Structure of GUN4 from *Chlamydomonas reinhardtii*

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The genomes uncoupled 4 (GUN4) protein stimulates chlorophyll biosynthesis by increasing the activity of Mg-chelatase, the enzyme that inserts magnesium into protoporphyrin IX (PPIX) in the chlorophyll biosynthesis pathway. One of the roles of GUN4 is in binding PPIX and Mg-PPIX. In eukaryotes, GUN4 also participates in plastid-to-nucleus signalling, although the mechanism for this is unclear. Here, the first crystal structure of a eukaryotic GUN4, from *Chlamydomonas reinhardtii*, is presented. The structure is in broad agreement with those of previously solved cyanobacterial structures. Most interestingly, conformational divergence is restricted to several loops which cover the porphyrin-binding cleft. The conformational dynamics suggested by this ensemble of structures lend support to the understanding of how GUN4 binds PPIX or Mg-PPIX.

1. Introduction

In plants and algae, tetrapyrrole biosynthesis occurs in the chloroplast, and the genes for light-harvesting proteins are encoded in the nuclear genome (Formighieri *et al.*, 2012). Retrograde signalling pathways between these two organelles were discovered through the *Arabidopsis thaliana* genomes uncoupled (GUN) mutants (Susek *et al.*, 1993; Willows, 2003). Five *gun* mutants have been reported (*gun1–gun5*), among which four (*gun2–gun5*) are on the same retrograde signalling pathway and encode proteins that function in tetrapyrrole metabolism. *gun2* codes for haem oxygenase, *gun3* for phytylchromobilin synthase and *gun5* for the H subunit of Mg-chelatase. Mg-chelatase is the enzyme complex which inserts the Mg²⁺ ion into protoporphyrin IX (PPIX), generating Mg-PPIX, in an ATP-dependent manner, and which constitutes the precursor for chlorophyll biosynthesis. Mg-chelatase consists of three protein subunits: ChlI/BchI (38–42 kDa), ChlD/BchD (60–74 kDa) and BchH/ChlH (140–150 kDa; Gorchein *et al.*, 1993; Jensen *et al.*, 1996; Papenbrock *et al.*, 1997; Wang *et al.*, 1974). These subunits are conserved from bacteria to higher plants and are responsible for different functions. The ChlI/BchI subunits are members of the AAA⁺ superfamily with ATPase activity required for catalysis. They bind Mg²⁺ and ATP and also function as molecular chaperones for the ChlD/BchD subunits. The ChlD/BchD subunits appear to have an inactive AAA⁺ ATPase. The ChlH/BchH subunits are the largest within the complex and form the core of the magnesium chelatase enzyme, and they bind both PPIX and Mg-PPIX.

The *gun4* gene was the first locus to be identified in the screen for genome uncoupled mutants (Susek *et al.*, 1993;

Table 1
Protein-production information.

Source organism	<i>C. reinhardtii</i>
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MRGSHHHHHGMSMTGGQQMGRDLYDDDDKDHPTFAMMRVTVAAGKLDVSLFGGDTASLMGGSQTVEKKKSGKEAVMEVQLSSTAGIDYTVLRDHLANGEFREAEDETRALLIKLAGPEAVKRNWVYFTEVKNISVTDFQTLNLWKASSNNKFGYSVQKEIWWQNQKRWPKFFKQIDWTQGENNNYRKWPM-EFIYSMDAPRGHLPLTNALRGTLFQAIMEHP-AFEKSSATAKLDQKAAEAAGRTQSLF

Willows & Hansson, 2003), and it has since been shown to be involved in both retrograde signalling and post-translational regulation of tetrapyrrole biosynthesis (Larkin *et al.*, 2003; Peter & Grimm, 2009). The *gun4* gene product (GUN4) has also been shown to be required for optimal Mg-chelatase activity through its interaction with the ChlH subunit (Davison *et al.*, 2005; Verdecia *et al.*, 2005). In doing so, it acts as a regulatory subunit and facilitates delivery of the PPIX substrate to ChlH, and also binds the product (Mg-PPIX; Chen *et al.*, 2015; Davison *et al.*, 2005; Larkin *et al.*, 2003; Verdecia *et al.*, 2005). Two crystal structures of GUN4 proteins have been elucidated from the cyanobacteria *Synechocystis* (PDB entries 1y6i, 4xkb and 4xkc; Verdecia *et al.*, 2005; Chen *et al.*, 2015) and *Thermosynechococcus elongatus* (PDB entries 1z3x and 1z3y; Davison *et al.*, 2005). The structures with PDB codes 4xkb and 4xkc are with porphyrin bound. Both are predominantly α -helical proteins comprised of a smaller N-terminal domain (five α -helices) connected to a larger C-terminal domain (eight α -helices). Structural conservation is stronger in the C-terminal domains, consistent with the high sequence similarity of the C-terminal domains among GUN4 family members across a broad spectrum of species. The N-terminal domain is more variable and is missing in some prokaryotic family members (Davison *et al.*, 2005; Verdecia *et al.*, 2005). Here, we report the crystal structure of GUN4 from *Chlamydomonas reinhardtii* at 3.5 Å resolution and hence reveal the first structural details of an eukaryotic GUN4 protein.

2. Methods and materials

2.1. Protein expression and purification

The GUN4 gene from *C. reinhardtii* was cloned from cDNA into expression vector pET-28a (Merck–Novagen) and was expressed in *Escherichia coli* strain BL21(DE3) as a fusion with an N-terminal poly-His tag (Table 1). The IPTG-induced cells were grown at 37°C in LB medium. His₆-tagged GUN4 was purified by immobilized Ni²⁺-affinity chromatography using a 5 ml HisTrap FF column (GE Healthcare). The supernatant from cleared lysate was loaded onto the column and washed with ten volumes of wash buffer (20 mM Tris–HCl pH 7.9, 0.5 M NaCl, 60 mM imidazole) and the immobilized proteins were eluted in elution buffer (80 mM Tris–HCl pH 7.9, 0.5 M NaCl, 1 M imidazole). The peak protein fractions

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	24-well (Cryschem plate, Hampton Research)
Temperature (°C)	25
Protein concentration (mg ml ⁻¹)	35
Buffer composition of protein solution	20 mM tricine pH 8.0, 2 mM β -mercaptoethanol
Composition of reservoir solution	1.0 M ammonium citrate tribasic pH 7.0, 0.1 M bis-tris propane pH 7.0
Volume and ratio of drop	2 μ l; 1:1 protein:reservoir
Volume of reservoir (μ l)	500

were determined using the Bradford reagent (Bio-Rad). Proteins in pooled fractions were immediately desalted using a PD-10 column (GE Healthcare) to a final buffer consisting of 20 mM tricine–NaOH pH 8.0, 2 mM β -mercaptoethanol. The GUN4 samples were concentrated to 20–35 mg ml⁻¹ using a 10K Amicon centrifugal filter device (Millipore) prior to crystallization experiments.

2.2. Crystallization and data collection

The GUN4 protein was screened for crystallization using a robotic liquid-handling dispenser (Phoenix, Art Robbins Instruments) at the University of New South Wales, Sydney, Australia. Crystals were grown using the vapour-diffusion technique and a sitting-drop format, in which equal volumes of protein and well solutions were combined in total drop volumes of 1–2 μ l. Crystallization was monitored by a microscope at various time intervals. Numerous crystallization hits (17) were observed from these sparse-matrix screens. The small (100 μ m in the longest dimension) rice-grain-like hexagonal crystals used in X-ray diffraction experiments appeared only after a prolonged period of room-temperature incubation, 44 weeks after the trays were established. The crystallization condition was solution B5 from the SaltRx HT screen (Hampton Research), which consisted of 1.0 M ammonium citrate tribasic pH 7.0, 0.1 M bis-tris propane pH 7.0. The protein for this sample was originally supplied at 35 mg ml⁻¹. Harvesting was achieved by simply looping crystals from the mother liquor and then plunge-cooling them in liquid nitrogen with no cryoprotection regime. Crystals were transported to the Australian Synchrotron for data collection. Diffraction data were collected at 100 K on beamline MX2. Dozens of crystals were investigated; however, useable diffraction was only obtained from several crystals using an unattenuated beam and 3 s exposures. Crystallization information is summarized in Table 2.

2.3. Structure determination and refinement

Diffraction data from two crystals (44 frames and 22 frames, respectively, each recorded over 1° oscillations with 3 s exposures to an unattenuated beam) were indexed and integrated to 3.5 Å resolution with *MOSFLM* (Leslie & Powell, 2007). The space group (*P*₃21) was confirmed with *POINTLESS* (Evans, 2011). Integrated reflections from these two crystals were scaled and merged with *AIMLESS* (Evans &

Table 3

Diffraction data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9537
Space group	<i>P</i> 3 ₂ 21
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 115.0, <i>c</i> = 141.2, α = β = 90, γ = 120
Resolution range (Å)	44.56–3.50 (3.83–3.50)
Observed reflections	48483 (11641)
Unique reflections	13965 (3267)
Completeness† (%)	99.1 (99.0)
Multiplicity†	3.5 (3.6)
<i>R</i> _{merge} †	0.21 (0.67)
<i>R</i> _{p.i.m.} †	0.19 (0.58)
Mean <i>I</i> /σ(<i>I</i>)†	5.2 (1.9)
Wilson <i>B</i> factor (Å ²)	78

† As calculated by *AIMLESS*.

Murshudov, 2013), accessed via the *CCP4* software interface (Winn *et al.*, 2011). The data from multiple crystals were combined to maximize the data completeness and multiplicity. Data-collection and processing statistics are summarized in Table 3.

The structure was determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007). The search model was taken from the *T. elongatus* wild-type structure (PDB entry 1z3x), which is 40% identical at the amino-acid sequence level over 165 residues. The more divergent first 88 residues of this structure (the N-terminal domain) were excluded from the search model. Molecular replacement initially identified four GUN4 molecules in the asymmetric unit, consistent with the crystal comprising 48% solvent (as calculated from the Matthews coefficient and full-length GUN4). However, incomplete crystal packing and inspection of electron-density maps suggested that additional unaccounted-for molecules might in fact be present. The search model was subsequently

Table 4

Structure refinement and model validation.

Protein molecules in asymmetric unit	6
Atoms modelled in asymmetric unit	6241
Ramachandran plot†	
Favoured (%)	92.5
Outliers (%)	0.71
R.m.s.d., bond lengths (Å)	0.011
R.m.s.d., bond angles (°)	1.46
<i>R</i> _{work} / <i>R</i> _{free}	0.26/0.31
Residues, chain <i>A</i> (side chains absent)	151 (40)
Residues, chain <i>B</i> (side chains absent)	147 (44)
R.m.s.d., chain <i>B</i> superposed on chain <i>A</i> (Å)	0.29 over 146 C ^α atoms
Residues, chain <i>C</i> (side chains absent)	146 (39)
R.m.s.d., chain <i>C</i> superposed on chain <i>A</i> (Å)	0.15 over 146 C ^α atoms
Residues, chain <i>D</i> (side chains absent)	148 (45)
R.m.s.d., chain <i>D</i> superposed on chain <i>A</i> (Å)	0.14 over 145 C ^α atoms
Residues, chain <i>E</i> (side chains absent)	145 (39)
R.m.s.d., chain <i>E</i> superposed on chain <i>A</i> (Å)	0.15 over 145 C ^α atoms
Residues, chain <i>F</i> (side chains absent)	141 (44)
R.m.s.d., chain <i>F</i> superposed on chain <i>A</i> (Å)	0.24 over 141 C ^α atoms
Average <i>B</i> factor (Å ²)	64.4
PDB entry	4ykb

† As calculated by *MolProbity*.

trimmed further (a further 18 residues were removed from the N-terminus, with the removal of several loops that were divergent in the *T. elongatus* and *Synechocystis* structures and with side chains truncated to the C^β position), which allowed *Phaser* to fit six non-clashing molecules in the asymmetric unit (solvent content of 53% for six molecules of ~150 residues). Hence, it appeared that the N-terminal domain of the molecule was missing.

Restrained *B*-factor refinement was performed with *REFMAC5* (Murshudov *et al.*, 2011), using local noncrystallographic symmetry (NCS) restraints. Between rounds of refinement, electron-density maps and their fit to the model were examined using *Coot* (Emsley *et al.*, 2010). Amino-acid side chains were added if suggested by difference map

<i>C. reinhardtii</i>	-----MLAQTHTASSRSACRGRAQRGQLAFSAPRPISQSRGSLIQQAPSMMAMRVTVAAAGK	55
<i>Synechocystis</i>	-----MSDNLTELSQQLHDASEKKQLTAIAALAEMGEGGQGIILLDYLAKNVPLEKP--V	52
<i>T. elongatus</i>	PEFMVTEPALADLQEQLYNGNEKSQLAAMSTLSTAGTEGYHLLQEFLKDSATFSPFPAP	60
	α1 α2	
<i>C. reinhardtii</i>	LDSVSLFGGDTASLMGGSQTVEKKKSGKEAVMEVQLSSTAGIDYTVLRDHLANGEFREAE	115
<i>Synechocystis</i>	LAVGNVYQ---TLRNLEQETITTLQQRNYPTGIFPLQSAQGIDYLLPLQEALGSQDFETAD	109
<i>T. elongatus</i>	WIRGQAYR---LLFHSPEASVQAFLLQHHYPQGVIPLRSDRGVDYQELAKLLVAEKFEAAD	117
	α2/α3 α3 α4 α5	
<i>C. reinhardtii</i>	DETRALLIKLAGPEAVKRNWVYFTEVKNISVTDFQTLNWLKASSNNKFGYSVQKEIWWQ	175
<i>Synechocystis</i>	EITRDKLCELAGPGASQRQWLYFTEVEKFPALDLHTINALWLLHSNGNFGFSVQRRLWLA	169
<i>T. elongatus</i>	RLTTQKLCELAGPLAQKRWLYFTEVEQLPIPDLTIDQLWLAFSLGRFGYSVQRQLWLW	177
	α6 α7	
<i>C. reinhardtii</i>	NQKRWPKEFKQIDWTQGENNNYRKWPMFEFIYSMDAPRGHLPLTNALRGTLQFQAIMHPA	235
<i>Synechocystis</i>	SGKEFTKLWPKIGWKS--NVWTRWPKGFTWDLAPQGHPLPLNQLRGVRVAESLYRHPV	227
<i>T. elongatus</i>	CGQNWDRLEWIKIGWRQG--KRWPRYPNEFIWDLAPRGHLPLTNQLRGVQVLNALLNHPA	235
	* * * *	
<i>C. reinhardtii</i>	FEKSSTAKTLDQKAAEAAGRTQSLF	260
<i>Synechocystis</i>	WSQYGW-----	233
<i>T. elongatus</i>	WTA-----	238

Figure 1

Sequence alignment of GUN4 performed with *ClustalW2*. Highlighted in yellow is the portion of *C. reinhardtii* GUN4 that is resolvable in the crystal structure. The underlined region shows peptide coverage from MSMS proteomic analysis of the crystals. Residues highlighted by stars are directly involved in porphyrin binding in the *Synechocystis* GUN4 structures and the blue highlighted residues are involved in secondary-structure rearrangements (Chen *et al.*, 2015). α-Helix sections are shown in bold, with the labelling above the sequences the same as used in the text and the other figures.

electron density. Difficult segments of the model were built with sensible geometry using *Buccaneer* (Cowtan, 2006). Structure validation was performed using the *MolProbity* web server (Chen *et al.*, 2010). Refinement statistics are shown in Table 4. The coordinates for the final model have been deposited in the Protein Data Bank (PDB entry 4ykb).

3. Result and discussion

3.1. Overall structure

Six GUN4 molecules were found in the crystallographic asymmetric unit (chains A–F). All of them are lacking the N-terminal domain (the first resolvable residues are 80–83), and all of them are lacking the last 22–25 residues (the last resolvable residues are 235–238) (see Fig. 1). The fold of the resolvable ~ 150 residues modelled is effectively the same in all chains (r.m.s.d. of 0.14–0.29 relative to chain A; see Fig. 2). Owing to the low resolution of the data, between 25 and 30% of the amino-acid side chains in the various chains have not been modelled (Table 4). Those unmodelled are predominantly on the surface of the domain, whilst those buried in the hydrophobic core of the domain are generally well ordered.

The fold is largely α -helical, as expected from the *T. elongatus* and *Synechocystis* structures (PDB entries 1z3x and 1y6i,

respectively). The A chain superposes with the 1z3x structure with an r.m.s.d. of 0.97 Å (over 143 C α positions) and with the 1y6i structure with an r.m.s.d. of 1.1 Å (over 131 C α positions) (Fig. 2).

The GUN4 molecules resolved in the crystal have quite clearly undergone proteolysis, as confirmed by MSMS proteomic analysis of tryptic digests of a number of crystals. The N-terminus of the resolvable domain immediately abuts a (missing) KKK sequence motif (analogous to the region linking the N- and C-terminal domains in related structures), whilst the C-terminal limit is adjacent to Lys238 (the last residue modelled in chain A). The C-terminal residues missing in our structure contain sites of phosphorylation in eukaryotes, but are not sequence features that are found in the cyanobacterial GUN4 molecules. The extended time taken for crystal growth presumably reflects the time taken for a contaminating protease within the crystallization drop to convert sufficient molecules to the doubly truncated form such that crystal growth could occur.

3.2. Comparison with cyanobacterial structures

Whilst the overall fold of the C-terminus of our *C. reinhardtii* GUN structure is highly similar to the cyanobacterial structures (Fig. 2; the *Synechocystis* structure is coloured light

Figure 2
Crystal structure of GUN4 from *C. reinhardtii*. (a) Three different perspectives are shown. The $\alpha 2/\alpha 3$ and $\alpha 6/\alpha 7$ loops are coloured pink and orange, respectively. The side chains of Pro216 and Thr218 within the $\alpha 6/\alpha 7$ loop are drawn as sticks. The N- and C-termini are also indicated. (b) Superposition of GUN4 from *C. reinhardtii* [tan, orange and pink; as in (a)] with cyanobacterial GUN4 structures from *Synechocystis* (light blue) and *T. elongatus* (dark blue).

Figure 3

Porphyrin-binding cleft of GUN4 from *C. reinhardtii*. GUN4 has been trimmed of the $\alpha 2/\alpha 3$ and $\alpha 6/\alpha 7$ loops to reveal the porphyrin-binding cleft. Both a front-on (left) and a side view (right) are presented. The side chains of numerous residues lining the channel can be viewed under the transparent surface. Tyr166 is positioned at the base and approximately in the middle of the cleft. Other residues are identified to help mark the extremities of the cleft.

blue and the *T. elongatus* structure dark blue), the largest differences, and also the largest differences between the cyanobacterial structures themselves, concern two ‘loop’ segments: the loop linking helices 2 and 3 of the *Synechocystis* structure and the loop linking helices 6 and 7. The $\alpha 2/\alpha 3$ loop is actually a short section of helix and the $\alpha 6/\alpha 7$ loop is a β -turn in the porphyrin-bound *Synechocystis* structures (Chen *et al.*, 2015), whilst these loops help to cover the hydrophobic ‘greasy palm’ in the unbound *Synechocystis* structure (Verdecia *et al.*, 2005) (Fig. 2; $\alpha 2/\alpha 3$ and $\alpha 6/\alpha 7$ loops). The *T. elongatus* structure and our *C. reinhardtii* structure are thus intermediate between the porphyrin-bound and unbound *Synechocystis* structures (Fig. 2).

The $\alpha 6/\alpha 7$ loop covers the greasy-palm surface but projects very few residues into the cleft, namely a proline residue (conserved in all three organisms) and a leucine residue (substituted by a threonine residue in the *T. elongatus* structure and in our *C. reinhardtii* structure) (Fig. 2; proline and threonine residues from our structure are drawn as sticks). The porphyrin-bound *Synechocystis* structures (Chen *et al.*, 2015) clearly show that this $\alpha 6/\alpha 7$ loop has a propensity for conformational dynamics to form a β -hairpin, making a more structured cleft which enables porphyrin binding.

4. Conclusion

We have solved the crystal structure of the GUN4 protein from *C. reinhardtii*. Proteolysis within the crystallization experiment removed the N-terminal domain and the last ~20 residues, leaving the bulk of the C-terminal domain intact; this domain is involved in binding PPIX and Mg-PPIX. Although

the structure is of relatively low resolution, the bulk of the side chains which line the ‘greasy palm’ cleft involved in binding the porphyrin ring can be resolved. When superposed with cyanobacterial GUN4 structures, whilst the α -helical scaffolds are conserved, the $\alpha 2/\alpha 3$ and $\alpha 6/\alpha 7$ loops previously predicted to undergo movement to accommodate porphyrin binding display markedly different conformations. This diversity of fold captured by multiple crystal structures supports the notion that these loops undergo conformational rearrangement to accommodate the insertion of PPIX or Mg-PPIX into the underlying hydrophobic cleft (Fig. 3), as observed in the recent porphyrin-bound *Synechocystis* structures (Chen *et al.*, 2015).

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