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C-terminal residues of *Oryza sativa* GUN4 are required for the activation of the ChlH subunit of magnesium chelatase in chlorophyll synthesis

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ABSTRACT

***Oryza sativa* GUN4 together with the magnesium chelatase subunits ChlI, ChlD, and ChlH have been heterologously expressed and purified to reconstitute magnesium chelatase activity in vitro. Maximum magnesium chelatase activity requires pre-activation of OsChlH with OsGUN4, Mg^{2+} and protoporphyrin-IX. OsGUN4 and OsChlH preincubated without protoporphyrin-IX yields magnesium chelatase activity similar to assays without OsGUN4, suggesting formation of a dead-end complex. Either 9 or 10 C-terminal amino acids of OsGUN4 are slowly hydrolyzed to yield a truncated OsGUN4. These truncated OsGUN4 still bind protoporphyrin-IX and Mg-protoporphyrin-IX but are unable to activate OsChlH. This suggests the mechanism of GUN4 activation of magnesium chelatase is different in eukaryotes compared to cyanobacteria as the orthologous cyanobacterial GUN4 proteins lack this C-terminal extension.**

Structured summary of protein interactions:
ChlH and **ChlH** bind by molecular sieving (View interaction)
ChlD and **ChlD** bind by molecular sieving (View interaction)

1. Introduction

Magnesium chelatase (Mg-chelatase), which catalyzes the first committed step of the chlorophyll biosynthesis pathway, involves insertion of magnesium into protoporphyrin IX (proto). It is also the most complex step of chlorophyll synthesis, as magnesium chelatase employs at least three subunits. In anoxygenic bacteriochlorophyll synthesizing organisms it consists of the proteins known as BchI, BchD, and BchH [1]. In oxygenic chlorophyll synthesizing organisms it consists of the orthologous proteins ChlI, ChlD and ChlH as well as the accessory protein GUN4 that is required for optimal activity [2–6]. BchH/ChlH is the porphyrin-binding subunit and is most likely to have the active site for chelation [7,8]. ChlI is a member of the AAA^+ superfamily and has ATPase activity [9,10]. The larger ChlD also has an AAA^+ domain at the N-terminal and a C-terminal integrin I domain containing a divalent metal ion dependent adhesion site (MIDAS) motif [11]. The BchI:BchD orthologues form a stable "motor" complex of two stacked hexameric rings [12].

GUN4 was identified from a screen for plastid-to-nucleus signaling mutants [4]. It is a positive regulator of chlorophyll biosynthesis but is not absolutely essential for the accumulation of chlorophyll in *Arabidopsis thaliana* [4]. All of the in vitro activity data for GUN4 comes from experiments with the cyanobacterial GUN4. GUN4 from *Synechocystis* (SynGUN4) stimulates the *Synechocystis* Mg-chelatase in vitro [13,14] and is necessary for optimal magnesium chelatase activity. The cyanobacterial GUN4 binds the porphyrin substrate, proto, and the product, Mg-protoporphyrin IX (Mg-proto) [4,14,15]. For the cyanobacterial enzyme the activation of ChlH by GUN4 is at least in part due to the porphyrin binding properties of GUN4. When *cch* and *gun5* mutations are introduced into *Synechocystis* ChlH, the *Synechocystis* GUN4 overcomes the block in magnesium chelation due to these mutations indicating that the subunits interact [16]. The X-ray crystallographic structures of GUN4 from two cyanobacteria have been determined but these have a different molecular architecture from the chloroplastic GUN4 [14,15]. The N-terminal 4 helix bundle found in cyanobacterial GUN4 proteins is absent from chloroplastic GUN4 which also have an additional ~40 amino acid C-terminal extension [15,17].

All of the in vitro studies of GUN4 function have been on the cyanobacterial proteins while only in vivo studies have been conducted on the eukaryotic GUN4 [4,18–20]. These in vivo studies include the membrane distribution of GUN4 under various conditions and its chromatographic behaviour. GUN4 co-chromatographs with the much larger ChlH under some conditions and is suggested to be

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involved in porphyrin solubilisation and channelling of the porphyrin substrate into chlorophyll synthesis [19,20]. One of the curious features of the plant GUN4s is that they all appear to be present in two different molecular weight forms in planta and in organello. These two GUN4 proteins differ by approximately 2–4 kDa [4,18–20].

Despite this recent progress, the mechanism of GUN4 activation of the Mg-chelatase reaction in higher plants remains unclear as no one has reported high activity from purified higher plant magnesium chelatase subunits. To address this we used a new model system for in vitro studies of Mg-chelatase from *Oryza sativa*. Using purified subunits of the rice Mg-chelatase we show that GUN4 is required for formation of the active enzyme complex in vitro by activating the ChlH with proto and magnesium. We define the conditions under which ChlH is activated by GUN4 and demonstrate that the GUN4 activated ChlH is the optimal chelatase substrate for the ATP dependent ChlI:ChlD complex. We also show that the C-terminal domain of GUN4 is important for this activation.

2. Materials and methods

2.1. Cloning of Rice ChlH, ChlD, ChlI, and GUN4

All the recombinant proteins were cloned from cDNA of *O. sativa l. japonica Nipponbare* into expression vector pET 28a (Merck-Novegen, Darmstadt, Germany) using standard cloning protocols. Primers were designed with BamHI or EcoRI and NotI or XhoI sites at the ends of the products (Supplementary Table 1).

2.2. Expression and purification

The proteins were expressed in *Escherichia coli* strains BL21(DE3) grown at 37 °C in 2×YT medium supplemented with 50 mg/l kanamycin. Cells were induced at OD600 = 0.6–0.8 with 0.1 mM isopropyl-β-D-thiogalactoside and incubation was continued overnight at 16 or 25 °C. The cells were harvested by centrifugation and stored at −20 °C.

His_6 tagged OsChlH, OsChlI, and OsGUN4 proteins were purified on a HisTrap FF column and immediately desalted by PD-10 column according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA). OschH was further purified by gel filtration using a Superdex™ HR 10/30 column (GE Healthcare, Piscataway, NJ, USA). All the proteins were desalted into exchange buffer (10 mM Tricine–NaOH, pH 8.0, 10% [w/v] glycerol, 2 mM EDTA, and 2 mM DTT).

His_6 tagged OsChlD protein was expressed as inclusion bodies, and these were purified on a HisTrap FF column under denaturing conditions in 6 M urea and desalted before further purification by anion-exchange chromatography using SOURCE 15Q resin (GE Healthcare, Piscataway, NJ, USA). OsChlD was then fractionated on a Superose™ 6 prep grade 16/60 column (GE Healthcare, Piscataway, NJ, USA) at a flow rate of 0.1 ml/min and 1.25 ml fractions were collected. Finally, the protein was desalted into 6 M urea, 10 mM Tricine–NaOH, pH 8.0, 10% [w/v] glycerol, 2 mM EDTA, and 2 mM DTT.

Protein determinations were performed with Bio-Rad protein assay reagent (Bio-Rad Laboratories Pty. Ltd., Hercules, CA, USA) according to the manufacturer's instructions, with BSA as a standard.

2.3. Magnesium chelatase assays

All assays were performed as previously described [6,21] with the following modifications. The final concentrations of assay components were 50 mM Tricine–NaOH (pH 8.0), 15 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM ATP (assay buffer), 500 nM Proto, 200 nM OsChlI, 500 nM OsChlH, 500 nM OsGUN4, and 20 nM OsChlD.

2.4. Porphyrin binding and absorbance spectra

Porphyrin induced tryptophan fluorescence quenching was used to estimate porphyrin binding constants to GUN4 isoforms [14]. Absorbance spectra were in exchange buffer and for each analysis, 2 μM Proto or 0.5 μM Mg-Proto was incubated with 2 μM or 0.5 μM OsGUN4 and/or OsChlH respectively at 30 °C for 20 min.

2.5. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis

Protein bands from SDS–PAGE were in-gel digested overnight with trypsin, peptides extracted with 0.1% TFA, concentrated on a C18 ZipTip (Eppendorf, Hamburg, Germany) and eluted onto the MALDI plate with 80% acetonitrile in 0.1%TFA containing 4 mg/ml cinnamic acid. Intact protein mass spectra were obtained by mixing 10–100 ng of purified protein with 4 mg/ml sinapinic acid matrix in 20% acetonitrile 0.1% TFA, spotted onto the MALDI plate. The dried spots were subjected to MALDI-TOF MS and/or MS-MS on an ABI-4800 (Foster City, CA). Mascot (Matrix Science, London, UK) and X-Tandem were used to search for matches. All spectra were an average of 500–2000 single-shot spectra to achieve a good signal-to-noise ratio.

3. Results and discussion

3.1. Purification

The rice *OsChlI*, *OsChlD*, *OsChlH*, and *OsGUN4* genes were cloned into pET 28a, which carry a N-terminal hexahistidine (His_6) affinity tag. Coomassie Blue-stained SDS–PAGE gels revealed that the OsChlI and OsGUN4 were pure after HisTrap FF purification (Fig. 1, lane 1 and lane 2), whereas the OsChlD and OsChlH still contained

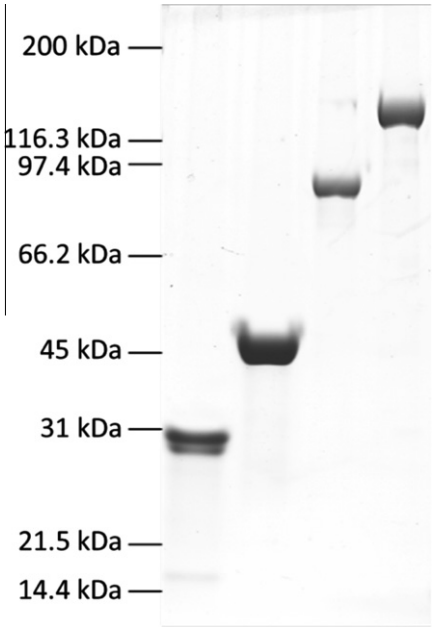

Fig. 1. SDS–polyacrylamide gel electrophoresis of the purified His_6 tagged proteins. Lane 1, OsGUN4; lane 2, OsChlI; lane 3, OsChlD; lane 4, OsChlH. The positions of molecular mass markers are indicated at the left.

many other protein bands. The OsChlD and OsChlH proteins were further purified on 10 × 1 cm SOURCE 15Q column using a linear salt gradient and followed by gel filtration chromatography (Fig. 1, lane 3 and 4). The OsChlD subunit eluted as a very large (molecular mass larger than 500 kDa) and stable complex even though it was solubilized in 6 M urea. The complex remains soluble when diluted or desalted into urea free buffer at 0.5 mg/ml and is active in magnesium chelatase assays. Since the homologous BchD forms a double hexameric ring complex with BchI [12] the size of this OsChlD is consistent with a hexamer.

Unlike the BchH protein from *R. capsulatus* [22] and the ChlH from *Synechocystis* [23], the OsChlH protein did not have any detectable proto bound after HisTrap FF purification (data not shown). OsChlH protein eluted as a monomer at an apparent molecular mass of 150 kDa by size-exclusion chromatography, when the protein was expressed at 16 °C (Fig. 1, lane 4). When expressed at higher temperatures the OsChlH eluted as a soluble aggregate suggesting partial misfolding.

3.2. GUN4 is required for active enzyme complex of Mg-chelatase

GUN4 stimulates Mg-chelatase by a mechanism that involves binding the ChlH subunit of Mg-chelatase, as well as proto and Mg-proto, substrate and product of Mg-chelatase reaction [4,14,15,24]. However, GUN4 is not absolutely required for Mg-chelatase activity in vitro [25] or in vivo [4].

Mg-chelatase activity in the absence of OsGUN4 is barely detectable in vitro and exhibits a very long lag phase before magnesium chelation begins (Fig. 2A). The preincubation of OsGUN4 with proto, magnesium and OsChlH eliminated the lag (Fig. 2A) and increased the maximum reaction rate 16-fold (Fig. 2B). These results suggest that OsGUN4 activates the OsChlH complex as previously described for *Synechocystis*.

3.3. Requirements for GUN4 activation of ChlH

The activation of OsChlH by OsGUN4 is dependent on OsGUN4, magnesium, proto and possibly ATP. We investigated this activation by doing a matrix of 128 experiments in which OsChlH and OsGUN4 were pre-incubated in 50 mM Tricine–NaOH pH 8.0, 2 mM DTT and all possible combinations of 15 mM Mg^{2+}, 1 mM ATP and/or 2 μM proto in a one-step or two-step pre-incubation procedure, as detailed below, prior to assaying for magnesium chelatase activity with the ChlI:ChlD complex as detailed in the methods.

In the one-step pre-incubations, OsGUN4 and OsChlH were pre-incubated separately in buffer and with all possible combinations of Mg^{2+}, ATP, and proto for 25 min at 25 °C. These eight pre-incubations with GUN4 and eight pre-incubations with OsChlH (see combinations in Table 1) were then mixed quickly together and immediately assayed for magnesium chelatase activity with ChlI:ChlD. This yielded 64 different assay progress curves from which maximum rates and lags could be calculated (see Supplementary Table 2). The progress curves for these assays were classified into one of three types of curves. Type 1. 8-12 min lag before reaching a rate of at least 0.6 nmol min^{-1} (nmol ChlD)$^{-1}$. Type 2. 12-21 minute lag before reaching a rate of at least 0.35 nmol min^{-1} (nmol ChlD)$^{-1}$. Type 3. Over 21 minute lag with a rate less than 0.35 nmol min^{-1} (nmol ChlD)$^{-1}$ (Fig. 3 and Supplementary Table 2).

In the two-step pre-incubations, the eight OsGUN4 and eight OsChlH pre-incubations described above for one step incubations, were mixed and incubated for a further 25 min at 25 °C before assaying for magnesium chelatase activity with ChlI:ChlD. This yielded an additional 64 different assay progress curves from which maximum rates and lags could be calculated (see Supplementary Table 2). The progress curves for these 64 two-step pre-incubation assays were classified into one of four types of curves. Type A. Less than 5 min lag and rate of 0.8–1.2 nmol min^{-1} (nmol ChlD)$^{-1}$. Type B. Less than 12 min lag and rate 0.6-0.9 nmol min^{-1} (nmol ChlD)$^{-1}$. Type C. Over 12 min lag with a rate above 0.3 nmol min^{-1} (nmol ChlD)$^{-1}$ Type D. Over 20 min lag and rate less than 0.3 nmol min^{-1} (nmol ChlD)$^{-1}$ (Fig 3 and Supplementary Table 2). The curve classification data from all 128 assays is summarized in Table 1 with actual maximum rates and lag times for individual assay conditions shown in Supplementary Table 2.

Two things are obvious from the pattern of data summarized in Table 1: Firstly, when proto is absent from preincubations of both OsChlH and OsGUN4 the magnesium chelatase activity is similar to that observed for magnesium chelatase assays without OsGUN4 (Table 1: dark shaded "3, D"). This indicates that proto needs to be bound to either OsChlH or OsGUN4 prior to them interacting otherwise activation is prevented. This suggests formation of a dead end complex between OsGUN4 and OsChlH. Secondly, pre-incubation of OsChlH with proto is a key step in the activation

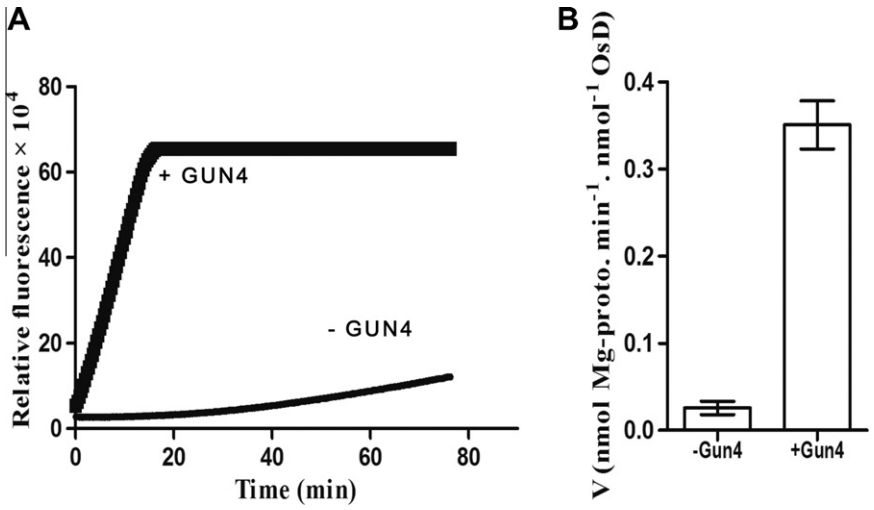

Fig. 2. OsGUN4 is required for active enzyme complex of Mg-chelatase. Assays conditions and final concentrations of components as described in Materials and Methods. The assay was started by mixing OsChlI and OsChlD that had been preincubated in assay buffer for 15 min together with OsChlH and proto that had been preincubated with or without OsGUN4 in assay buffer for 15 min. (A) Assay time course with fluorescent detection of Mg-proto (E_x 420 nm, E_m 595 nm). (B) Magnesium chelatase activity as means of triplicates ± standard deviation of the maximum rates of time course data shown in A.

Table 1
Summary of the different magnesium chelatase activity progress curve profiles using the 128 different one- and two-step preincubations of OsGUN4 and OsChlH. Numbers refer to progress curve types in Fig 3 for one-step incubation and letters refer to progress curve types in Fig 3 for two-step incubations.

		OsGUN4 preincubation additions							
		None	Mg^{2+}	ATP	Mg^{2+} ATP	Proto	Proto Mg^{2+}	Proto ATP	Proto Mg^{2+} ATP
OsChlH preincubation additions	None	3, D	3, D	3, D	3, D	2, C	2, B	2, C	2, B
	Mg^{2}	3, D	3, D	3, D	3, D	2, B	2, B	2, B	2, B
	ATP	3, D	3, D	3, D	3, D	2, B	2, B	2, A	2, A
	Mg^{2+} ATP	3, D	3, D	3, D	3, D	1, A	2, A	1, A	2, A
	Proto	1, B	1, A	1, B	1, A	1, B	*, A	1, B	1, A
	Proto Mg^{2}	1, A	1, A	1, A	1, A	1, A	1, A	1, A	1, A
	Proto ATP	1, B	1, A	1, B	1, A	1, B	1, A	1, B	1, A
	Proto Mg^{2+} ATP	1, A	1, A	1, A	1, A	1, A	1, A	1, A	1, A

* Not determined. Mg^{2+} at 15 mM, ATP at 1 mM and proto at 2 μM in the preincubations. Shading from light to dark indicates suboptimal to no activation, respectively.

because maximum magnesium chelatase activity is achieved in all single step assays in which OsChlH was pre-incubated with proto (Table 1: bottom four rows) and in all of the two step assays except for those in which magnesium was absent. This suggests that OsGUN4, although it has been shown to bind proto, may not be involved in delivery of proto per se but perhaps is involved in binding to proto on the OsChlH subunit. In addition to protoporphyrin, magnesium is an important component for the preactivation as slightly lower rates and/or longer lags are observed (curve types 1B in Table 1) when it is absent from the pre-incubations. However, pre-incubation of OsChlH in the absence of proto or ATP (Table 1: rows 1–3, columns 5–8) prevents maximum activation when these OsChlH pre-incubations are subsequently mixed with OsGUN4 indicating that proto and ATP have a stabilizing effect on OsChlH.

3.4. C-terminal residues of GUN4 are required to activate ChlH

A. thaliana GUN4 have two different molecular weight forms in vivo when examined by western blotting, and three processed forms when labeled GUN4 was imported into pea chloroplasts [4,18–20]. The OsGUN4 expressed in *E. coli* was also found as two different molecular weight forms by SDS–PAGE (Fig. 1). The larger Mwt form predominated and was separable from the smaller form on an anion exchange column (Fig. 4). Moreover, both truncated and full length forms of OsGUN4 bound Mg-proto, K_D of 685 ± 140 nM and 700 ± 155 nM, respectively, and proto, K_D of 259 ± 14 nM and 146 ± 10 nM, respectively (Supplementary Fig. 1). In addition, porphyrin binding to both truncated and full length OsGUN4 gave similar spectral shifts of the porphyrin Soret bands (Supplementary Fig. 2). However, only the full length OsGUN4 was able to activate the OsChlH subunit of the magnesium chelatase, as shown by the differences in activity between different OsGUN4 fractions (Fig. 4) and also when compared with activity differences with and without OsGUN4 (Fig. 2). Intact mass analysis confirmed the larger form was the expected Mwt of OsGUN4 without the start methionine of 26503 ± 30 mz. The smaller form had two predominant peaks in the MS spectra of 25364 ± 30 *m*/*z* and 25192 ± 30 *m*/*z* (Supplementary Figs. 3 and 4). These truncated OsGUN4 mass peaks correlate with the expected molecular weights of the full length OsGUN4 without the start methionine which have had either 9 amino acids hydrolysed (25355 Da) or 10 amino acids hydrolysed (25199 Da) respectively from its C-terminus. Tryptic digest and MALDI-TOF MS-MS analysis revealed that the truncated OsGUN4 form had all of the ions attributable to the full length OsGUN4 except for the predominant C-terminal peptide ion observed in the MS spectrum for DFFKPDYSF at *m*/*z* 1165.520 (Supplementary Fig. 4). Thus the truncated OsGUN4 observed by SDS–PAGE consists of two smaller Mwt forms that are 9 amino acids and 10 amino acids shorter at the C-terminal than the full length GUN4.

The orthologous C-terminal peptide VFKTNYpSF has been identified as a phospho-peptide in *A. thaliana* [26] and all plant and algal GUN4 have the YSF as a conserved sequence. The recombinant OsGUN4 is not phosphorylated as the expected DFFKPDYpSF peptide at *m*/*z* 1245.49 has not been observed in any of our OsGUN4 MS spectra (see Supplementary Fig. 4 as example). This suggests that phosphorylation, if it occurs in rice, is due to a specific chloroplastic kinase. We have been unable to form an OsGUN4-OsChlH complex in vitro that is stable during gel filtration as observed for the ChlH-GUN4 isolated from stromal extracts [4]. Given that the OsGUN4 without this C-terminal peptide is unable to activate OsChlH the possibility is that phosphorylation of OsGUN4 is required for formation of a stable OsGUN4-OsChlH complex.

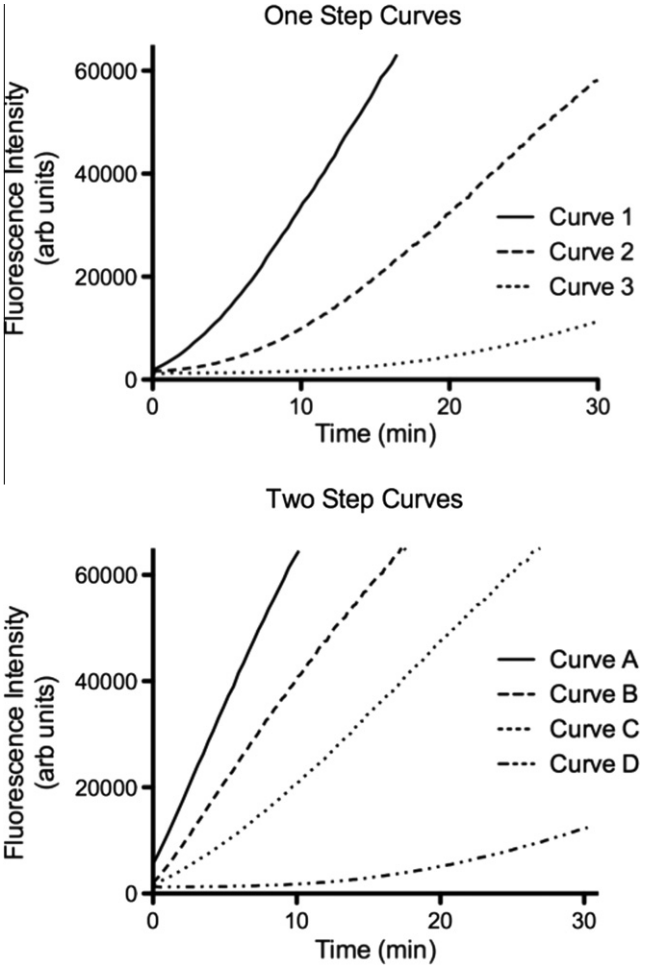

Fig. 3. Types of magnesium chelatase time course progress curves, with fluorescent detection of Mg-proto (E_x 420 nm, E_m 595 nm), observed using OsChlH and OsGUN4 that has been activated in different ways. See Table 1 for details.

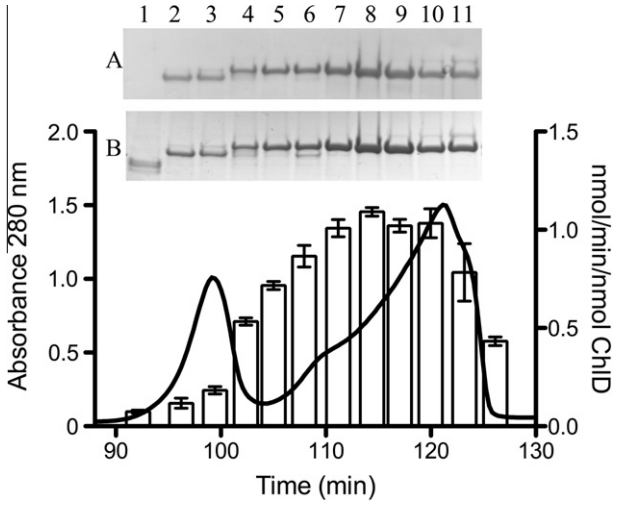

Fig. 4. Anion exchange separation trace at 280 nm of truncated OsGUN4 from full length OsGUN4. The bar graph shows the corresponding magnesium chelatase activity measured when 100 pmol OsGUN4 protein equivalent of the fraction was used to activate 100 pmol of OsChlH in the magnesium chelatase reaction. The two SDS-PAGE gel image panels above the chromatogram are of the corresponding fractions shown in the bar graph beneath the gel lanes. Image A is the western blot to detect the N-terminal 6xHis tag and image B is the stained blot.

4. Conclusions

The GUN4 activation of magnesium chelatase has been biochemically dissected and requires proto and Mg^{2+}. Optimal activation requires proto preincubation with OsChlH and inhibition of activation occurs when proto is omitted from pre-incubations of OsGUN4 and OsChlH. OsGUN4 which is missing 9–10 residues from the C-terminal still binds porphyrin but does not activate the OsChlH subunit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.12.026.

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