etc. The best-fitting model has the highest R square and F values and the lowest Akaike’s AIC value.

In Cleland’s nomenclature, substrates are designated by the letters A, B, C in the order which they add to the enzyme, and products by the letters P, Q, and R in order in which they leave the enzyme (21). In the Bi-Bi reaction, product inhibition studies can enable one to tell A from B and P from Q (29). Basically, the inhibition types of each product against each of the substrates are determined. By comparison with the theoretical patterns, it is possible to determine the reaction mechanism and the order of the substrate binding and product release (29).

2.2 Materials and Methods

2.2.1 Cloning of the \( bciA \) gene and construction of the expression plasmid

The \( bciA \) gene and flanking regions were cloned into pET-28a (Novagen, Madison, MI) by former labmate Dr. Aline Gomez Maqueo Chew as described (4). This plasmid was named pET28-BciA. However, BciA expressed in either BL21(DE3) or NovaBlue (Novagen) strain of \( E. \) coli using this plasmid was not very soluble (data not shown). Therefore, the \( bciA \) gene was recloned into the pET-42b expression vector (Novagen). The \( bciA \) gene was amplified by PCR from pET28-BciA plasmid provided by Dr. Gomez Maqueo Chew using primers:

5’ GCCCGCGCGCCAGCCATATGTCATCTC 3’ (forward)
5’ AGAGGCTTTATGCCCTCGAGGAACATCGCG 3’ (reverse)

The forward DNA primer generated an \( NdeI \) site (underlined), whereas the reverse primer generated an \( XhoI \) site (underlined). The resulting PCR product was
subcloned into plasmid pCR2.1-TOPO using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, Catalog # K4500-40) to produce plasmid pTA-BciA. The plasmid pTA-BciA and pET42b were then both digested with the enzymes NdeI and XhoI. The products of these reactions were separated by agarose gel electrophoresis, and fragments corresponding to linearized pET42b and \( bciA \) were excised from the gel, purified with QIAquick gel extraction kit (QIAGEN, Hilden, Germany, Catalog # 28704) and ligated together to produce plasmid pET42-BciA. The \( bciA \) gene sequence in this clone was verified by DNA sequencing. In this plasmid construct, the polyhistidine tag was moved from the N-terminus to the C-terminus of the polypeptide.

### 2.2.2 Overproduction of BciA in \( E. coli \)

The expression plasmid containing the \( bciA \) gene was introduced into \( E. coli \) NovaBlue (Novagen) cells by electroporation. The colonies that grew on a kanamycin-containing (kanamycin concentration, 30 µg/ml) LB (1 L LB medium contains 10 g NaCl, 10 g tryptone and 5 g yeast extract) growth medium were selected, and the presence of the \( bciA \) gene was verified by plasmid isolation and PCR amplification of the insert. For a large scale overproduction of BciA, single colonies were inoculated into 100 ml of sterilized LB medium containing kanamycin as overnight preculture. The preculture was inoculated into 6 L sterilized LB growth medium containing kanamycin. The cells were grown at 37 °C until \( \text{OD}_{600 \text{ nm}} \) reached ~ 0.6. The expression of \( bciA \) gene was then induced with IPTG at a final concentration of 0.4 mM. The cells were incubated for an
additional 8 h period at 28 °C and were harvested by centrifugation. The pellets were stored at -20 °C until required.

2.2.3 Purification of BciA from *E. coli*

The pellets were thawed and resuspended in buffer (50 mM sodium phosphate buffer, pH 7.5), and the cells were disrupted by three passes through a chilled French pressure cell at 138 MPa. The lysate was clarified by centrifugation at 4,300 x g for 15 min in a Sorvall SS 34 rotor (Sorvall, Milford, MA). The supernatant containing soluble BciA was collected and purified using a column loaded with TALON™ metal affinity resin (Clontech, Mountain View, CA). The TALON™ metal affinity resin was equilibrated with 10 bed volumes of equilibration buffer (50 mM sodium phosphate buffer, pH 8.0, and 300 mM NaCl). The supernatant was allowed to pass through the column by gravity flow. The column was washed with 5 bed volumes of wash buffer (equilibration buffer plus 30 mM imidazole). BciA was eluted from the column with 3 bed volumes of elution buffer (equilibration buffer plus 250 mM imidazole). Samples were prepared from each fraction to be analyzed by SDS-PAGE.

The elution fractions were desalted and concentrated using Amicon centrifugal filter devices with YM-50 MW (50,000 nominal molecular weight limit) membranes (Millipore, Billerica, MA). Some smaller molecules were also removed in this process. The protein solution was changed to different buffers when necessary using Spectra/Por dialysis membrane tubing (Thomas Scientific, Swedesboro, NJ).
Protein concentrations were determined by assuming molar absorptivities ($\varepsilon_{280}$) of 25,900 M$^{-1}$cm$^{-1}$, as calculated by the method of Gill and von Hippel (11).

2.2.4 Polyacrylamide gel electrophoresis

The expression level in *E. coli* and purification of His$_6$-BciA were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) according to the method of Laemmli (20). Samples were mixed with loading buffer (0.25 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and trace amount of bromophenol blue) and boiled for 1 min before being loaded on an SDS-PAGE gel. The proteins were separated by electrophoresis through a 12% (w/v) polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R250 (Pierce Biotechnology, Rockford, IL).

Non-denaturing polyacrylamide gel electrophoresis was performed using the method modified from the blue native electrophoresis developed by Schägger and von Jagas (26). It is similar to the SDS-PAGE except that SDS is removed from all buffers and Coomassie brilliant blue is added to the running buffer to induce a charge shift on the proteins. A gradient separation gel was made from 6% and 13% polyacrylamide stock solutions (26).

2.2.5 Peptide mass fingerprinting

Peptide mass fingerprinting (PMF) was carried out to confirm the identity of the heterologously produced protein at the Proteomics and Mass Spectrometry Core Facility of The Pennsylvania State University, University Park. A protein band corresponding to
the expected size of BciA was excised from a Coomassie blue-stained SDS-PAGE gel and was cut into small pieces with a sterile scalpel blade. The gel pieces were destained with 50 mM ammonium bicarbonate and 50% (v/v) acetonitrile for 10 min with sonication in a water bath and the supernatant was removed and discarded. This step was repeated twice before the gel pieces were dehydrated in acetonitrile for 5 min. The acetonitrile was removed and the gel pieces were allowed to dry completely in air (30 min). They were then rehydrated, reduced, alkylated and digested by trypsin. An aliquot (1 µl) of the digest was mixed with 1 µl MALDI matrix solution (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% (v/v) ethanol and 50% (v/v) acetonitrile) and dried onto a MALDI target plate. The dried target was transferred to a mass spectrometer (M@LDI L/R system, Waters, Milford, Massachusetts) and was analyzed by MALDI-TOF MS in reflectron mode using standard operating parameters. Briefly, the instrument used a N₂ laser at 337 nm, the source voltage was set at 15,000 V, the microchannel plate detector voltage was set at 1950 V, the pulse voltage was set at 2450 V, the reflectron voltage was set at 2000 V and the coarse laser energy was set to low with the fine adjustment used to optimize the signal. At least 100 laser shots were accumulated and combined to produce a raw spectrum over the m/z range 800-3500. The set of monoisotopic peptide masses was used to search the National Center for Biotechnology Information non-redundant (NCBI) databases using the Mascot search engine (Matrix Science).

2.2.6 Purification of divinyl and monovinyl protochlorophyllide

2.2.6.1 Culture of bchJ mutant of Chlorobium tepidum
The DV- and MV-PChlide were extracted from spent medium of \textit{bchJ} mutant. The \textit{bchJ} mutant was provided as a frozen stock by Dr. Aline Gomez Maqueo Chew. The procedure to make the mutant was described (3, 4). The frozen cells were thawed, spun down to remove glycerol as well as old media and added to fresh media. Liquid media for \textit{C. tepidum} were prepared as described (9). Cell cultures were grown at 42 °C in a Coy anaerobic chamber (Grass lake, MI) with an atmosphere composed of 10% CO\textsubscript{2} and 10% H\textsubscript{2} balanced with N\textsubscript{2} for 48 to 36 h. Cells were centrifuged in a Sorvall SLC-4000 rotor (Sorvall, Milford, MA) at 10,000 \( \times \) g for 20 min. The supernatant was then extracted with 1/10 volume of ethyl ether. The yellowish green ether phase was collected, placed in 5 ml glass vials and dried under nitrogen gas. The dried pigments were stored at -20 °C until needed.

2.2.6.2 HPLC purification of DV- and MV-PChlide from the extract of \textit{bchJ} mutant spent medium

The DV- and MV-PChlide were purified using reverse phase high performance liquid chromatography (HPLC) (Agilent Model 1100 equipped with a diode array detector [model G1315B] and controlled with Agilent ChemStation software; Agilent Technologies, Palo Alto, CA) on a 25 cm x 4.6 mm ProntoSIL 5 μm C30 column (Bischoff Chromatography, Leonberg, Germany). The solvent system used was isocratic and was modified from the described method of Kruk \textit{et al.} (19). Solvent A (95% methanol, 5% hexane, 10 mM ammonium acetate) was used to equilibrate the column 10 min prior to injection. The dried pigments were dissolved in solvent A and filtered over
0.2 µm polytetrafluoroethylene filter (Whatman, Florham Park, NJ) immediately before injection. Solvent A was allowed to run another 40 min. Finally solvent B (50% methanol, 20% acetonitrile, 30% acetate) was used to run 15 min to clean the column. Pigments were identified based on their retention time, absorption spectra and mass. The DV-and MV-PChlide segments were collected in 10 ml glass vials and dried under nitrogen gas. The dried pigments were stored at -20 °C until needed.

2.2.7 UV-Vis spectroscopy

In-line spectra were recorded with a diode-array detector during HPLC runs. Absorption spectra were collected from 200 to 900 nm with a step of 1 nm. 37.5 spectra were collected per minute for the duration of the run. In-line spectra of compounds were extracted from the data at a time point corresponding to the peak maximum of the compound in the chromatogram.

Absorbance of NADPH and pigments was measured using a modified Cary-14 spectrophotometer (On-Line Instrument Systems, Bogart, GA) and/or a Genesys 10 UV scanning spectrophotometer (ThermoSpectronic, Rochester, NY).

2.2.8 BciA divinyl reductase activity assay

The concentrations of the substrates were determined using the following extinction coefficients in aqueous solution: NADPH, 6.22 mM⁻¹cm⁻¹ at 340 nm; PChlide, 23.95 mM⁻¹cm⁻¹ at 630 nm (2, 18). Protein concentration was measured as described in 2.2.3. A 1-ml assay mixture included 0.05 µM BciA in assay buffer (20 mM MOPS, pH 7.2), 0.2 mM NADPH, 30 µM DV-PChlide. The reaction was allowed to proceed at room
temperature for 15 min. Acetone (500 µl) was added to terminate the reaction and the mixture was centrifuged at 4 °C to pellet the precipitated protein. The pigments were extracted from the supernatant and analyzed by HPLC using the same isocratic method as described in 2.2.6.2.

2.2.9 Kinetic characterization of BciA as a DVR

BciA activity was also assayed spectrophotometrically by continuously monitoring the absorbance of NADPH at 340 nm. The concentrations of NADPH and DV-PChlide were calculated using extinction coefficients as described in 2.2.5. For the temperature studies, bovine serum albumin (10 µg/ml final concentration) was added to the assay buffer. For the pH studies, the MOPS buffer was replaced by a Tris/acetate/MES buffer (100 mM Tris, 50 mM glacial acetic acid, and 50 mM MES), and the pH was adjusted with HCl or NaOH. The ionic strength of this buffer was calculated to be 0.1 at all pH values (8). Initial velocities patterns were obtained by measuring initial velocities at optimal conditions found in the temperature and pH studies. A grid of concentrations of the two substrates was used, in which the pattern can be plotted with either substrate as the variable. The NADPH concentrations measured were: 10 µM, 20 µM, 40 µM, 160 µM. At each NADPH level, the DV-PChlide concentrations measured were 0.4 µM, 0.8 µM, 1.2 µM, 3 µM, 6 µM. Three replicates were made at each concentration pair. 0.025 µM BciA was added to the assay mixture to initiate the reaction and the assay was allowed to proceed for 3 min. The data were fit non-linearly to the two equations corresponding to the sequential and Ping-Pong mechanisms using
SPSS (SPSS, Inc.). The two equations (equations 2.14-2.15) that were used are not reciprocally transformed.

\[
\text{Sequential Bi Bi} \quad v = \frac{V_{\text{max}}[A][B]}{[A][B] + K_{M}^{A}[A] + K_{M}^{B}[B] + K_{S}^{A}K_{M}^{B}} \quad (\text{eq. 2.14})
\]

\[
\text{Ping Pong Bi Bi} \quad v = \frac{V_{\text{max}}[A][B]}{[A][B] + K_{M}^{A}[A] + K_{M}^{B}[B]} \quad (\text{eq. 2.15})
\]

Product inhibition studies were carried out to determine further the order of substrate binding (12). Assays were performed with varying concentration of one substrate at fixed non-saturating concentrations of the second substrate. Different levels of both products were used as shown in Figure 2.11 and 2.12. Inhibition types were determined by plotting and model fitting using Enzyme Kinetics Module of SigmaPlot (Systat Inc).

2.3 Results

2.3.1 Overproduction and Purification of BciA

The recombinant BciA protein, with a C-terminal His-tag, has a calculated molecular mass of 39 kDa. The recombinant BciA protein was found in both the soluble fraction and inclusion bodies as determined by SDS-PAGE. The soluble fraction was about half of the total BciA protein produced (data not shown). As purified by TALON™ metal affinity chromatography, BciA was estimated to be greater than 95% pure (Figure 2.1). Approximately 20 mg of purified protein was routinely obtained from 1 L of \textit{E. coli} culture.
Peptide mass fingerprinting of the overproduced BciA excised from a SDS-PAGE gel slice matched the CT1063 protein with a high score (202) and high coverage (70%) (Figure 2.2). This confirmed the identity of the overproduced protein as the expression product the gene bciA (CT1063).

Non-denaturing PAGE was performed to test the oligomeric state of BciA. It can be seen from the Figure 2.3 that the BciA mostly exists as monomers, and that about 10% of the protein exists as dimers. A small amount of higher aggregation products was also observed.

2.3.2 Purification of divinyl-and monovinyl-protochlorophyllide

DV-and MV-PChlide were well separated as shown in Figure 2.4. The MV-PChlide content was estimated from the chromatogram to be about 10% of the DV-PChlide amount in the extract of spent medium of bchJ mutants. Absorption spectra of DV-and MV-PChlide were extracted from in-line data of HPLC and plotted as shown in Figure 2.5. As has been reported, MV-PChlide has a blue shift in the Soret band compared with DV-PChlide (33).

2.3.3 Temperature dependence of the specific activity of BciA

The specific activity of BciA was assayed over a range of temperatures. As shown in Figure 2.6, the maximal enzyme activity was achieved between 40-42 ºC which is consistent with the natural growth conditions of C. tepidum (C. tepidum grows optimally at 48 ºC). It suggests that the temperature dependence of the enzyme activity reflects the prevailing conditions of the living environment of this organism as in the case of another
thermophilic enzyme, protochlorophyllide oxidoreductase (POR) found in the *Thermosynechococcus elongatus* (24).

2.3.4 pH dependence of the specific activity of BciA

The effect of pH on the specific activity of BciA is illustrated in Figure 2.7. Maximal activity was achieved at about pH 7.4. At lower pH values, the rate decreased much more rapidly than at higher pH values.

2.3.5 DVR activity of purified BciA confirmed by HPLC

Purified BciA was found to convert DV-PChlide to MV-PChlide utilizing NADPH as the reductant. NADH was not able to substitute for NADPH, as shown in Figure 2.6. The HPLC chromatogram showed the existence of a MV-PChlide peak after DV-PChlide was incubated with assay mixture containing NADPH and BciA. The peak of MV-PChlide was absent in the assays from which BciA or NADPH was omitted. In the assay mixtures in which NADPH was replaced by NADH, the MV-PChlide peak was also absent.

2.3.6 Initial velocity patterns

Initial velocities were measured at each pair of substrate concentration and the results were analyzed graphically to allow for a preliminary evaluation. Double reciprocal plots produced by varying the concentration of PChlide at fixed concentrations of NADPH showed a series of lines which converged. When fixed, non-saturating levels of DV-PChlide were used and the concentration of NADPH was varied, a converging
pattern of lines was also formed. These patterns are indicative of a sequential (ternary complex) mechanism in which both substrates must bind to the enzyme before any product can be released. In contrast, a ping-pong mechanism is typically characterized by a parallel line pattern as discussed in 2.1.

Model fitting using the entire dataset also confirmed that that the sequential model is a better fit to the data because the fitting using the sequential model has a higher R square value and F value. The results of data fitting of the two models are shown in Table 2.1. Data fitting using the sequential model provided a $K_m$ value for DV-PChlide, as estimated by the non-linear equation fitting, that was 1.7 ± 0.5 µM. The $K_m$ value of NADPH was calculated to be 20.1 ± 7.8 µM. The $V_m$ value was determined to be 1.1 ± 0.1 nmol/min (Table 2.1). $k_{cat}$ (assuming one active site) was calculated to be about 0.7 s⁻¹.

2.3.8 Product inhibition analysis

To distinguish between the random and ordered sequential mechanisms, initial rate studies were also carried out in the presence of products, NADP⁺ and MV-PChlide, which were used as reaction inhibitors. The types of inhibition were determined graphically and by fitting the data to various models using the Enzyme Kinetics module of SigmaPlot (SPSS Inc.). Overall, inhibition types obtained from statistical analyses were able to match those indicated by plotting. When the concentration of NADPH was varied in the presence of NADP⁺, lines with various inhibitor concentrations intersect at $1/v$ axis (Figure 11 A). When the concentration of NADPH was varied in the presence of MV-PChlide and DV-PChlide was varied in the presence of NADP⁺ and MV-PChlide,
lines intersect to the left of the $1/v$ axis. Therefore, NADP$^+$ was indicated to be a competitive inhibitor of NADPH ($K_i = 51.7$) and was a mixed-type inhibitor of DV-PChlide ($K_i = 254.164$, alpha = 0.5611). MV-PChlide was found to be a mixed-type inhibitor of both NADPH ($K_i = 12.6$, alpha = 0.9889) and DV-PChlide ($K_i = 14.2$, alpha = 1.1). The results of fitting and a comparison with alternative models are shown in Table 2.2. Product inhibition studies indicated that the mechanism may be an ordered sequential mechanism with NADPH binding first and NADP$^+$ leaving last (12, 29). Alternatively, the product inhibition pattern of a random sequential Bi-Bi mechanism investigated with one substrate varied at the constant concentration of the other substrate should have at least two competitive inhibitions (P vs A, Q vs B) (21).

### 2.4 Summary and discussion

In this thesis project, BciA protein was purified and DVR assay was performed using purified protein. It was confirmed that purified BciA was able to convert DV-PChlide into MV-PChlide with the cofactor NADPH serving as the reductant.

Kinetic studies using purified BciA showed that the kinetic parameters of BciA were comparable to those of other enzymes in the (B)Chl biosynthesis pathway. POR catalyzes the key regulatory step in the Chlorophyll biosynthesis pathway, the reduction of the C17-C18 double bond of protochlorophyllide as discussed in 1.2.3. The light-driven POR from *Thermoysnechococcus elongatus* overproduced in *E. coli* has $K_m$ values for NADPH and PChlide (a mixture of MV- and DV-PChlides) of 0.013 and 1.8 µM, respectively (24). A more recent study from the same group studied the kinetic properties of the light-dependent POR using purified monovinyl and divinyl substrates. The $K_m$
value for MV-PChlide was calculated to be 1.36±0.34 µM and the \( K_m \) for DV-PChlide was 0.92±0.33 µM (14). POR from pea (\textit{Pisum sativum} L.) overproduced in \textit{E. coli} was reported to have \( K_m \) values for NADPH and protochlorophyllide of 8.7±0.34 µM and 0.27±0.04 µM, respectively (23). The magnesium protoporphyrin IX methyltransferase (ChlM) catalyses the second step in the (B)Chl biosynthesis, converting MP-IX to MPE utilizing S-adenosyl-l-methionine (SAM). Kinetic studies of ChlM from \textit{Synechocystis} sp. PCC 6803 overproduced in \textit{E. coli} was carried out using the water-soluble analogue magnesium deutero-porphyrin IX (MgD). The \( K_m \) for MgD was 2.37 µM and that for SAM was 38 µM (32). Another enzyme in the (B)Chl synthesis pathway which has been kinetically characterized is magnesium chelatase which catalyzes the first committed step in the (B)Chl biosynthesis. Magnesium chelatase is composed of three subunits, BchI, BchD and BchH. The most recent data from \textit{Rhodobacter sphaeroides} was with BchD purified free of contamination with the chaperone GroEL, which could make the ATP utilization measurement unreliable (10). The \( K_m \) value for protoporphyrin was 0.15µM, for Mg\(^{2+}\) was 3.3 mM, and for ATP was 0.12 mM. The \( K_m \) values for Mg\(^{2+}\) and ATP for the \textit{Synechocystis} sp. PCC 6803 enzyme were 4.9 mM and 0.49 mM, respectively. Representative kinetic values of these enzymes are summarized and compared with those of BciA in Table 2.3. Overall, the \( K_m \) values for the tetrapyrroles are very low among all the enzymes studied. This is consistent with the fact that the cellular concentrations of tetrapyrroles are most likely to be very low because of the extreme toxicity of these metabolites.

The measured \( k_{cat} \) value of BciA (0.7 s\(^{-1}\)) was low, which was also the case for other enzymes in (B)Chl biosynthesis pathways. POR from \textit{Synechocystis} sp. PCC 6903
and *Thermosynechococcus elongatus* has $k_{\text{cat}}$ values of $\sim 0.175 \text{ s}^{-1}$ (calculated from the reported $V_m$ and total enzyme concentration) (15, 24). The $k_{\text{cat}}$ for ChlM was 3.5 min$^{-1}$ (32). Transient kinetics of ChlM found that the formation and decay of the pre-steady state intermediate are much faster than $k_{\text{cat}}$, and it was proposed that the rate-limiting reaction may be the product-release steps (31). The low $k_{\text{cat}}$ values may also be due to improper folding of these enzymes when overproduced in *E. coli* or the absence of a partner protein in the assay mixture.

NADH was not able to substitute for NADPH for BciA. NADH and NADPH are nearly analogous in their redox chemistry, as the redox-active group is the nicotinamide moiety, whereas the adenosine moiety is the one that carries the extra phosphate in NADPH. However, specific charge interactions between several amino acids residues and the 2’-phosphate of NADPH may be responsible for the coenzyme specificity among these enzymes (7, 30). NAD$^+$ is used almost exclusively in oxidative degradations producing ATP, whereas NADPH is confined, with few exceptions, to the reactions of reductive biosynthesis (28). Changes in amino sequence of an enzyme can lead to changes in the cofactor specificity of the enzyme for NADPH or NADH (7, 17, 27, 28).

Steady-state kinetic analyses provided evidence of a ternary complex mechanism for the BciA reaction, as indicated by the clear intersecting line pattern in the initial rate studies. Product inhibition studies indicated the binding sequence of the reactants is that NADPH binds first and NADP$^+$ is released at last. A scheme of the reaction is shown in Figure 2.13. Before this thesis study, among all the enzymes involved in the (B)Chl biosynthesis pathway, only the reaction mechanism of ChlM had been reported. ChlM was shown to react via a random sequential mechanism (32). Transient kinetics of the
same reaction was performed more recently by the same group. It was concluded that a rapid binding step is preceded by a slower isomerization of the enzyme and pre-steady state turnover numbers were provided (31).

The reaction mechanism analyzed in this thesis study could be supported by further evidence from the use of dead-end inhibitors and isotope exchange experiments. Rapid reaction techniques such as stopped-flow and quenched-flow could provide microscopic rate constants. One could then compute the theoretical steady-state kinetic constants ($K_m$ and $k_{cat}$) and compare the values calculated with the measured values in this thesis (16).

2.5 References


Figure 2.1 Overexpression and purification of recombinant BciA. Protein samples from various stages of purification were separated by SDS-PAGE (12% (w/v) acrylamide) and stained with Coomassie Blue. Lane 1: whole-cell extract; Lane 2: supernatant after removal of cell debris; Lane 3: unbound fraction; Lane 4: elution fraction of BciA protein; Lane 5: marker proteins (size in kDa is shown)
Figure 2.2 Peptide mass fingerprinting identified BciA as the expression product of the gene CT1063
Figure 2.3 Non-denaturing polyacrylamide gel electrophoresis. Lane 1: carbonic anhydrase monomer MW 29,000; dimer MW 58,000. Lane 2: purified BciA. Lane 3: Albumin MW 66,000. MW standards were purchased from Sigma (St. Louis, MO)
Figure 2.4 HPLC separation of DV-PChlide and MV-PChlide from bchJ spent medium. The peaks of DV- and MV-PChlide are indicated by the arrow.
Figure 2.5 The absorption spectrum of DV-and MV-PChlide (drawn from the in-line recording of respective peaks of HPLC chromatogram)
Figure 2.6 BciA activity confirmed by HPLC. A: Assay mixture with NADPH and BciA. B: Assay mixture without BciA. C: Assay mixture without NADPH. D: Assay mixture with NADPH replaced by NADH.
Figure 2.7 Temperature dependence of the specific activity of BciA at pH 7.2
Figure 2.8 pH dependence of the specific activity of BciA at 42 °C.
Figure 2.9  Initial velocity patterns: 1/velocity versus 1/NADPH] at different non-saturating concentrations of DV-PChlide. Concentrations of DV-PChlide used were: 0.4 (◆), 0.8 (█), 1.2 (▲), 3(*) µM. Assays were performed in triplicate and the average of three measurements of initial velocities was used.
Figure 2.10 Initial velocity patterns: 1/velocity versus 1/[DV-PChlide]. Concentrations of NADPH used were: 10(◆), 20(█), 40(▲), 160(*) µM. Assays were performed in triplicate and the average of the three measurements of initial velocities was used.
Figure 2.11 Initial velocity patterns in the presence of the product NADP⁺. The NADP⁺ concentrations used were 0 (*), 30 (▲), 90 (■), 120 (◆) µM. A: [DV-PChlide] was held constant at 3 µM with [NADPH] varied. B: [NADPH] was held constant at 40 µM and [DV-PChlide] was varied.
Figure 2.12 Initial velocity patterns in the presence of the product MV-PChlide. The MV-PChlide concentrations used were 0 (X), 6 (▲), 12 (█) µM. A: [DV-PChlide] was held constant at 3 µM with [NADPH] varied. B: [NADPH] was held constant at 40 µM and [DV-PChlide] was varied.
Figure 2.13 The proposed reaction scheme for BciA which depicts an ordered sequential mechanism. A: NADPH, B: DV-PChlide P: MV-PChlide Q: NADP⁺
Table 2.1: Initial rate model fitting using the program SPSS 14 (SPSS Inc.). The data were fit to the indicated enzymatic mechanisms and the values of the critical parameters are shown.

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<th>Mechanism</th>
<th>$K^A_M$ μM</th>
<th>$K^A_S$ μM</th>
<th>$K^B_M$ μM</th>
<th>$V_{max}^i$ (nmol/min⁻¹)</th>
<th>$R^2$</th>
<th>F value</th>
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<td>Ping-Pong</td>
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<td>1.4 ± 0.2</td>
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</table>
Table 2.2 Product inhibition studies. A: Inhibition of BciA by NADP⁺ with DV-PChlide constant at 3 µM. B: Inhibition of BciA by NADP⁺ with NADPH constant at 40 µM. C: A: Inhibition of BciA by MV-PChlide with DV-PChlide constant at 3 µM. B: Inhibition of BciA by MV-PChlide with NADPH constant at 40 µM.
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<th>POR</th>
<th>ChlM</th>
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<td><em>Synechocystis</em> Sp. PCC6803</td>
<td><em>Rhodobacter sphaeroides</em></td>
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<td>20.1 (NADPH)</td>
<td>0.012 (NADPH)</td>
<td>38 (SAM)</td>
<td>3300 (Mg$^{2+}$) 120 (ATP)</td>
</tr>
</tbody>
</table>

Table 2.3 Comparison of $K_m$ values for enzymes in the (B)Chl biosynthesis pathway