GUN4-Protoporphyrin IX Is a Singlet Oxygen Generator with Consequences for Plastid Retrograde Signaling*

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The consequence of organisms relying on oxygen for metabolism is the continual generation of reactive oxygen species (ROS) including superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (·OH), and singlet oxygen (O$_2$(a$_1^g$)) (1–3). In plants, the reaction center of PSII and the antenna system of the chloroplast is the major source of ROS generation (4). A low to medium concentration of ROS triggers an acclimation response, whereas a high level initiates cell death (5). In either case, a signal is delivered from the chloroplast to the nucleus (retrograde signaling) (3, 5). In vivo sub-lethal O$_2$(a$_1^g$) levels have been generated by a Arabidopsis flu mutant, which resulted in protochlorophyllide accumulation (6, 7) and triggered signaling mechanisms, which led to changes in nuclear gene expression (6). This effect was suppressed by the double mutant EXECUTER 1 and 2, thus implicating these two proteins in O$_2$(a$_1^g$)-dependent plastid-to-nucleus signaling (8, 9).

The GUN1–5 (genomes uncoupled) proteins are also implicated in retrograde signaling because mutant gun plants in Arabidopsis still allow transcription of photosynthesis-associated nuclear-encoded genes (PhANG), for example the genes for light-harvesting chlorophyll a/b-binding (LHCB) proteins, under conditions when transcription is normally repressed (10). Exogenous feeding of Mg-PPIX resulted in reduced light-harvesting chlorophyll a/b-binding proteins; therefore this porphyrin was suggested as a negative retrograde signaling molecule (11). However, intracellular concentrations of PPIX and Mg-PPIX never reach the levels required for this to occur (12–14).

GUN4 mutants in Arabidopsis and Chlamydomonas reinhardtii show a pale-green phenotype indicating reduced chlorophyll biosynthesis (15, 16). In vitro it is a regulatory tetrapyrrrole-binding protein involved in enhancing Mg-chelatase activity in the chlorophyll biosynthetic pathway presumed to be via substrate delivery and/or release (15, 17–19). Mg-chelatase subunit ChlH, also known as GUN5, binds PPIX and has been proposed to receive its PPIX from GUN4 (15, 17–19). This PPIX bound to ChlH has Mg$^{2+}$ inserted during ATP hydrolysis by the ChlI-ChlD Mg-chelatase motor complex (20). Free porphyrins act as photosensitizers, with O$_2$(a$_1^g$) being generated in the light under aerobic conditions, and it has been proposed that all porphyrins in vivo exist in complex with a protein partner such as GUN4 and/or ChlH to prevent O$_2$(a$_1^g$) being generated (21, 22). This model is widely accepted in part because GUN4 appears to be exclusively found in oxygenic photosynthetic organisms, and because it appears to play a direct role or an indirect role in oxygen-related stress (15, 16, 23). It is generally presumed that GUN4 plays a photo-protective role within the chloroplast, which may protect plants from ROS that are produced by collisions between O$_2$ and triplet excited porphyrins formed in the light (15, 17). Furthermore, it was suggested that GUN4 participates in Mgx-PPIX trafficking or shields PPIX and Mg-PPIX from collisions with O$_2$ that might yield ROS...
(15). Additionally, a Mg-chelatase-deficient strain chlD-1/GUN4 that accumulates PPIX and overexpresses GUN4 implicates GUN4 in retrograde signaling through sensing and binding tetrapyrrole metabolites with the suggestion that it may prevent O$_2$(a$\Delta_g$) production (24).

The crystal structure of ligand-free GUN4 suggested that its porphyrin-binding domain offered protection from collisions with molecular O$_2$ (18). However, recent structural data with PPIX-bound GUN4 (25) show a half-open porphyrin-binding pocket, which is presumably important for delivery of the substrate to ChlH or ChlM or in retrograde signaling pathways. This cleft is similar in the C. reinhardtii GUN4 structure (26). The PPIX bound to GUN4 is thus exposed and contrasts with a proposed deep hydrophobic porphyrin-binding cleft in ChlH (27).

Here we show that PPIX bound to GUN4 or oxidatively damaged ChlH results in a significant increase in the rate of singlet oxygen production over PPIX or PPIX bound to other proteins including undamaged ChlH. Combined with kinetic data on the assembly of a functional Mg-chelatase, it appears that GUN4 acts as a sensor of Mg-chelatase activity and PPIX availability. When PPIX is partitioned to GUN4 rather than ChlH or when ChlH is damaged, singlet oxygen is produced in the light. The diffusion limit of singlet oxygen requires that GUN4-PPIX interact directly with the singlet oxygen sensor, such as one of the EXECUTER proteins, producing a negative signal inhibiting specific PhANG gene expression. This negative retrograde signaling system would work in concert with the bilin- or phycobiliprotein-based positive retrograde signaling systems found in Chlamydomonas and plants, respectively (28).

**Experimental Procedures**

**Porphyrin Solutions**—Stock solutions were prepared in the dark wherein a small amount of PPIX or Mg-PPIX powder was dissolved in a few microliters of 1 M NaOH, diluted 100-fold with water, and centrifuged at 16,000 × g for 5 min. The supernatant was transferred to a fresh tube, and the concentration was measured in 5% HCl at 407.5 nm using a molar extinction coefficient of 278,000 M$^{-1}$ cm$^{-1}$ (29). Mg-PPIX was synthesized from PPIX according to the procedure of Ref. 30. Each porphyrin solution was prepared fresh daily.

**Cloning of chlI1, chlI2 chlD, chlH, and gun4**—RNA was extracted from C. reinhardtii cells using a MasterPure yeast RNA purification kit (Epicentre) according to the manufacturer’s instructions. mRNA was converted to cDNA using a SuperScript VILO cDNA synthesis kit using random primers. Details of chlH cloning into pET-28a was previously described (31). The chlD and gun4 genes from C. reinhardtii were cloned from cDNA into expression vector pET28a (Merck-Novagen), whereas chlI1 and chlI2 were cloned into pGEX-6P-1. Transformation of each construct into Escherichia coli strain BL21 (DE3) from Life Technologies was performed according to the manufacturer’s instructions.

**Protein Expression and Purification**—Each protein was expressed and purified separately with an N-terminal poly-His tag (ChlD, ChlH, GUN4) or a GST tag (ChlI1 and ChlI2), which was later cleaved with PreScission protease (GE Healthcare). Protein expression was as described previously (31) with the exception that PPIX and light were not used during induction for ChlD, GUN4, ChlI1, or ChlI2 proteins. His$_6$-tagged ChlD, ChlH, and GUN4 were purified by immobilized metal ion affinity chromatography as described for ChlH (31). ChlH and GUN4 were additionally purified by gel filtration chromatography, using a Superose 6 10/300 GL column (GE Healthcare) for ChlH or a Superdex 200 HR 10/30 column for GUN4. ChlH separated into the minor oligomeric and major monomeric components as described previously (31). Coomassie Blue-stained SDS-PAGE gel indicated that the ChlH and GUN4 proteins were >95% pure after purification.

GST-tagged ChlI1- or ChlI2-expressed lysates in PBS were applied to a 5-ml GST-trap column, and then washed with PBS until no protein was detected via Bradford assay. Protein was eluted with 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione, desalted into PreScission cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.5), and concentrated to 5 ml. 1 unit of PreScission protease was added to 100 µg GST-tagged ChlI1 or ChlI2, and the digest was allowed to continue for 15 h at 4 °C. The mixture was reapplied to the GST-trap column, and the run-through containing ChlI1 or ChlI2 was desalted into exchange buffer, concentrated, and stored at −80 °C in small aliquots.

**Reconstitution of ChlH and GUN4 with PPIX for Singlet Oxygen Measurements**—Reconstitution of ChlH or GUN4 with PPIX was achieved by mixing an equal concentration of PPIX with protein, at a minimum final concentration of 10 µM, pre-incubating at 22 °C for 20 min, and desalting into reconstitution buffer (20 mM Tricine-NaOH, pH 8.0, 2 mM MgCl$_2$) using a 5-ml HiTrap desalting column (GE Healthcare). The peak elution fractions identified by assaying with Bradford reagent (BioRad) also contained the PPIX and were pooled and used in further experiments.

**Mg-chelatase Enzyme Assay**—Preparation of each protein solution was in assay buffer consisting of 50 mM Tricine-NaOH (pH 8.0), 15 mM MgCl$_2$, 2 mM DTT, 4 mM ATP (31). Preparation of the ChlI1-ChlI2-ChlD complex involved an initial preparation of a 4-fold concentrated composition of 200 nM ChlD, 200 nM ChlI2, and 200 nM ChlI1, which was incubated for 20 min at room temperature prior to use. Assays were started by adding 12.5 µl of ChlI1-ChlI2-ChlD to a microtiter plate and then adding 25 µl of ChlH-PPIX-Gun4 and 12.5 µl of 1400 nM ChlI1. Final concentrations of Mg-chelatase subunits used in the assays were: 50 nM ChlD, 400 nM ChlI1, 50 nM ChlI2, 500 nM ChlH, together with 500 nM GUN4 and 2000 nM PPIX. Fluorescence was measured immediately in a BMG Labtech PHERaStar plate reader using excitation at 420 nm and emission at 600 nm, with time intervals of 30 s for a duration of 30 min to determine the maximum rate in nM Mg-PPIX min$^{-1}$ assay using a Mg-PPIX standard curve. Experiments using ChlH and GUN4, preincubated with or without PPIX, involved preincubating these proteins for 20 min at 22 °C. For these assays, these preincubated GUN4 and ChlH proteins were rapidly mixed to a concentration of 2 µM and then added to an equal volume of 8 µM PPIX for final concentrations of 1 µM protein and 4 µM PPIX. 25 µl of this mixture was used in an Mg-chelatase assay within 1 min. Oligomeric ChlH from rice and barley has variable activity, typically very low, when compared with the monomer, but in some preparations, it was up to
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25% (31). The same variable results were observed using C. reinhardtii oligomeric ChlH.

SOSG Fluorescent Singlet Oxygen Production Measurements—The rate of O$_2$(a$_1^g$) production was measured using Singlet Oxygen Sensor Green (SOSG) reagent, a ROS-selective fluorescent sensor (Life Technologies/Molecular Probes) (32). PPIX and Mg-PPIX were used as photosensitizers to generate O$_2$(a$_1^g$) at a final concentration of 10 $\mu$M unless otherwise stated in the figure legends. All assays were measured under the exposure of direct incandescent light 12 cm from the plate through a glass sheet to prevent heating with a measured light intensity of 132 $\mu$mol m$^{-2}$ s$^{-1}$. Assays were performed in triplicate in buffer consisting of 50 mM Tricine-NaOH, pH 8.0, 10% w/v glycerol, 2 mM MgCl$_2$, and 2 mM DTT. Concentrations of proteins in presence of 5 $\mu$M PPIX, ChlH-PPIX, GUN4-ChlH-PPIX, and BSA were used during exposure of the assay mixture to medium intensity and Mg-PPIX. The kinetics of the SOSG fluorescence development was measured in the presence of photosensitizers PPIX and Mg-PPIX. The SOSG method using histidine, which reacts with O$_2$(a$_1^g$) to produce a short-lived peroxide species, which in turn forms oxidized histidine, resulting in concentration-dependent O$_2$(a$_1^g$) removal from solution that can be measured using an oxygen electrode (33, 35–37). Using this method, the rate of O$_2$(a$_1^g$) removal using PPIX (O$_2$(a$_1^g$) sensitizer) was saturated above 10 $\mu$M PPIX as might be expected under light-limiting conditions. The O$_2$(a$_1^g$) production rates indicate that the O$_2$(a$_1^g$) production is dependent on PPIX concentration up to ~4 $\mu$M. In all further titration experiments, 2.5 $\mu$M PPIX was used as this is close to concentrations found for PPIX in vivo and it is above the $K_d$ values for porphyrin binding to the proteins tested. Using this method, GUN4 protein significantly enhances the rate of O$_2$(a$_1^g$) production from PPIX (Fig. 1D) in a concentration-dependent manner, and this was ~2.5-fold greater than BSA-PPIX. BSA was used as a control as it is known to bind PPIX (38) and the titration saturation observed (Fig. 1D) is consistent with reported $K_d$ values for PPIX binding to GUN4 (0.2–0.5 $\mu$M) and BSA (2 $\mu$M). A comparison of the direct effect of PPIX bound to GUN4 and ChlH proteins in O$_2$(a$_1^g$) production was tested by reconstituting ChlH and GUN4 with PPIX. The O$_2$(a$_1^g$) production level of ChlH-PPIX monomer and oligomer and GUN-PPIX proteins is increased significantly in a concentration-dependent manner (Fig. 1E). The rate of O$_2$(a$_1^g$) production is enhanced in the presence of protein as found using the SOSG method. The oxidatively damaged form of ChlH-PPIX (oligomer) produces O$_2$(a$_1^g$) at two times the rate of GUN4-PPIX, whereas ChlH (monomer) had half the GUN4-PPIX O$_2$(a$_1^g$) production rate (Fig. 1, E and F). A comparison of these O$_2$(a$_1^g$) production rates with PSI1 (Fig. 1F) shows that these porphyrin complexes can produce O$_2$(a$_1^g$) at much greater rates, which has implications for signaling.

GUN4 with PPIX Bound Slows the Assembly of Mg-Chelatase—Mg-PPIX is formed at a constant rate with no lag phase when ChlH is preloaded with PPIX and assayed by combining with GUN4 that does not have PPIX prebound as shown in Fig. 2A. Similarly, when Mg-chelatase is assayed in the absence of GUN4, Mg-PPIX is produced at a constant but lower rate (Fig. 2B) as has been reported previously, indicating that GUN4 stimulates Mg-chelatase activity. However, when GUN4 is preincubated with PPIX and used in Mg-chelatase assays, a very long 5-min lag phase results as shown in Fig. 2A, indicating an inhibition of the assembly of a functional Mg-chelatase complex. This lag phase is eventually overcome to obtain a maximum rate that is still greater than ChlH alone but less than the
lag-free rate. The experiment with mixing PPIX-free ChlH and GUN4 also shows a long lag but has a lower maximum rate than ChlH alone, which may suggest a dead-end complex between ChlH and GUN4. These results suggest that GUN4 modulates Mg-chelatase activity depending on PPIX availability and the PPIX-bound state of ChlH.

Discussion

GUN4 is involved in modulating ALA biosynthesis in response to chlorophyll biosynthesis pathway intermediates and sensing PPIX and Mg-PPIX (23, 24). However, this involvement is not related to any significant measurable changes in the intracellular levels of these tetrapyrroles (12–14). As GUN4 accentuates PPIX-generated \( O_2(a^1\Delta_g) \) rather than attenuating it as has been suggested in the mutant child-1/GUN4 (24), the increased production of \( O_2(a^1\Delta_g) \) is an important factor that needs to be considered in the context of retrograde signaling. As such, we postulate a new framework for the role of GUN4 and ChlH (GUN5) in plastid-to-nucleus communication. We have shown that GUN4 and oxidatively damaged ChlH (GUN5) in complex with PPIX are singlet oxygen generators. Given that GUN4 and ChlH have been localized to both membrane and soluble components within chloroplasts (39, 40) and that they have a function in retrograde signaling, we postulate a new singlet oxygen-initiated model of retrograde signaling involving GUN4 and ChlH (Fig. 3). In this model, an \( O_2(a^1\Delta_g) \) signal is generated from GUN4-PPIX, and possibly oxidatively damaged ChlH-PPIX, when these molecules interact directly with a singlet oxygen-sensing system such as the membrane-associated EXECUTER 1 and 2 system. Due to the reactivity and short half-life of \( O_2(a^1\Delta_g) \), the singlet oxygen producer must be in close proximity (10–20 nm) to the singlet oxygen sensor (2, 41, 42). The proximity dependence is limited by the lifetime of singlet oxygen, which is \( \sim 1.6 \) \( \mu s \) in vivo, with three times the lifetime corresponding to a root mean square radial diffusion distance of less than 20 nm (2, 41, 42). Thus the model must take into account the relative location of the singlet oxygen producer to the singlet oxygen sensor and this must be within 20 nm.
require the ability of GUN4-PPIX to change its location within the chloroplasts to interact with the \( O_2(\alpha^1\Delta_g) \) sensor, possibly the EXECUTOR 1 or 2 singlet oxygen-sensing system. The nature of the signaling from EXECUTOR 1 and 2 is unknown, but several candidates have been identified (5, 43). However, this signaling would trigger differential regulation of target genes that include PPIX/chlorophyll biosynthetic enzymes, PhANG genes, and other stress-related genes.
The kinetic data also support the model of GUN4 as a sensor of the flux of intermediates through to chlorophyll. Here ChlH is the main acceptor of PPIX, possibly directly from protoporphyrinogen oxidase (27) with GUN4 acting as Mg-PPIX acceptor after chelation. When PPIX is not channeled to ChlH, GUN4-PPIX is formed, which inhibits Mg-chelatase assembly and further chlorophyll synthesis. This is not a problem in the dark, but in the light, it results in singlet oxygen production, producing a negative retrograde signal.

The model requires the movement of GUN4 and damaged ChlH to the envelope membrane in response to PPIX availability. This has been shown to occur in organello for GUN4, which relocates from the soluble phase to the membrane (39, 40) when PPIX concentration is artificially increased by ALA feeding. In addition, the C terminus of GUN4 is phosphorylated in plants (44) and this phosphorylation may also influence location of GUN4 within the chloroplast and its response to the flux of chlorophyll intermediates. Importantly, this model also explains much of the in vivo data for mutant (24) and artificially perturbed systems in which high endogenous concentrations of tetrapyrrole intermediates accumulate. The effect of tetrapyrrole accumulation would depend on the location and quantity of the tetrapyrrole and the quantity and location of tetrapyrrole-binding proteins as both could potentially disrupt or complicate signaling by producing large quantities of singlet oxygen as well as other ROS species.

In conclusion, our data assign a role of GUN4-PPIX and oxidatively damaged ChlH-PPIX protein complexes as generators of $O_{2}({\alpha^1\Delta_g})$ signals in the presence of light. We suggest that GUN4 is involved in sensing the flux of chlorophyll biosynthetic intermediates, primarily through sensing PPIX delivery to Mg-chelatase. Both a decrease in the flux of intermediates and a low Mg-chelatase activity are sensed by GUN4 binding PPIX and moving to the envelope membrane and singlet oxygen being generated to transmit a signal to the nucleus, possibly via EXECUTOR 1 or 2, to alter nuclear gene expression. This leads to further questions to determine the impact of this signal in vivo in chlorophyll biosynthesis and in chloroplast biogenesis. We anticipate that the $O_{2}(\alpha\Delta_g)$ signal must be either dealt with specifically through dedicated signaling mechanisms or reacted with particular antioxidant molecules to produce a secondary signaling molecule. The second possibility is attractive as these types of potential secondary plastid-to-nucleus signals have been identified, but the source of the ROS that generates them has not been identified (43, 45).

Author Contributions—S. T. T. conducted most of the singlet oxygen experiments, analyzed these results with the help of R. D. W., and drafted that section of the paper with help from R. D. W. and A. S. Z. and M. L. designed and produced the expression clones and helped with the final version of the manuscript. S. Z and A. S. developed the protein purification protocols. S. T. T. purified the GUN4 and ChlH proteins used in assays, and A. S. and S. Z. purified the ChlD, ChlI, and ChlI2 proteins used in the assay data. R. D. W. conceived the idea for the project, conducted the enzyme assays and analysis, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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