Recombinant Arabidopsis SQD1 Converts UDP-Glucose and Sulfite to the Sulfolipid Head Group Precursor UDP-sulfoquinovose In Vitro

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Running Title: Sulfolipid Precursor Synthesis
SUMMARY

The sulfolipid sulfoquinovosyldiacylglycerol is a component of plant photosynthetic membranes and represents one of the few naturally occurring sulfonic acids with detergent properties. Sulfolipid biosynthesis involves the transfer of sulfoquinovose, a 6-deoxy-6-sulfo-glucose, from UDP-sulfoquinovose to diacylglycerol. The formation of the sulfonic acid precursor, UDP-sulfoquinovose, from UDP-glucose and a sulfur donor is proposed to be catalyzed by the bacterial SQDB proteins or the orthologous plant SQD1 proteins. To investigate the underlying enzymatic mechanism and to elucidate the de novo synthesis of sulfonic acids in biological systems, we developed an in vitro assay for the recombinant SQD1 protein from Arabidopsis thaliana. Among different possible sulfur donors tested, sulfite led to the formation of UDP-sulfoquinovose in the presence of UDP-glucose and SQD1. An SQD1 Thr145Ala mutant showed greatly reduced activity. The UDP-sulfoquinovose formed in this assay was identified by co-chromatography with standards and served as substrate for the sulfolipid synthase associated with spinach chloroplast membranes. Approximate $K_m$ values of 150 $\mu$M for UDP-Glucose and 10 $\mu$M for sulfite were established for SQD1. Based on our results, we propose that SQD1 catalyzes the formation of UDP-sulfoquinovose from UDP-glucose and sulfite, derived from the sulfate reduction pathway in the chloroplast.
INTRODUCTION

The sulfolipid 6-sulfo-α-D-quinovosyl diaclyglycerol (SQDG) is a unique nonphosphorous lipid found in the photosynthetic membranes of plants and bacteria (1, 2). The head group of SQDG is sulfoquinovose, an anionic sulfonic acid derivative of glucose (6-deoxy-6-sulfo-glucose). Therefore, SQDG contributes a negative charge to the thyalkoid membrane along with the other major anionic thylakoid lipid phosphatidylglycerol (PG). Sulfolipid-deficient bacterial mutants are impaired in growth following phosphate deprivation (3, 4). Based on this result and other evidence it was proposed that SQDG is essential to maintain a balance of thylakoid membrane charge by substituting for PG under phosphate limiting conditions (1).

The elucidation of the reactions of sulfolipid biosynthesis by biochemical means has been recalcitrant in the past, but recently powerful new experimental tools became available with the isolation of sulfolipid-deficient mutants of the purple bacterium *Rhodobacter sphaeroides* and the cloning of the first genes encoding enzymes of sulfolipid biosynthesis, *sqdA*, *sqdB*, *sqdC*, and *sqdD* (5-7). The sulfonic acid precursor giving rise to the sulfolipid head group was originally postulated by A.A. Benson to be UDP-sulfoquinovose (UDP-SQ) (8) and synthetic UDP-SQ was later shown to specifically stimulate sulfolipid biosynthesis in isolated chloroplast membranes (9, 10). Only recently, the existence of UDP-SQ in living cells has been demonstrated using a sulfolipid-deficient mutant of *R. sphaeroides* that accumulates UDP-SQ (11). Subsequently, UDP-SQ was discovered also in other photosynthetic organisms (12). Indirect clues towards the elucidation of sulfolipid biosynthesis could be deduced from...
the sequences of putative sqd gene products. In particular, the SQDB protein of \textit{R. sphaeroides} has sequence similarity to sugar nucleotide modifying enzymes, and orthologous proteins in bacteria and plants are highly conserved (13). It was suggested that these proteins catalyze a reaction between UDP-Glc and a suitable sulfur donor leading to the formation of UDP-SQ (5, 14). The SQD1 protein of \textit{A. thaliana} is an orthologue of the bacterial SQDB proteins (15) and its crystal structure has been elucidated (16). Recombinant SQD1 lacking the chloroplast transit peptide has a mass of 45.5 kDa, forms a dimer, and contains a buried active site with tightly bound \textit{NAD}^+ (16, 17). Co-crystallization with UDP-Glc demonstrated directly the binding of this presumed substrate in the active site. Furthermore, in place of the sulfur donor, water molecules were present. Labeled sulfate is incorporated into SQDG by isolated chloroplasts (18-20), and it seemed likely that the sulfur donor is derived from the sulfate reduction pathway in the chloroplast. Intermediates of this pathway, adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), were indeed incorporated into SQDG by isolated chloroplasts (21). Another intermediate of the sulfate reduction pathway, sulfite, has not been tested in this system, but was shown to be incorporated into SQDG by extracts of \textit{Chlamydomonas reinhardtii} (22). However, this reaction was linear over time and therefore thought to be non-enzymatic. Here, we provide evidence for the SQD1 catalyzed formation of the sulfolipid head group donor UDP-SQ from UDP-Glc and sulfite \textit{in vitro}.
EXPERIMENTAL PROCEDURES

Substrates—The following sulfur containing substrates were used: Sulfate (JT Baker, Phillipsburg, NJ), sulfite (Merck Co., Rahway, NJ), sulfide (Sigma, St. Louis, MO), adenosine 5′-phosphosulfate (APS) (Sigma, St. Louis, MO), 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Sigma), glutathione (reduced and oxidized forms) (Sigma), thiosulfate (Mallinckrodt Chemical Works, St. Louis, MO). Sulfo-glutathione was synthesized according to (23), and purified by TLC and visualized as described (24). Labeled UDP-Glc [glucose-\textsuperscript{14}C(U)] and UDP-Gal [galactose-\textsuperscript{14}C(U)] were purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. Labeled UDP-sulfoquinovose [\textsuperscript{35}S-6-sulfoquinose] was prepared using the sqdD mutant of \textit{R. sphaeroides} and analyzed by TLC as described (11). Labeled [\textsuperscript{35}S]APS was prepared from [\textsuperscript{35}S]PAPS (New England Nuclear, Boston, MA) by phosphatase treatment (25). Unless otherwise specified below, common buffer ingredients were obtained from general commercial sources.

\textit{Assay Conditions for SQD1 and HPLC Analysis—}Recombinant \textit{A. thaliana} SQD1 protein excluding the transit peptide was purified on Ni-NTA columns (Qiagen, Valencia, CA) as described by Essigmann et. al. (15). The column was eluted with 200 mM imidazole which was subsequently removed by use of a Millipore Co. (Bedford, MA) Ultrafree 4 concentrator. The SQD1 protein was stored in 20% glycerol, 300 mM NaCl, and 25 mM NaH\textsubscript{2}PO\textsubscript{4} (pH 7.5) at -20°C. Basic activity assays were carried out at 37°C in a buffer containing 10 µg SQD1, 100 µM Na\textsubscript{2}SO\textsubscript{3}, 500 µM UDP-Glc [\textsuperscript{14}C(U)-glucose] (89 Bq nmol\textsuperscript{-1}) and 50 mM Tris (pH7.5) in a total volume of 100 µl for 40 min.
unless otherwise stated. For some experiments as indicated, we increased the UDP-Glc concentration to 1.6 mM to fully saturate (10 X $K_m$) the enzyme with this substrate. The coupled APS reductase/SQD1 assay contained 50 mM Tris pH 8.5, 10 mM dithiothreitol (DTT), 25 µM $^{35}$SAPS (500 Bq nmol$^{-1}$), 250 mM Na$_2$SO$_4$, 1 mM EDTA, 500 µM UDP-Glc, 66 µg SQD1, and 12 µg APR1 from Arabidopsis thaliana (25). This reaction was incubated at 30°C for 10 min. In all cases, samples were heat denatured for 5 min at 95°C, centrifuged at 10,000 X g for 5 min, and analyzed by HPLC (Waters Corporation, Milford, MA) employing a Beckman (Fullerton, CA) Ultrasphere ODS column (4.6 mm X 25 cm, particle size 5 µm) kept constantly at 42°C. Substrates and products were separated by applying a linear gradient of 30 mM KH$_2$PO$_4$, 2 mM tetrabutylammonium hydroxide (Fisher Scientific, Fair Lawn, NJ), adjusted to pH 6.0 with KOH, to HPLC grade acetonitrile (EM Science, Gibbstown, NJ) with a flow rate of 1 ml per min over 45 min. The column was allowed to re-equilibrate for 17 min (acetonitrile to phosphate buffer) after each run. Labeled compounds were detected using a $\beta$-Ram Model 2 Flow Through Monitor (INUS Systems, Tampa, Florida).

Sulfolipid Synthase Assay—Chloroplasts were isolated from spinach (locally available produce) according to Rossak et al. (11). Chlorophyll was quantified by the method of Lichtenthaler (26). The assay consisted of 35 µg chlorophyll with 10,000 dpm of radioactive compound U$_2$ in 50 µl reaction buffer (50 mM Tricine/KOH, 30 mM MgCl$_2$, pH 7.5). For control purposes, galactolipid synthesis was monitored using UDP-Gal [galactose-$^{14}$C(U)] (0.4 µM, 1200 Bq nmol$^{-1}$) as substrate. The reactions were incubated at room temperature for 1 h. Assays were stopped by the addition of 100 µl
chloroform:methanol:formic acid (1:1:0.1, vol/vol/vol) and 50 µl 1 M KOH, 0.2 M phosphoric acid. Samples were mixed and centrifuged for 3 min at 10,000 X g. Lipids in the lower chloroform phase were analyzed by thin layer chromatography on ammonium sulfate-impregnated plates by the method of Benning and Somerville (6) with the substitution of toluene for benzene in the mobile phase. Auto-radiography was used to visualize labeled sulfolipid.

*Site-directed Mutagenesis*—An SQD1 mutant derivative was constructed by PCR mutagenesis employing a method by Ito et al. (27). Threonine 145 was changed to Alanine using the primers 5’-ATACTCACCATCGCCCCAAGTTTTAC-3’ and 5’-ATCACCATCAAGCCTCCGTGTTAT-3’. PCR products were cloned into pPCR-Script Amp SK(+) (Stratagene, LaJolla, CA) and mutant plasmids were sequenced at the MSU sequencing facility. The mutant open reading frame was inserted into pQE30 (Qiagen, Valencia, CA), expressed in *E. coli*, and the protein was purified as described (15).
RESULTS

In vitro Reaction of UDP-Glc and Sulfite Mediated by SQD1—An enzyme assay was developed to measure the conversion of UDP-Glc to UDP-SQ as predicted for SQD1 activity. SQD1 protein was estimated to be at least 95% pure by gel analysis (Fig. 1). To broadly analyze the reaction mixtures for substrates and reaction products, we employed an HPLC system optimized for the separation of sugar nucleotides. Sample through-put was limited, in particular because columns had to be regenerated extensively after few runs. Therefore, we also explored different TLC systems and filter based assays to separate substrates and products with the goal to process large sample numbers in parallel. However, none of the alternative procedures we designed, thus far, were satisfactory with regard to sample recovery or reproducibility. Because of the limitation in sample through-put, data points shown are representative for at least three different independent experiments, instead of averages of multiple repeats in a single experiment. We routinely used labeled UDP-Glc [\(^{14}\)C(U)-glucose] as tracer for the radio assay. Incubation of the SQD1 protein with labeled UDP-Glc in a simple Tris buffer, as described in the experimental procedures, resulted in the formation of two compounds with unique retention times as compared to UDP-Glc (Fig. 2A, B). At the end of the assay period, the protein was routinely denatured to release any products or intermediates still bound in the active site of the protein. Filtration of the reaction mixture using Amicon filters (MW cut off 10,000; Millipore Co., Bedford, MA) without denaturation revealed that 77% of compound U₂ (Fig. 2B) was free in solution as compared to 35% of compound U₁ (average of three samples). Adding sulfite to the
reaction mixture eliminated compound U₁ completely and stimulated the formation of compound U₂ (Fig. 2C). The relative amounts of compounds U₁ and U₂ were variable in assays to which no sulfite was added, depending on the SQD1 protein preparation. This effect may have been caused by varying amounts of contaminating compounds carried over from the E. coli extract in SQD1 preparations, a hypothesis that was not further investigated.

**Compound U₂ has UDP-SQ like Properties**—To this time, it has not been possible to identify compound U₁. Because of the lack of suitable standards, a *de novo* structural elucidation would have been required for which we could not obtain sufficient amounts of material. However, compound U₂ co-chromatographed in the HPLC system with authentic UDP-SQ isolated from the *sqdD* mutant of *R. sphaeroides* (Fig. 2C, D) indicating that this compound may be the proposed intermediate of sulfolipid biosynthesis, UDP-SQ. To obtain corroborating evidence, labeled compound U₂ purified by HPLC was analyzed by TLC together with extracts from [³⁵S]sulfate labeled *R. sphaeroides* wild type and *sqdD* mutant cells. The latter are known to accumulate UDP-SQ (11). Compound U₂ co-chromatographed with UDP-SQ also in this system (data not shown).

The sulfolipid synthase of spinach chloroplast envelopes is highly discriminatory towards UDP-SQ (9, 10). We took advantage of the substrate specificity of this enzyme and incubated compound U₂ with spinach chloroplast membranes and observed the formation of a [³⁵S]-labeled compound co-chromatographing with sulfolipid (Fig. 3). Taken together, these three independent lines of evidence identified compound U₂ as
Confirmation of Sulfite as the Sulfur Donor—Thus far, the greatest mystery in the elucidation of the biosynthetic pathway for sulfolipid biosynthesis has been the nature of the sulfur donor for the formation of UDP-SQ. The establishment of the SQD1 in vitro assay described above gave us the opportunity to directly address this problem. It seemed most likely that a metabolite or its derivative of the sulfur assimilation pathway in bacteria and plants - sulfate, APS, PAPS, sulfite, thiosulfate, sulfide, or sulfoglutathione - would provide the sulfonic acid group in the formation of UDP-SQ. We therefore tested these compounds unlabeled at concentrations ranging from 0.1- to-10 mM in the UDP-Glc \(^{14}C(U\text{-glucose})\) based SQD1 assay to determine if they could stimulate the formation of UDP-SQ. Addition of sulfate, APS, and PAPS had no affect on the absolute amounts or ratios of the reaction products. Addition of 0.1 mM thiosulfate, sulfide, and sulfoglutathione resulted in a decrease in the relative amount of compound U\(_1\) and an increase in the amount of compound U\(_2\) as shown for sulfite in Figure 1C. We assumed that in all three instances this effect was due to sulfite, which was either produced by chemical reaction from thiosulfate, sulfide, or sulfoglutathione in aqueous solution or was already present in the respective compound preparations as contaminant.

To corroborate this hypothesis, it was necessary to directly test the incorporation of labeled sulfite into UDP-SQ. Because labeled sulfite was not commercially available and because it is fairly reactive in solution (28), we decided to synthesize sulfite directly in the assay mixture from \(^{35}\text{S}\)APS and DTT as reductant using recombinant
APS reductase 1 from *A. thaliana* (APR1) (25). The second SQD1 substrate, UDP-Glc, was provided unlabeled. Incubating APS, DTT, UDP-Glc, and APR1 alone followed by HPLC analysis of the reaction products resulted in the conversion of APS to sulfite (Fig. 4A, B). When SQD1 was present in the APR1 reaction mixture, sulfite was converted to compound U₂ previously identified as UDP-SQ (Fig. 4C, D). The formation of UDP-SQ from labeled APS in this APR1/SQD1 coupled assay was further confirmed using the spinach sulfolipid synthase assay described above (data not shown). Incubating labeled APS with SQD1 alone did not lead to the formation of labeled UDP-SQ (result not shown, but essentially indistinguishable from Fig. 4A).

*A Thr145Ala Mutant of SQD1 with Strongly Decreased Activity*—Sulfite had been previously suggested as a substrate for sulfolipid synthesis using extracts of *C. reinhardtii* (22). However, sulfite incorporation was not saturable as expected for enzyme catalyzed reactions in this system. To rule out a nonenzymatic reaction of sulfite as the cause for the observed UDP-SQ formation and to demonstrate directly that SQD1 activity is required in the *in vitro* assay system described above, we constructed a point mutant of SQD1 (Thr145Ala) by exchanging threonine 145 with alanine (for gel see Fig. 1C). From the crystal structure of the SQD1/UDP-Glc complex, it was obvious that threonine 145 coordinates a water along with the C-4 and C-6 hydroxyl groups of the glucose moiety of UDP-Glc in a high energy conformation (16). Therefore, it was predicted that threonine 145 plays a critical role for catalytic activity. Indeed, when the Thr145Ala mutant was incubated for 40 min in the presence of sulfite and labeled UDP-Glc, no product was formed in comparison to the wild type reaction.
(Fig. 5A, B). Only after 46 hours of incubation, a very small product peak was visible in the mutant sample (Fig. 5C). This result suggested that the activity of the mutant enzyme is reduced by several orders or magnitude, thereby confirming that SQD1 enzymatic activity is essential for the observed conversion of UDP-Glc and sulfite to UDP-SQ.

**Characterization of SQD1 Activity**—Basic enzymatic properties of SQD1 were determined using the standard assay as described in experimental procedures. Enzyme activity was linear from 5 to 50 µg of SQD1 protein as tested (Fig. 6A). The reaction was also linear with respect to the assay time of up to 60 min (data not shown). The optimal pH for activity was between 7.5 and 9.5 (Fig. 6B). Subsequently, all standard assays were performed at pH 7.5 with 10 µg of protein for 40 min. To determine the kinetic constants for UDP-Glc, increasing amounts of this substrate were added at a concentration of 100 µM sulfite (Fig. 6C). The reaction was saturable and the Michaelis-Menten constant, $K_m$, for UDP-Glc was estimated to be 150 µM, the specific activity 2.6 nmoles UDP-SQ min$^{-1}$ mg$^{-1}$ protein, and the turnover number, $k_{cat}$, 0.1 min$^{-1}$. To examine the specificity of the enzyme, we added equal amounts of ADP-Glc and UDP-Glc (500 µM each) with UDP-Glc as the labeled tracer. However, no inhibition of the reaction by ADP-Glc was observed (data not shown). Because SQD1 normally contains NAD$^+$ in its binding site, this nucleotide was added to the reaction, but did not affect product formation (data not shown). Keeping the UDP-Glc concentration at 1.6 mM and varying the concentration of sulfite (Fig. 6D), the reaction was saturable, with a similar $V_{max}$ as observed for UDP-Glc. However, the $K_m$ for sulfite
was approximately 10 μM, an order of magnitude lower as compared to UDP-Glc. Increasing the sulfite concentration beyond 100 μM inhibited the reaction (Table I). This effect was specific to sulfite, because other salts as shown in Table I did not inhibit the reaction.
DISCUSSION

Unlike sulfonic acids such as taurine, which represent oxidation products of sulfur amino acids in animals, the sulfolipid head group donor UDP-sulfoquinovose (UDP-SQ)(11) is synthesized de novo in bacteria and plants. Studying the recombinant protein SQD1 of *A. thaliana*, we could observe the enzyme catalyzed formation of UDP-SQ in vitro. Although no direct structural elucidation of the reaction product was feasible, three independent lines of indirect evidence confirmed the identity of the product as UDP-SQ: first, co-chromatography with authentic UDP-SQ by HPLC; second, co-chromatography by TLC; and third, conversion of the product by spinach SQDG synthase to sulfolipid. The formation of UDP-SQ was dependent on the presence of UDP-Glc and sulfite. Based on this result, we propose a tentative model of sulfolipid biosynthesis with UDP-Glc and sulfite as the precursors as shown in Figure 7.

*UDP-Glc as Substrate*—Sulfolipid biosynthesis is a function of chloroplasts (18-20) and SQD1 has previously shown to be imported into the plastid (15). This poses a theoretical problem, because it is unclear whether the precursor UDP-Glc is actually available in the plastid and, at least, one would have to assume that its concentration is very low compared to ADP-Glc (29). However, ADP-Glc which is involved in photosynthetic starch biosynthesis in plastids, was not a substrate for the reaction. Furthermore, UDP-Glc provided a perfect fit within the active site based on the crystal structure of SQD1 (16). Therefore, we postulate that UDP-Glc is present in the plastid in sufficient amounts to support sulfolipid biosynthesis. Whether UDP-Glc is imported
from the cytosol as indicated in Figure 7, or generated inside the plastid remains unclear.

*Sulfite is the Sulfur Donor*—Of all the possible sulfur donors tested, none was more active than sulfite. Compounds which could spontaneously give rise to sulfite in aqueous solution, such as thiosulfate and sulfoglutathione, did stimulate the activity of the enzyme to the same extent as sulfite directly. It is still debated whether APS-reductase generates sulfite directly or first sulfoglutathione which subsequently hydrolyzes to release sulfite (30, 31). Therefore, the question arises whether sulfoglutathione may be a precursor for UDP-SQ biosynthesis in vivo. Two arguments speak against this hypothesis: first, glutathione could not be modeled into the structure of SQD1 in a catalytically sensible way and second, sulfoglutathione was not more effective in stimulating UDP-SQ biosynthesis than sulfite itself. Therefore, we propose that sulfite derived from the APS reductase reaction is the precursor for UDP-SQ biosynthesis in vivo. The APS-reductase is part of the sulfur assimilation machinery in the chloroplast. At least three isoforms exist (32) and it seems possible that SQD1 directly and specifically interacts with one of these. Sulfite is a reactive and cytotoxic compound, e.g. (33-35) and substrate channeling between one of the APS reductase isoforms and SQD1 would allow sulfolipid biosynthesis without build-up of sulfite as an intermediate. The $K_m$ of approximately 10 $\mu$M for sulfite is relatively low in accordance with a high affinity of SQD1 for the substrate. Interestingly, concentrations above 100 $\mu$M sulfite inhibit the reaction. Whether this inhibition reflects a biologically meaningful regulatory process or a toxic effect on the protein due to reactive radicals derived from
sulfite oxidation (28) requires further investigation.

The SQD1 Catalyzed Formation of UDP-SQ Is Very Slow—Contrary to experiments with extracts of *C. reinhardtii* (22), the formation of UDP-SQ from sulfite and UDP-Glc was saturable with increasing amounts of sulfite as would be expected for an enzyme catalyzed reaction. The reaction depended on the presence of SQD1 enzyme and evidence for the crucial role of SQD1 in the formation was derived from the Thr145Ala mutant of SQD1, which was virtually inactive while retaining a native structure (Theisen et al. in preparation). Although clearly measurable, the reaction of the wild-type protein is already very slow with 0.1 turnovers per min. This value is among the lowest turnover values for reported enzymes (http://www.brenda.uni-koeln.de/). It seems unlikely that the activity of SQD1 is this low in vivo because it would presumably not suffice to produce enough sulfolipid during rapid leaf growth. Assuming that UDP-Glc and sulfite are the correct substrates at least three explanations can be found for the low in vitro activity of SQD1: First, the recombinant enzyme has been truncated at the N-terminus to remove the predicted transit peptide (15). The prediction of the cleavage site may be incorrect and the truncation may have affected activity. However, the bacterial SQDB proteins are similar in size as compared to the recombinant SQD1 protein and seem to work properly in vivo. Second, an allosteric factor is missing which would normally activate the enzyme in vivo. Third, SQD1 is part of a larger protein complex and requires, thus, direct and proper contact with an APS reductase and possibly other enzymes. At this time, we
cannot distinguish between these possibilities. Another unusual feature of SQD1 activity is that it shows a broad pH optimum, between 7.5 and 9.5. One possibility for the high activity close to pH 7.5 is that it arises from the change in protonation state of Tyr 182 and/or His 183. The amino acid Tyr 182 is thought to initiate catalysis by abstracting a proton from the 4'hydroxyl group of UDP-Glc. A homologous residue in the structurally related enzyme UDP-galactose 4'-epimerase, Tyr 149, has an estimated pK$_a$ of 6.08 (36). If Tyr 182 of SQD1 has a similar pK$_a$, SQD1 would lose activity as the pH decreased. Moreover His 183 is considered to be the general base in the dehydration step which requires the removal of the C5' proton from glucose (16). Unless perturbed by the local environment, His 183 should have a pK$_a$ ~ 7. The origin of the high activity close to pH 9.5 remains an enigma.

In summary, we have shown that recombinant SQD1 of *A. thaliana* catalyzes the formation of UDP-SQ, the sulfolipid head group donor and one of the few biological sulfonic acids, *in vitro* from UDP-Glc and sulfite. The reaction showed all features expected for an enzymatic reaction. However, the turnover rate was very low, and further analysis will be required to demonstrate that SQD1 catalyzes the proposed reaction also *in vivo.*
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REFERENCES


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FIGURE LEGENDS

FIG. 1: **Purification of SQD1.** SDS-PAGE analysis of (A) crude *E. coli* cell culture extract expressing SQD1 protein and nickel column purification of (B) SQD1 and (C) Thr145Ala mutant. (4 µg of each)

FIG. 2: **Conversion of UDP-Glc by SQD1.** The chromatographic analysis of $^{14}$C-labeled substrate and reaction products by HPLC is shown (A-C). (A) UDP-Glc without SQD1 protein, (B) UDP-Glc and SQD1 protein, (C) UDP-Glc, SQD1 protein, and sulfite, (D) authentic $^{35}$S-labeled UDP-SQ isolated from the *sqdD* mutant of *R. sphaeroides*. $U_1$ and $U_2$, products as described in the text.

FIG. 3: **Incubation of spinach chloroplast membranes with reaction product $U_2$.** Assay of sulfolipid synthase associated with thylakoid membranes which specifically converts UDP-SQ and diacylglycerol to SQDG. (A) Thin-layer chromatography of lipids following the incubation of spinach thylakoid membranes with labeled reaction product $U_2$ or, for control purposes, $^{14}$C-labeled UDP-Gal the substrate for galactolipid biosynthesis. Lipids were visualized by autoradiography. (B) Iodine staining of the $U_2$ lane. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.
FIG. 4: **Coupled APS reductase/SQD1 assay.** HPLC chromatograms of reaction products and standards are shown. (A) $^{35}$S-labeled substrate APS without enzymes; (B) $^{35}$S-labeled reaction products following the incubation with APS reductase alone, or (C) in the presence of APS reductase and SQD1; (D) $^{14}$C-labeled UDP-SQ ($U_2$) from the standard SQD1 assay.

FIG. 5: **Comparison of SQD1 wild type and Thr145Ala mutant.** HPLC chromatograms of reaction products are shown. (A) Standard 40 min assay in the presence of SQD1 wild-type enzyme. (B) Incubation with mutant enzyme for 40 min and (C) 46 hrs.

FIG. 6: **Exploring different assay conditions for SQD1.** (A) Addition of varying amounts of SQD1 protein. (B) Dependence on pH. MES buffer was used for pH values of 6.0, 6.5, and 7.0, Tris buffer for 7.5 - 9.5, and CAPS for 10. (C) Substrate saturation for UDP-Glc at 100 µM sulfite. (D) Substrate saturation for sulfite at 1.6 mM UDP-Glc. 13 µg SQD1 protein was used for analysis in B, C, and D. Error bars represent standard deviation for five samples.

FIG. 7. **Proposed model for sulfolipid biosynthesis in plant chloroplasts.** Enzymes involved are SQD1, ATP sulfotransferase (ATS), APS reductase (APR), and SQDG synthase. UDP-Glc is proposed to be imported from the cytosol as indicated. GSH,
reduced glutathione; GSSG, oxidized glutathione; DAG, diacylglycerol. Other abbreviations are standard or have been defined already in the text.
TABLE I

SQD1 assay with excess sulfite and added salt. Mean values ± standard deviation (n=5). All assays contained 0.1 mM Na$_2$SO$_3$, 1.6 mM UDP-Glc, and 13 µg SQD1 protein but were otherwise conducted under standard conditions.

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<th>Concentration (mM)</th>
<th>SQD1 Activity (pmoles min$^{-1}$)</th>
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Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfite to the sulfolipid head group precursor UDP-sulfoquinovose in vitro
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