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# Inducing the oxidative stress response in *Escherichia coli* improves the quality of a recombinant protein: Magnesium chelatase ChlH

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ABSTRACT

The ~150 kDa ChlH subunit of magnesium chelatase from *Oryza sativa, Hordeum vulgare* and *Chlamydomonas reinhardtii* have been heterologously expressed in *Escherichia coli*. The active soluble protein is found as both a multimeric and a monomeric form. The multimeric ChlH appears to be oxidatively damaged but monomer production is favoured in growth conditions that are known to cause an oxidative stress response in *E. coli*. Inducing an oxidative stress response may be of general utility to improve the quality of proteins expressed in *E. coli*. The similar responses of ChlH's from the three different species suggest that oligomerization of oxidatively damaged ChlH may have a functional role in the chloroplast, possibly as a signal of oxidative stress or damage.



## Introduction

Magnesium chelatase is one of the enzymes located at the branchpoint between hemes and chlorophylls in the synthesis of tetrapyrroles. The enzyme inserts magnesium into protoporphyrin IX in an ATP hydrolysis dependent manner. The product, magnesium protoporphyrin IX, is the first committed intermediate in the synthesis of chlorophyll or bacteriochlorophyll. Magnesium chelatase consists of three subunits, BchI/ChlI (40 kDa), BchD/ChlD (70 kDa) and BchH/ChlH (150 kDa) [1] with the prefixes Bch and Chl used to distinguish the proteins from bacteriochlorophyll or chlorophyll synthesizing organisms respectively. The BchI/ChlI and BchD/ChlD subunits form a double hexameric AAA + motor complex, $BchI_6BchD_6$, and show conformational rearrangements depending on the nucleotide present [2]. The BchH/ChlH subunit has no ATPase activity but binds the substrate and product of the reaction [3,4]. This lead to the proposal of a mechanism where ATP is bound and hydrolyzed by $I_6D_6$ causing a conformational change in BchH/ChlH that subsequently drives chelation of magnesium into protoporphyrin IX [5]. When studying the kinetics of the reaction the BchH/ChlH and BchI/ChlI subunits act like "substrates" in the magnesium chelatase reaction with the BchD/ChlD subunit treated as the "enzyme" [6,7].

In addition to its direct role in chlorophyll synthesis, ChlH has further been implicated in plastid to nucleus signalling as point mutants in *chlH* have a so-called *gun* (genomes *un*coupled) phenotype with disrupted control of nuclear gene expression [8]. The ChlH subunit has also been suggested to be an abscisic acid receptor [9–11]. Although its direct receptor role has been challenged [12,13], it does still appear to be involved in some way in abscisic acid signaling [13].

In chlorophyll synthesizing organisms, an accessory protein called GUN4 is required for optimal magnesium chelatase activity. GUN4 was discovered in a mutant screen of plants with defects in plastid control over nuclear gene expression [8]. Both cyanobacterial and eukaryotic GUN4 binds protoporphyrin IX and magnesium protoporphyrin IX, interacts with ChlH and stimulates the activity of magnesium chelatase [14–17].

Here we report on the heterologous expression of ChlH from three different eukaryotic organisms, each giving a monomeric and a multimeric state by size exclusion chromatography. The monomeric state gave consistent specific activity in enzymatic assays while the multimeric state varied from batch to batch. The ratio of these two states depended primarily on oxidative conditions during induction of expression.

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## Materials and methods

### *Expression clones of ChlH from Chlamydomonas reinhardtii, Hordeum vulgare and Oryza sativa*

Expression constructs for ChlH used in this work were prepared from cDNA of *H. vulgare* cultivar Svalöfs Bonus, *O. sativa l. japonica Nipponbare* and *C. reinhardtii* cc124. The *O. sativa l. japonica Nipponbare* and *H. vulgare* expression plasmids, pET28a-Os-ChlH and pET15bXanF respectively, have been described previously [12,17]. The *Chlamydomonas* expression construct pET28a-Cr-ChlH was constructed by cloning the PCR product of the *chlH* from cDNA (Accession XM_001700843 between 141 and 4416 bp) between the BamHI and EcoRI sites of pET28a. The *Chlamydomonas*, *H. vulgare* and *O. sativa* proteins are differentiated by the prefixes "Cr", "Hv" and "Os" respectively.

### *Expression of ChlH*

The expression vectors described above were used to transform *Escherichia coli* strains BL21(DE3) Star, or BL21(DE3) pLysS or BL21(DE3) Rosetta 2 (Novagen). All three strains yielded soluble expressed protein. 20% glycerol stocks were prepared from single colony transformants from solid LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 20 g/l agar; with appropriate antibiotics: either 50 mg/l kanamycin or 100 mg/l ampicillin; and 20 mg/l chloramphenicol when using pLysS or Rosetta strains) grown to mid-late log phase. The glycerol stock (10 μL) was spread on LB agar containing the appropriate antibiotics and colonies grew overnight at 37 °C. Fresh LB (10 mL) was added to the plate and slowly shaken at 150 rpm for 5 min to resuspended the colonies. The resuspended colonies were used to inoculate 250 mL–1 L of LB media with antibiotics to $OD_{600}$ = 0.05. After growth to $OD_{600}$ = 0.6 at 15 °C (Os- and Cr-ChlH) or 30 °C (Hv-ChlH), the protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to 0.1 mM and incubated for 24 h at 15 °C.

For anoxic expressions, a 30 mL culture in LB (with ampicillin and chloramphenicol) was grown from $OD_{600}$ = 0.05 to $OD_{600}$ = 1 at 30 °C (shaken at 200 rev. $min^{-1}$) and then diluted to 300 ml with LB (with 10 mM glucose and antibiotics) in a 300 ml screw cap bottle containing a magnetic stirring flea. The bottle was capped tightly with no air and stirred for 1 h at 30 °C, then induced by addition of 0.1 mM IPTG and stirred for 24 h at 18 °C. Other variations to expression conditions are as described in the results section.

Cells were harvested (10,000×*g*, 15 min, 4 °C), washed in binding buffer (50 mM Tricine-NaOH pH 8.0, 250 mM NaCl, 50 mM $MgCl_2$, 20 mM imidazole) and pelleted again (10,000×*g*, 15 min, 4 °C) to be resuspended in binding buffer for lysis by two runs through a French press. Soluble and insoluble lysis fraction were separated by centrifugation (15,000×*g*, 15 min, 4 °C).

### *Purification of ChlH*

ChlH was purified by immobilized metal ion affinity chromatography (IMAC)[4] using a 5 mL HisTrap column using $Ni^{2+}$ as the immobilized metal ion (GE Healthcare). The supernatant was loaded onto the column and washed with 10 volumes wash buffer (50 mM Tricine-NaOH pH 8.0, 250 mM NaCl, 50 mM $MgCl_2$, 40 mM imidazole) and the protein was eluted with elution buffer (50 mM Tricine-NaOH pH 8.0, 250 mM NaCl, 50 mM $MgCl_2$, 250 mM imidazole). The peak elution fractions were determined by Bradford Reagent (BioRad) and pooled. The yield of ChlH per liter of culture at this purification stage ranged from a low yield of 0.5 mg/L when methylviologen was added during expression to a high yield of 15 mg/L with no additives. The ChlH was either further purified and buffer exchanged directly by gel filtration chromatography as detailed below or the samples were buffer exchanged on PD-10 desalting columns (GE Healthcare) into an imidazole-free buffer (50 mM Tricine-NaOH pH 8.0, 250 mM NaCl, 50 mM $MgCl_2$, 2 mM dithiothreitol). The buffer exchanged ChlH was concentrated to between 5–10 mg/mL using a 30 K Amicon centrifugal filter device (Millipore).

Deviations from this standard procedure are detailed in the results.

### *Gel filtration chromatography*

A SMART system (Pharmacia) or an ÄKTA system (GE Healthcare) system was used for analytical size exclusion chromatography with Superose 12 (3.2/300) column. 50 μL samples were run on these Superose columns in a buffer containing 15 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, 1 mM EDTA.

For larger scale purifications an ÄKTA system (GE Healthcare) with a Superdex 200 (10/300) column was used with 50 mM Tricine-NaOH, pH 8.0, 250 mM NaCl, 50 mM $MgCl_2$, 2 mM dithiothreitol. This was later modified to 50 mM Tricine-NaOH, pH 8.0, 250 mM NaCl, 2 mM dithiothreitol to allow for better control of the $MgCl_2$ concentration in assays.

### *Magnesium chelatase activity measurements*

The assay utilized *O. sativa* ChlI, ChlD and GUN4 subunits as described previously [17]. Briefly, the assay consisted of 50 mM Tricine-NaOH, pH 8.0, 2 mM dithiothreitol, 15 mM $MgCl_2$ and 4 mM ATP with 10 nM Os-ChlD, 100 nM Os-ChlI and 500 nM Os-GUN4, 500 nM protoporphyrin IX and 500 nM ChlH. The reaction was started by mixing equal volumes of H-premix (1000 nM ChlH, 1000 nM GUN4 and 1000 nM protoporphyrin IX mixed in 50 mM Tricine-NaOH, pH 8.0, 2 mM dithiothreitol, 15 mM $MgCl_2$ and 4 mM ATP, preincubated at ambient temperature at 30 °C for 30 min prior to starting the assay) with ID premix (50 mM Tricine-NaOH, pH 8.0, 2 mM dithiothreitol, 15 mM $MgCl_2$ and 4 mM ATP with 20 nM Os-ChlD, 200 nM Os-ChlI). The fluorescence emission at 600 nm upon excitation at 420 nm was monitored in a BMG-Pherstar plate reader at 15 s intervals for 30 min. The maximum rate over a 2 min interval was determined from the linear part of the progress curves and activity units are in nmoles of magnesium protoporphyrin formed per minute per nmole of ChlD.

## Results

### *Expression, purification and assay of ChlH proteins*

The ChlH proteins were purified as described in the methods. Although the proteins appeared to be more than 90% pure as estimated by SDS–PAGE (Fig. 1) the gel filtration profiles on Superdex (Fig. 2) and Superose 12 (see an example in Fig. 3) indicated that the ChlH proteins had two major components - a multimeric component eluting at 8 mL on Superdex 200 and 0.9 mL on Superose 12 and a monomeric component eluting at ~12.5 mL on Superdex 200 and 1.25 mL on Superose 12. (see Figs. 2 and 3A) The major peak at 0.9 mL is close to the exclusion limit determined with blue dextran for Superose 12 of 0.85 mL. The smaller peak at 1.25 mL eluted at the same position as standard IgG indicating a monomeric Hv-ChlH.

[4] *Abbreviations used:* IMAC, immobilized metal ion affinity chromatography.