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Abolition of magnesium chelatase activity by the *gun5* mutation and reversal by Gun4

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ABSTRACT

The chlorophyll-deficient *gun5-1* and *cch Arabidopsis* mutants carry single point mutations in the CHLH subunit of the magnesium chelatase enzyme, which catalyses the first committed step of chlorophyll biosynthesis. Recombinant *Synechocystis* ChlH subunits carrying the *gun5-1* or *cch* mutations are inactive in Mg-chelatase assays, despite being able to bind both substrate and product, and retaining a capacity to form a ChlH–ChlI–ChlD Mg-chelatase complex. These mutant subunits act as inhibitors of ChlH, showing that the ChlH-porphyrin complex associates reversibly with the ChlI and D subunits during the catalytic cycle. This inhibition is reversed upon addition of Gun4.

1. Introduction

In plants chlorophyll biosynthesis takes place in the chloroplast, whilst expression of genes encoding light-harvesting apoproteins occurs in the nucleus. Uncoupling of these two processes is detrimental to the cell because free chlorophyll can lead to photooxidative damage. Such an uncoupling was discovered in the *Arabidopsis thaliana* Genome Uncoupled (GUN) mutants, *gun2-5*, and was ascribed to a deficient developmental signalling pathway between the chloroplast and the nucleus [1]. Three of the *gun* mutants have pale phenotypes and lesions in genes that encode enzymes in the haem/chlorophyll biosynthetic pathway [2], including *gun5* which has a lesion in the porphyrin binding subunit of magnesium chelatase [3] the enzyme that catalyses the first committed step of chlorophyll biosynthesis [4,5], the conversion of protoporphyrin IX (P_{IX}) to magnesium protoporphyrin IX (MgP_{IX}). GUN4 has a pale phenotype and in *Arabidopsis* is required in vivo for normal levels of chlorophyll accumulation but is not essential for chlorophyll synthesis [6], unlike in *Synechocystis* [7]. *Synechocystis* Gun4 stimulates Mg-chelatase in vitro [8]. GUN4 was localised to the chloroplast in *Arabidopsis* and pea [6,9] and co-purifies with CHLH in *Arabidopsis* [6] and ChlH in *Synechocystis* [7,10].

Previous work showed that in vitro studies of the Mg-chelatase enzyme from the cyanobacterium *Synechocystis* act as a useful model system for the mechanistic studies of magnesium chelation [5,11,12] and the analysis of *gun* mutations [6,8,13]. This enzyme consists of ChlH (148 kD), ChlI (39 kD) and ChlD (73 kD) subunits [5]. ChlI is the ATPase subunit, containing a Mg^{2+} binding site and a fold characteristic of a member of the AAA^{+} superfamily [12,14,15]. ChlD forms a stable complex with ChlI [12] and a structure for the homologous BchI-BchD complex in *Rhodobacter capsulatus* has been proposed [16]. ChlH binds porphyrins and presumably contains the active site for chelation [17].

Using purified subunits of the *Synechocystis* Mg-chelatase engineered to duplicate the native and *gun*-related proteins of *Arabidopsis* we show that the residues A942 and P595 in *Synechocystis* ChlH are of general importance for the catalytic function of Mg-chelatase, but not for substrate binding, since the H-gun5 and related H-cch proteins have unchanged porphyrin binding capacities but no activity in Mg-chelatase assays with wild-type ChlI and D subunits. However, the addition of Gun4 restores Mg-chelatase activity, suggesting an explanation for continued chlorophyll biosynthesis in *gun5* mutants of *Arabidopsis*.

2. Materials and methods

2.1. Protein purification

The expression vectors pET9a-ChlI, pET9a-His_6ChlD and pET9a-His_6ChlH were used to produce recombinant *Synechocystis* sp. PCC6803 magnesium chelatase subunits as described previously

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[5,11]. Non-His-tag protein was prepared by removal of the tag using thrombin. The expression vector pGEX-4T-1 (GE Healthcare) was used to produce recombinant glutathione-S-transferase (GST) tag fusion *Synechocystis* Gun4 protein in *Escherichia coli* as described previously [8].

2.2. Mutagenesis

The *Synechocystis* H-gun5 (A942V) and H-cch (P595L) mutations were generated in pET9a-His$_6$ChlH using the QuikChange mutagenesis kit (Stratagene) with the following primers:

H-gun5 forward: 5′ CAGGAAAAAATATCCACGTCCTCGATCCCCA ATCCA 3′
H-gun5 reverse: 5′ TGGATTGGGGATCGAGGACGTGGATATTTTT TCCTG 3′.
H-cch forward: 5′ CTCCCGTTCCGCCAGTCTCCATCACGGTTTTGC CGC 3′
H-cch reverse: 5′ GCGGCAAAACCGTGATGGAGACTGGCGGAA CGGGAG 3′.

2.3. Porphyrin binding

Porphyrin binding was detected using quenching of tryptophan fluorescence as described previously [17].

2.4. Magnesium chelatase in vitro enzyme assay

Assay conditions and detection systems were as described previously [8].

2.5. Binding assays and Western blotting

Chelatase subunit in vitro binding assays were carried out using 1 ml Ni^{2+} agarose columns (1 mg binding capacity) as in the manufacturer's instructions (ProPur Mini MC columns; Fisher). Western blotting was carried out using the Hybond-ECL system (GE Healthcare) as described in the manufacturer's instructions.

3. Results

The *gun5-1* and *cch* mutants isolated in *Arabidopsis* both show defects in the signalling pathway between the chloroplast and the nucleus but also exhibit a pale phenotype indicating a defect in chlorophyll biosynthesis. They both carry single point mutations in a conserved region of the H subunit of Mg-chelatase (A990V in *gun5-1* and P642L in *cch*) [3] though there are currently no data pinpointing the effect of these mutations on the Mg-chelatase reaction. Although there is no in vitro assay using purified recombinant chelatase subunits from *Arabidopsis*, there is a kinetically well-established system for the *Synechocystis* enzyme [5,11,18].

The *gun5-1* and *cch* mutations (A942V and P595L respectively in *Synechocystis*) were introduced into the DNA sequence of 6×His-tagged wild-type *Synechocystis* ChlH subunit using PCR, the mutant proteins (designated H-gun5 and H-cch) overexpressed in *E. coli* and purified on a Ni^{2+} affinity column. The mutant subunits were assessed for their activity in an in vitro Mg-chelatase assay containing purified wild-type *Synechocystis* ChlI and D subunits, the more water-soluble porphyrin substrate deuteroporphyrin IX (D_{IX}), free Mg^{2+} and ATP; formation of the product Mg deuteroporphyrin IX (MgD_{IX}) was measured as an increase in fluorescence emission at 575 nm. Fig. 1A illustrates a Mg-chelatase reaction run with wild-type enzyme subunits showing MgD_{IX} product formation after 1 h. In contrast, reactions containing either H-gun5 (Fig. 1B) or H-cch subunit (Fig. 1C) show no product formation. To see whether

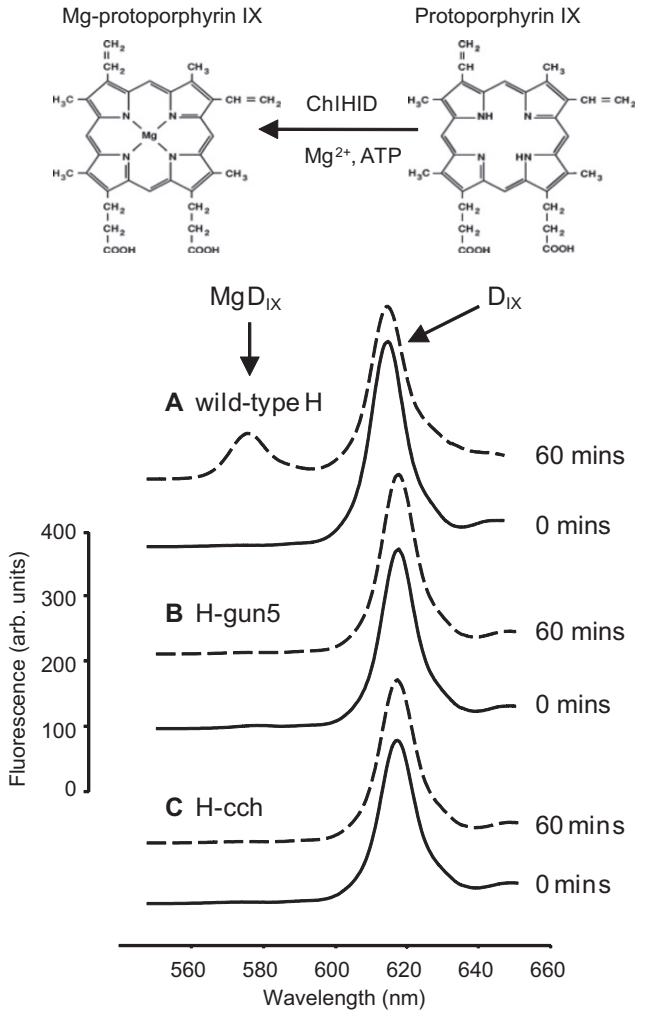

Fig. 1. In vitro stopped fluorimetric assay of *Synechocystis* wild-type and mutant Mg-chelatase activity. Pure *Synechocystis* wild-type ChlH (A), H-gun5 (B) or H-cch (C) protein was mixed with ChlI and D protein in the ratio 0.4 ChlH:0.2 ChlI:0.1 ChlD μM with 8 μM D_{IX}, 5 mM $MgATP^{2-}$ and 10 mM free Mg^{2+}. Product formation (MgD_{IX}) was measured at 575 nm at 0 min (solid line) and after 60 min (broken line).

this was due to the point mutations affecting porphyrin binding, tryptophan fluorescence studies were carried out to assess binding of the purified proteins to both the substrate, $D_{IX,}$ and product, $MgD_{IX,,}$ of this reaction. As can be seen from Table 1 the K_d values of the H-gun5 and H-cch subunits for both D_{IX} and MgD_{IX} are very similar to those of the wild-type. Hence, although one should be cautious when extrapolating from in vitro work in *Synechocystis* to the in vivo situation in *Arabidopsis*, it is at least possible that neither the lesion in chlorophyll biosynthesis, nor lack of Mg-chelatase activity, resulting from the *gun5-1*/*cch* mutations, are due to impaired porphyrin binding by the mutant ChlH subunits.

Another possibility is that the H-gun5 and H-cch subunits show no activity due to an inability to associate with ChlI and D to form a

Table 1
Dissociation constants (K_d) of several purified wild-type (ChlH) and mutant proteins from *Synechocystis* for deuteroporphyrin IX (D_{IX}) and magnesium deuteroporphyrin IX (MgD_{IX}) as determined by tryptophan fluorescence quenching.

Protein	D_{IX} K_d(μM)	MgD_{IX} K_d(μM)
ChlH	4.37 ± 0.33	5.28 ± 0.76
H-gun5	4.68 ± 0.28	6.08 ± 0.94
H-cch	4.97 ± 0.33	5.32 ± 0.48