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Purification and Crystallization of the I subunit of Magnesium Chelatase

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Master project in Protein Science, 30 credits.

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Abstract: The I subunit of magnesium chelatase is an AAA+ protein which binds and hydrolyzes ATP to drive the insertion of Mg^{2+} into protoporphyrin IX during bio-synthesis of bacteriochlorophyll and chlorophyll. The purification method and co-crystallization conditions of the *Rhodobacter capsulatus* BchI (bacteriochlorophyll Magnesium Chelatase I subunit) protein with either ADP or AMPPNP and crystallization conditions of *Synechocystissp sp 6803* ChlI (chlorophyll Magnesium Chelatase I subunit) protein are described. This is the first report of a successful preparation of the crystals of BchI-ADP complex, the crystals of BchI-AMPPNP complex and crystals of ChlI protein. The crystals of both proteins will be used for further structure determination.

Key words: Magnesium chelatase; BchI; ChlI; AAA+ protein.
Abbreviations: AAA, ATPases associated with various cellular activities; ADP, Adenosine diphosphate; AMPPNP, non-hydrolysable ATP-analogue; ATP, Adenosine triphosphate; PEG, Polyethylene Glycol.

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Introduction:

Insertion of metal ions into tetrapyrroles by chelatases play an important role during bio-synthesis of many essential small molecules such as chlorophyll, bacteriochlorophyll, factor 430, heme and vitamin B12, in cells [1, 2]. Chlorophyll and bacteriochlorophyll are essential components of light harvesting complexes in photosynthetic organisms. Insertion of Mg^{2+} into protoporphyrin IX by an ATP dependent magnesium chelatase is the first dedicated step in bio-synthesis of (bacterio)chlorophyll (Figure 1) [3-5]. The magnesium chelatase is composed of three subunits BchI, BchD and BchH in bacteria and ChlI, ChlD and ChlH in plants. The molecular weight of I, D and H subunits are approximately 40 kDa, 70 kDa and 140 kDa, respectively [4, 6].

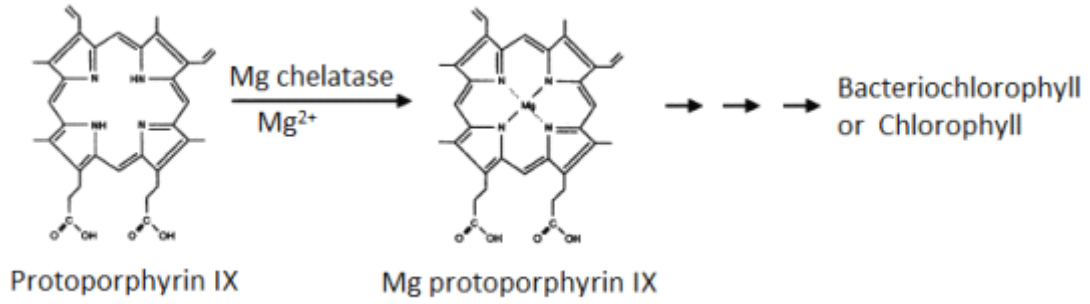

Figure 1: Bio-synthesis of bacteriochlorophyll or chlorophyll [4].

The I subunit is the smallest subunit of magnesium chelatase ranging in length from 324 to 426 amino acids in different organisms. It is the only subunit of magnesium chelatase that binds and hydrolyzes ATP [7]. Sequence analysis could identify the I subunit as a member of the extended class of the AAA family, the so-called AAA+ family, this was confirmed by the X-ray crystallographic structure of the I subunit from *Rhodobacter capsulatus* [7]. In the AAA+ family, all members are Mg^{2+} dependent ATPases with a highly conserved N-terminal nucleotide-binding domain and an alpha-helical C-terminal domain (Figure 2A) [2]. Like many other AAA proteins, the I subunit forms oligomers when ATP or ADP is present. For example, the I subunit from *R. Capsulatus* can form a hexamer and the I subunit from *Synechocystis sp* can form a heptamer [2, 8].

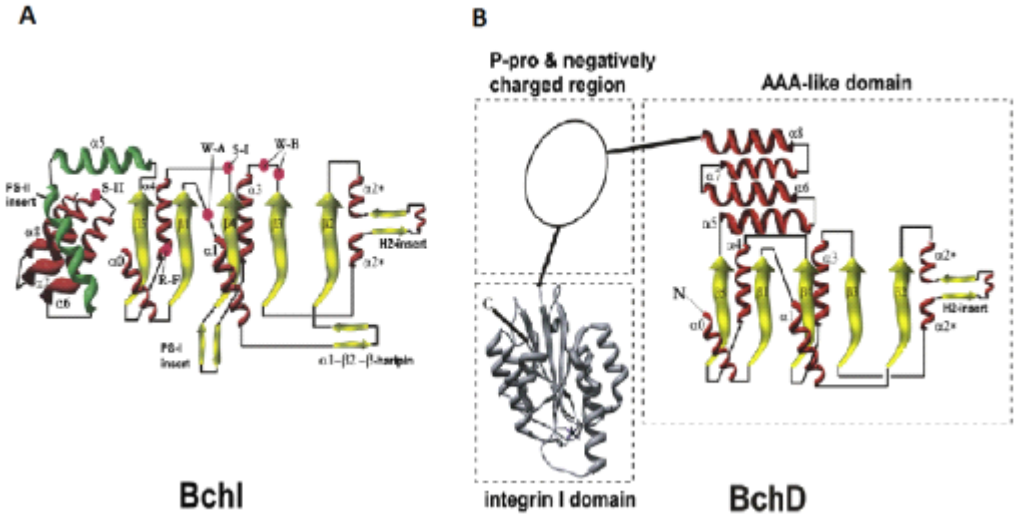

Figure 2: Topology diagram of (A) BchI and (B) BchD [8].

The D subunit of magnesium chelatase contains three domains: an AAA like domain, a proline and acidic residue-rich domain and an integrin I domain (Figure 2B). The AAA like domain is homologous to the I subunit. However, the walker A (P-loop) and Walker B motifs, which are necessary for ATP hydrolysis, are poorly conserved.

Indeed, no ATPase activity has been reported for the D subunit, yet it still forms oligomeric ring structures [8].

Using single-particle cryo-electron microscopy, the three dimensional structures of the complex between *R. capsulatus* BchD and BchI in the presence of ADP, AMP-PNP, and ATP were reconstructed to 7.5Å, 14Å and 13Å resolution, respectively [8] (Figure 3) . Large conformational changes could be seen between the different nucleotide bound states. A three-dimensional model of BchD was made using homology modeling with the structures of BchI and the integrin I domain. The three dimensional structures of BchI and BchD were then docked into the reconstructed electron microscopy structure of the complex. Thereby, a model for the function of magnesium chelatase was proposed [8]. The I subunit was found to be arranged as a trimer of homodimers, and the functional ATP binding sites were formed at the interface between each homodimer. Thus a total of three ATP molecules were bound in each ID complex. It has also been suggested that the D subunit may serve as a stabilizing plattform in formation of the ID complex. And that the integrin I domain play an important role in the interactions between the I- and D subunit [8, 9].

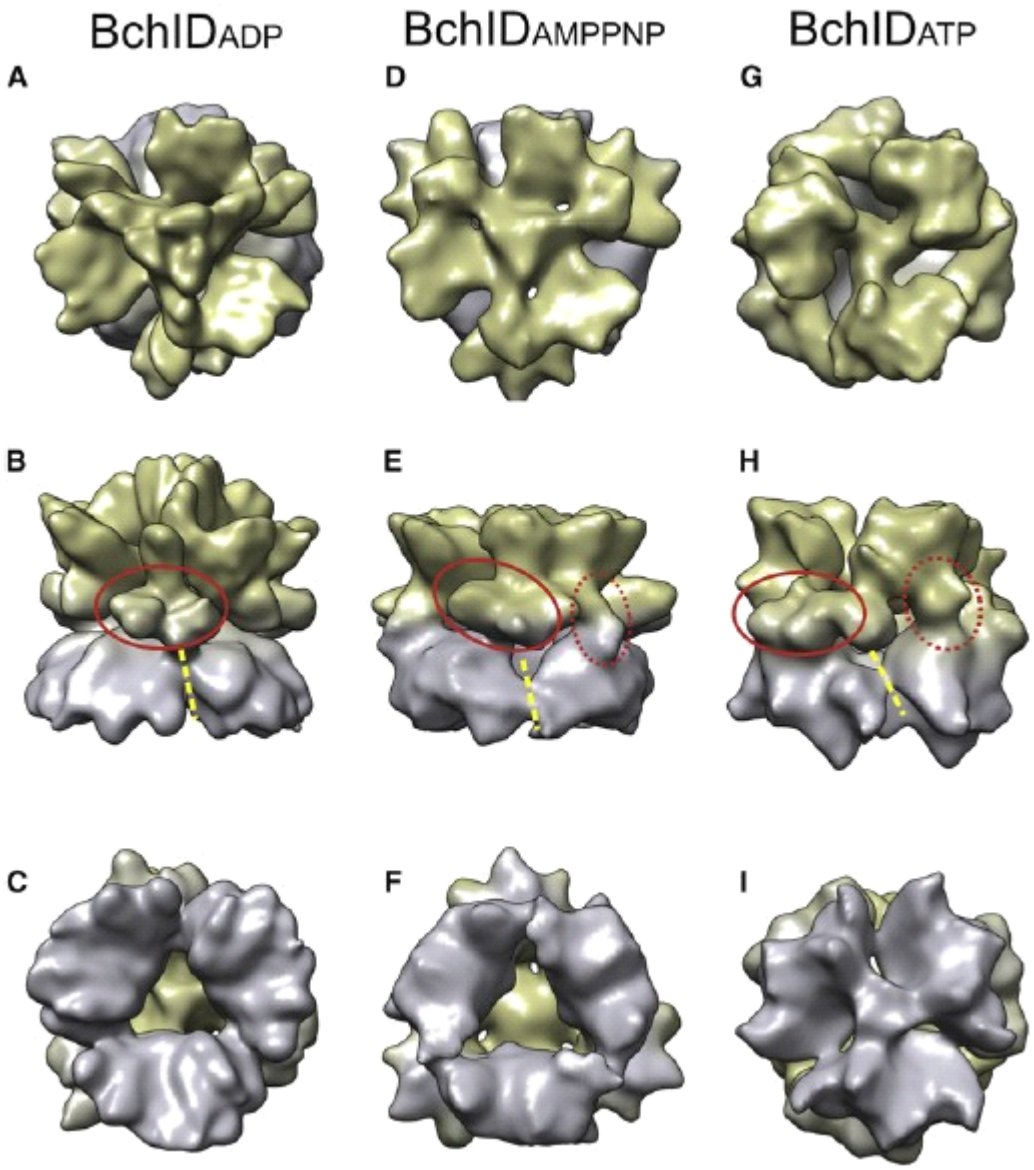

Figure 3: The BchI:BchD Complex of Magnesium Chelatase in Different Nucleotide States [8]. (A) Top, (B) side, and (C) bottom views of the complex in the presence of ADP, filtered to 13A° resolution for the comparison with the other reconstructions. (D) Top, (E) side, and (F) bottom views of the complex in the presence of the nonhydrolyzable ATP analog AMPPNP. (G) Top, (H) side, and (I) bottom views of the complex in the presence of ATP.

The biggest subunit of magnesium chelatase, the H subunit, binds porphyrin. Sequence analysis of the H subunit show no homology to structurally determined proteins. A 25 Å resolution negative stain electron microscopy single particle reconstruction of BchH both with and without porphyrin bound, demonstrated that BchH contains three domains. Also, a big conformational change took place when porhyrin bound [10].

Although great efforts has been made in understanding the function of magnesium chelatase, the molecular mechanism is not well known. The lack of high resolution structural models of the D- and H- subunits, but also of I in complex with nucleotides, prevent a more detailed discussion of exactly how energy is transducted from ATP hydrolysis to insertion of Mg^{2+} into porphyrin.

Aiming to reveal the molecular mechanism in detail, the BchI from *R. sphaeroides* was crystallized with ADP and AMPPNP present in the buffer, also ChlI from *Synechocystis sp.* was crystallized in a nucleotide free state.

Materials and methods

Cells for BchI protein and ChlI protein purification were obtained from labs in Carlsberg and Sheffield University, respectively. PACT premier™ Crystallization Screen kit and JCSG-plus™ Crystallization Screen kit were purchased from Molecular Dimensions Limited. Ni/NTA Agarose and High-trap Q columns were purchased from Qiagen and GE healthcare, respectively.

His binding buffer contains 50mM MOPS buffer pH 7.7, 500mM NaCl, 4mM DTT and 20mM imidazole.
His washing buffer contains 50mM MOPS buffer pH 7.7, 500mM NaCl, 4mM DTT and 50mM imidazole.
His elution buffer contains 50mM MOPS buffer pH 7.7, 500mM NaCl, 4mM DTT and 500mM imidazole.
Gel filtration buffer 1 contains Tricine-NaOH pH 8.0, 15 mM $MgCl_2$, 4 mM DTT.
Gel filtration buffer 2 contains 50mM MOPS pH 7.7 and 4mM DTT.

Purification and co-crystallization of BchI with either ADP or ATP

The 25ml of crude cell extract of BchI was centrifuged at 10000 rpm for 30min and the supernatant was used for protein purification. The purification was carried out on 2ml Nickel column with the force of gravity. After flow through of the supernatant, the column was washed with 20ml of His washing buffer. 10ml His elution buffer was used for protein elution and different fractions (12 drops per faction) were collected in 2ml Eppendorf tubes.

The Gel filtration purification was carried out on a HPLC system equipped with an UV–Vis detector. The Gel filtration column used was a Superdex 200 column. The wavelength for UV detection was 280 nm. The injection volume of the protein fraction purified by Nickel column was 0.5 ml. To compare which is the more favorable buffer for crystallization, both gel filtration buffer 1 and 2 were used in different purified batches. The flow rate was 0.5 ml/min and 0.5 ml fractions were collected.

The protein concentration was measured using Bradford assay. The purity of BchI was analyzed by SDS-PAGE with Coomassie Brilliant Blue staining. The monodispersity of the proteins was analyzed using dynamic light scattering.

PACT premier™ Crystallization Screen kit and JCSG-plus™ Crystallization Screen kit were used for Crystallization Screen BchI with ADP. The initial protein concentrations were 3.4mg/ml with 0.5 mM ADP and 1.7mg/ml with 0.25 mM ADP in a buffer contains Tricine-NaOH pH 8.0, 15 mM $MgCl_2$, 4 mM DTT. The drops were set by robot and the plates were incubated at a constant temperature of 20°C for crystal growth.

The conditions of the most promising drops were further optimized by changing PEG concentrations, drop size and temperature (15°C and 20°C). We also tried to use MOPS buffer (pH 7.7) instead of using Tricine-NaOH buffer (pH 8.0). $MgCl_2$ concentration was reduced to 8mM due to salt crystals appearing in the drops when 15 mM $MgCl_2$ was used.

Purification and crystallization of ChlI protein

The cell pellet for ChlI was dissolved in His binding buffer and the cells were disrupted using Frenchpress. The purification procedures on Nickel columns were similar to the purification of BchI. The ChlI protein seems to aggregate in high protein concentration. To overcome this problem, ChlI was eluted (10 drops per fraction) in 2 ml Eppendorf tubes containing 1.5ml of gel filtration buffer 2 to quickly dilute the protein concentration.

The further purification of ChlI was carried out on a HPLC system with High-trap Q column. 2ml ChlI protein was loaded and the elution was carried out at a flow rate of 1.0ml/min. The mobile phase A consisted of Gel filtration buffer 2 and the mobile phase B was Gel filtration buffer 2 with 1M NaCl. Bound protein was eluted using a 40ml linear gradient of 0% to 100% B. The protein concentration was monitored using UV detector at the wavelength of 280nm and 0.5 ml fractions were collected.

After purification on an ion exchange column, the ChlI protein was run again on a filtration column as described in BchI purification. The concentration, purity and monodispersity of ChlI protein were analyzed with the methods as for the BchI protein.

The pre-crystallization kit was used to find a starting protein concentration for crystallization. The crystallizations were initially screened using sitting drop method at a constant temperature of 20°C. 2µl drops were set by mixing 1µl of 1mg/ml ChlI protein with 1µl buffer from PACT premier™ Crystallization Screen kit. The mixture was allowed to equilibrate against 0.5 ml reservoir solution. The conditions with needle crystals were further optimized using different PEG concentration, different

size of drops or different temperature (4°C, 15°C and 20°C).

Results

Purification of proteins

The BchI protein was successfully bound to the Nickel column and after elution a significant purification had been achieved (Figure 4A). Further purification using gel filtration gave more pure BchI protein (Figure 4B). BchI monomer molecular weight is approximately 45 kDa, the hexamer molecular weight is 270 kDa. Dynamic light scattering studies estimated the molecular weight of the nucleotide free BchI, BchI with ADP and BchI with AMPPNP to 72.0 ± 9.4, 212.3 ± 54.7 and 190.6 ± 33.6 (Table 1), respectively.

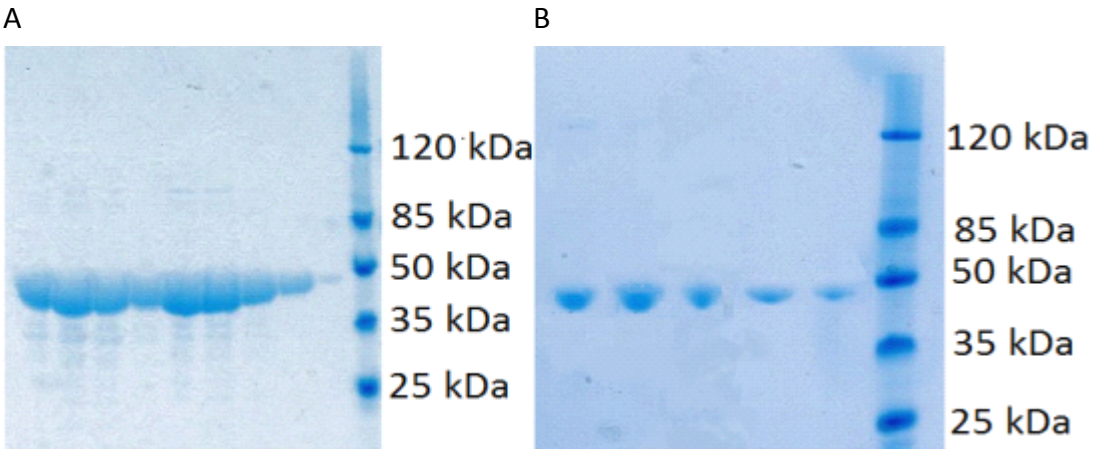

Figure 4. The SDS-PAGE gel of BchI protein after purification on the Nickel column. The molecular weight of His tagged BchI is approximately 50kDa.

Table 1: Summary of dynamic light scattering studies of BchI with and without ADP/AMPPNP, and ChlI with and without dilution of eluate from Nickel column. The molecular weight is estimated assuming a compact globular protein. Distribution of the estimated molecular weight with respect to intensity and mass is also presented.

Sample	Molecular weight (kDa) (Mode±SD)	% Intensity	% Mass
BchI nucleotide free	72.0 ± 9.4	31.5	99.5
BchI with ADP	212.3 ± 54.7	67.9	99.9
BchI with AMPPNP	190.6 ± 33.6	50.4	99.7
ChlI[a]	776.9 ± 193.4	73.8	98.7
ChlI[b]	111.0 ± 55.0	39.1	97.9

[a]without dilution after elution from Nickel column, [b]diluted directly after elution from Nickel column (see material and methods)

The ChlI protein was successfully purified using gel filtration after elution from the Nickel column (Figure 5A). The dynamic light scattering studies reveal that the ChlI

protein may aggregate, possibly due to high protein concentration (Table 1). The molecular weight was ~770 kDa and it could not be crystallized. When we diluted the protein immediately after elution from the Nickel column we got protein that could be crystallized. Also the molecular weight estimated by dynamic light scattering was reduced to ~110 kDa (Table 1). SDS-PAGE verified we had prepared a pure protein (Figure 5B).

A B

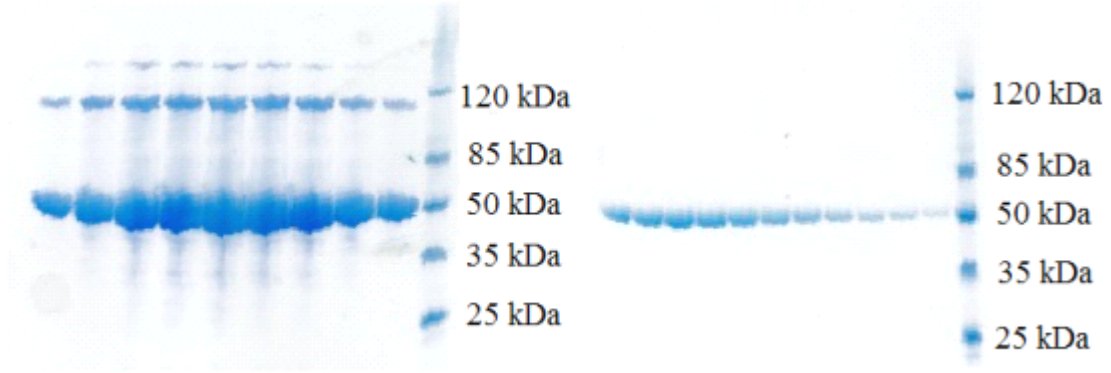

Figure 5: The SDS-PAGE image of ChlI protein after purification on the Nickel column. (A) SDS-PAGE gel of ChlI protein after elution from the Nickel column. (B) SDS-PAGE gel of ChlI after gel filtration. The molecular weight of His tagged ChlI is approximately 50kDa.

Crystallization

Small crystals or macro crystals of BchI with ADP formed in four drops from the PACT premier™ Crystallization Screen kit two days after setting the drops. The initial concentration of the protein was 1.7 mg/ml. However drops with the same conditions but using 3.4 mg/ml protein concentration were found to contain precipitated protein. This indicates that 1.7 mg/ml is a preferable concentration for BchI with ADP co-crystallization. We also found that Gel filtration buffer 2 (50mM MOPS pH 7.7 and 4mM DTT) with 8 mM $MgCl_2$ is more suitable than Gel filtration buffer 1 (Tricine-NaOH pH 8.0, 15 mM $MgCl_2$, 4 mM DTT). The reduced $MgCl_2$ concentration can also help to avoid salt crystal formation in the condition P2-10 (Table 2). The four conditions which gave crystals of BchI in the presence of ADP were also suitable to co-crystallize BchI with AMPPNP. The crystallization optimization results show that P2-32 (0.2 M sodium sulfate, 0.1 M Bis Tris propane pH 7.5 and 20 % w/v PEG 3350) is the best condition for co-crystallization of BchI with both ADP and AMPPNP (Figure 6).

Table 2: Results from crystallizing BchI in the presence of ADP using PACT premier™ Crystallization Screen kit. The protein solution used contained 1.7 mg/ml of BchI, 15mM $MgCl_2$ 0.2mM ADP in 50 mM Tricine-NaOH (pH 8.0). SC means Small Crystals and MC means Macro Crystals.

Tube No.	Salt	Buffer	Precipitant	Result
P2-8	0.2 M sodium sulfate	None	20 % w/v PEG 3350	SC
P2-10	0.2 M sodium/potassium phosphate	None	20 % w/v PEG 3350	MC
P2-32	0.2 M sodium sulfate	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	MC
P2-44	0.2 M sodium sulfate	0.1 M Bis Tris propane pH 8.5	20 % w/v PEG 3350	MC

Table 3: Results from crystallizing BchI in the presence of ADP using JCSG-plus™ Crystallization Screen kit. The protein solution used contained 1.7 mg/ml of BchI, 15mM $MgCl_2$ 0.2mM ADP in 50 mM Tricine-NaOH (pH 8.0) buffer.

Tube No.	Salt	Buffer	Precipitant	Result
P1-20	None	0.1 M sodium acetate pH 4.5	1.0 M di-ammonium hydrogen phosphate	Crystals

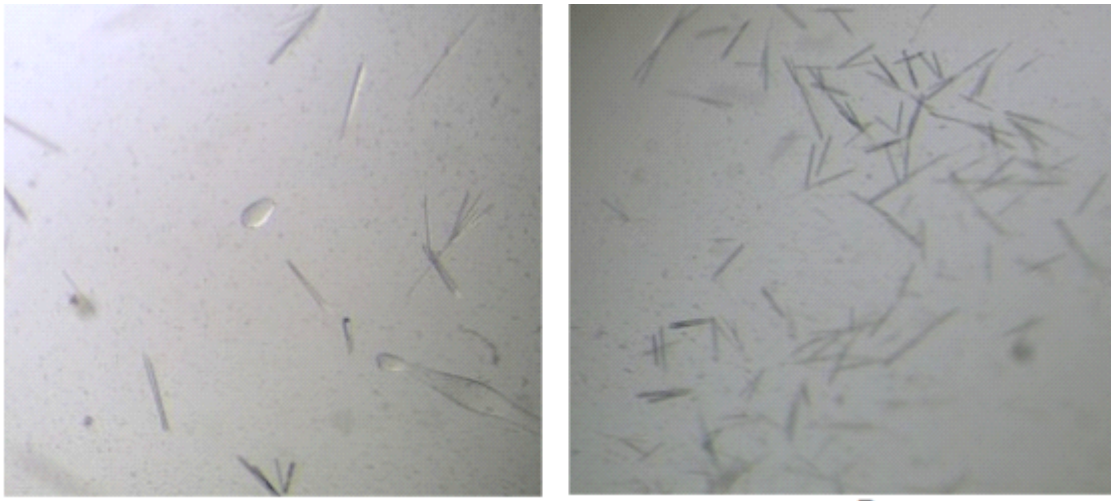

Figure 6: Crystals of BchI co-crystallized with (A) ADP and (B) AMPPNP

Needle crystals were found in 16 different conditions with ChlI protein using PACT premier™ Crystallization Screen kit. We tried to optimize the P2-32 (0.2 M sodium sulfate , 0.1 M Bis Tris propane pH 7.5 and 20 % w/v PEG 3350 condition which showed the biggest needle crystals. The crystals appeared 3 days after setting the drops and grew to their maximum size after 5 days. The crystals are big enough for data collection (Figure 7).

Table 4. Results from crystallizing ChlI using PACT premier™ Crystallization Screen kit. The protein solution used contained 1mg/ml of ChlI in 50mM MOPS (pH 7.7) buffer. NC means Needle Crystals

.Tube No.	Salt	Buffer	Precipitant	Result
P1-20	0.2 M ammonium chloride	0.1 M MES pH 6.0	20 % w/v PEG 6000	NC
P1-21	0.2 M lithium chloride	0.1 M MES pH 6.0	20 % w/v PEG 6000	NC
P1-28	None	0.1 M PCTP buffer pH 7.0	25 % w/v PEG 1500	NC
P1-29	None	0.1 M PCTP buffer pH 8.0	25 % w/v PEG 1500	NC
P1-33	0.2 M lithium chloride	0.1 M HEPES pH 7.0	20 % w/v PEG 6000	NC
P2-20	0.2 M sodium sulfate	0.1 M Bis Tris propane pH 6.5	20 % w/v PEG 3350	NC
P2-21	0.2 M potassium/sodium tartrate	0.1 M Bis Tris propane pH 6.5	20 % w/v PEG 3350	NC
P2-25	0.2 M sodium fluoride	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-26	0.2 M sodium bromide	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-30	0.2 M sodium formate	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-31	0.2 M sodium acetate	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-32	0.2 M sodium sulfate	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-36	0.2 M sodium malonate	0.1 M Bis Tris propane pH 7.5	20 % w/v PEG 3350	NC
P2-38	0.2 M sodium bromide	0.1 M Bis Tris propane pH 8.5	20 % w/v PEG 3350	NC
P2-42	0.2 M sodium formate	0.1 M Bis Tris propane pH 8.5	20 % w/v PEG 3350	NC
P2-43	0.2 M sodium acetate	0.1 M Bis Tris propane pH 8.5	20 % w/v PEG 3350	NC

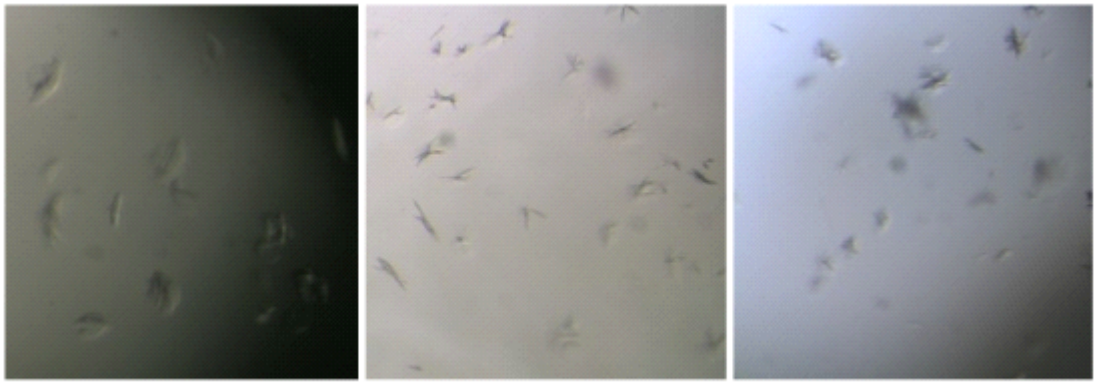

Figure 7: The crystals of ChlI protein at different temperatures (A) 20°C, (B) 15°C and (C) 4°C.

Discussion

Magnesium chelatase catalyses the first committed step in the biosynthesis of chlorophyll, which is the most important light harvesting pigment in the photosynthesis [1]. A high-resolution structure of magnesium chelatase with different nucleotides bound can reveal how the conformation changes during ATP binding and hydrolysis. Thereby the molecular mechanism can be understood in more detail. However, obtaining crystals of x-ray diffraction quality is the prerequisite for crystallographic structure determination. In the present study, large crystals of BchI with either ADP or AMPPNP were produced for the first time and the crystals can be reproduced easily. The equilibration rate between drops and reservoir solution is one of the most important parameters, which affects the size of the crystals. Big drops that have a relatively smaller surface can grow bigger crystals. To reduce the equilibration rate, we used a combination of increasing drop size (20µl drops), reducing the concentration of reservoir solution and adding oil to the reservoir solution. After formation of crystals, the drops were transferred to a higher concentration of reservoir solution. With such strategy, we could produce crystals that were big enough for X-ray diffraction study at synchrotron beamlines with micro focus capability. Some crystals are even big enough for data collection at conventional synchrotron beamlines. We also noticed the temperature can also affect the size of the crystals. The same condition in 15°C can produce fatter crystals than that in 20°C.

Unpublished data suggested that *Synechocystis sp* ChlI protein form heptamers. It is interested to crystallize ChlI and determine its structure. However, aggregation was a main problem during purification of ChlI. According to the experience from our collaborators, ChlI can aggregate or oligomerize when protein concentration is higher than 1mg/ml in 50 mM MOPS buffer (pH 7.7). We noticed the problem even arises during elution of the ChlI protein from the Nickel column. Dilution of ChlI fractions immediately after elution from the nickel column reduced aggregation (Table 1). We conclude that it is best to keep the protein concentration below at least

2mg/ml during all steps of purification. The crystallization trials performed in this work demonstrated that ChlI readily form needle crystals. However most of the crystals appeared within 24 hrs after setting the drops, and they were too many to allow formation of bigger crystals more suitable for x-ray diffraction. The condition with 0.2 M sodium sulfate, 0.1 M Bis Tris propane (pH 7.5) and 20 % w/v PEG 3350 (20°C) turned out as the preferred condition for further optimizations of crystallization of ChlI. In such condition. In addition, we found out crystallization at 20 °C can give bigger crystals than that at 15°C, while needle clusters formed at 4°C.

To summarize, aggregation problems in purification of ChlI protein have been overcome and the BchI and ChlI protein for crystallization were purified successfully. In addition, this is the first report for producing crystals of nucletotide free ChlI, BchII with ADP and BchI with AMPPNP.

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