Recombinant Arabidopsis SQD1 Converts UDP-glucose and Sulfite to the Sulfolipid Head Group Precursor

UDP-sulfoquinovosyldiacylglycerol (SQDG) 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-sulfoglucose, 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 T145A 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 μM for UDP-glucose and 10 μM 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.

The sulfolipid 6-sulfo-α-D-quinovosyl diacylglycerol (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-sulfoglucose). Therefore, SQDG contributes a negative charge to the thylakoid membrane along with the other major anionic thylakoid lipid phosphatidylglycerol. 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 phosphatidylglycerol 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 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 toward the elucidation of sulfolipid biosynthesis could be deduced from the sequences of putative sqd gene products. In particular, the SQDB protein of 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-glucose (UDP-Glc) and a suitable sulfur donor leading to the formation of UDP-SQ (5, 14). The SQD1 protein of Arabidopsis 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 NAD^+. 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 Chlamydomonas reinhardtii (22). However, this reaction was linear over time and therefore thought to be nonenzymatic. Here, we provide evi-
dence for the SQD1-catalyzed formation of the sulfolipid head group donor UDP-SQ from UDP-Glc and sulfite in vitro.

**EXPERIMENTAL PROCEDURES**

**Substrates—**The following sulfur-containing substrates were used: sulfate (J. T. Baker Inc.), sulfite (Merck), sulfide (Sigma), APS (Sigma), PAPS (Sigma), glutathione (reduced and oxidized forms) (Sigma), and thiosulfate (Malinckrodt Chemical Works, St. Louis, MO). Sulfoglutatione was synthesized according to Ref. 23 and purified by TLC and visualized as described (14). Labeled UDP-[14C(U)]galactose and UDP-[14C(U)]glucose were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Labeled UDP-[35S]sulfinoquinone was prepared using the sqdD mutant of *R. sphaeroides* and analyzed by TLC as described (11). Labeled [35S]APS was prepared from [35S]PAPS (PerkinElmer Life Sciences) by phosphatase treatment (25). Unless otherwise specified below, common buffer ingredients were obtained from general commercial sources.

**Assay Conditions for SQD1 and HPLC Analysis—**Recombinant *A. thaliana* SQD1 protein excluding the transit peptide was purified on Ni2+—nitrilotriacetic acid 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 Corp. (Bedford, MA) Ultrafree 4 concentrator. SQD1 protein was stored in 20% glycerol, 300 mM NaCl, and 25 mM NaH2PO4 (pH 7.5) at 20 °C. Basic activity assays were carried out at 37 °C in a buffer containing 10 μg of SQD1, 100 mM Na2SO4, 500 μM UDP-[14C(U)]glucose (89 Bq nmol−1), and 50 mM Tris (pH 7.5) 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 (30 × *Km*) enzyme with this substrate. The coupled APS reductase/SQD1 assay contained 50 mM Tris, pH 8.5, 10 mM dithiothreitol, 25 μM [35S]APS (500 Bq nmol−1), 250 mM Na2SO4, 1 mM EDTA, 500 μM UDP-Glc, 66 μg of SQD1, and 12 μg of APR1 from *A. 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 × g for 5 min, and analyzed by HPLC (Waters Corp., Milford, MA) employing a Beckman (Fullerton, CA) Ultrasphere ODS column (4.6 mm × 55 cm; particle size 5 μm) kept constantly at 42 °C. Substrates and products were separated by applying a linear gradient of 30 mM KH2PO4, 2 mM tetrabutyrammonium hydroxide ( Fisher), adjusted to pH 6.0 with KOH, to HPLC grade octanitrile (EM Science, Gibbstown, NJ) with a flow rate of 1 ml/min over 45 min. The column was allowed to re-equilibrate for 17 min (octanitrile to phosphate buffer) after each run. Labeled compounds were detected using a β-Ram model 2 Flow Through Monitor (INUS Systems, Tampa, FL).

**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 μl of chloroplasts with 10,000 dpm of radioactive compound U1 in 50 μl of reaction buffer (50 mM Tricine/KOH, 30 mM MgCl2, pH 7.5). For control purposes, galactolipid synthesis was monitored using UDP-[14C(U)]galactose (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 of chloroform/methanol/formic acid (1:1:0.1, v/v/v) and 50 μl of 1 M KOH, 0.2 M phosphoric acid. Samples were mixed and centrifuged for 3 min at 10,000 × 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. Autoradiography was used to visualize labeled sulfolipid.

**Site-directed Mutagenesis—**An SQD1 mutant derivative was constructed by PCR mutagenesis employing a method of Ito et al. (27). The resulting 216 bp change was introduced into the primers 5′-ATACCACCCCTACGCCCCAAAGATTTATCA-3′ and 5′-ATCACCACATCCGGCTC-CGGTTGATT-3′. PCR products were cloned into pPCR-Script Amp SK+ (Stratagene, La Jolla, CA), and mutant plasmids were sequenced at the MSU sequencing facility. The mutant open reading frame was amplified from *A. thaliana* cDNA (Qiagen, Valencia, CA), expressed in *Escherichia 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 throughput 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 of processing large sample numbers in parallel. However, none of the alternative procedures designed, thus far, were satisfactory with regard to sample recovery or reproducibility. Because of the limitation in sample throughput, 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 as tracer for the radioassay. Incubation of the SQD1 protein with labeled UDP-Glc in a simple Tris buffer, as described under “Experimental Procedures,” resulted in the formation of two compounds with unique retention times as compared with UDP-Glc (Fig. 2, A and B). 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 (M cut-off 10,000; Millipore) without denaturation revealed that 77% of compound U2 (Fig. 2B) was free in solution as compared with 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—**Until 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. 2, C and 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]sulfite-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 toward UDP-SQ (9, 10). We took advantage of the substrate specificity of this enzyme and incubated...

![Fig. 1. Purification of SQD1.](image-url)
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 UDP-SQ.

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-based SQD₁ assay to determine whether they could stimulate the formation of UDP-SQ. The addition of sulfate, APS, and PAPS had no affect on the absolute amounts or ratios of the reaction products. The addition of 0.1 mM thiosulfate, sulfide, and sulfoglutathione resulted in a decrease in the relative amount of compound U₁ and an increase in the amount of compound U₂ as shown for sulfite in Fig. 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 35S]APS and dithiothreitol as reductant using recombinant APS reductase 1 from A. thaliana (APR₁) (25). The second SQD₁ substrate, UDP-Glc, was provided unlabeled. Incubating APS, dithiothreitol, UDP-Glc, and APR₁ alone followed by HPLC analysis of the reaction products resulted in the conversion of APS to sulfite (Fig. 4, A and B). When SQD₁ was present in the APR₁ reaction mixture, sulfite was converted to compound U₂ previously identified as UDP-SQ (Fig. 4, C and 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 T145A 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 (T145A) 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 T145A mutant was incubated for 40 min in the presence of sulfite and labeled UDP-Glc, no product was formed in comparison with the wild type reaction (Fig. 5, A and B). Only after 46 h 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 under “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, *Km*, for UDP-Glc was estimated to be 150 μM, the specific activity 2.6 nmol of UDP-SQ min⁻¹ mg⁻¹ protein, and the turnover number, *kcat*, 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 it 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 *Vmax* similar to that observed for UDP-Glc. However, the *Km* for sulfite was ~10 μM, an order of magnitude lower as compared with 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-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 Fig. 7.

**UDP-Glc as Substrate**—Sulfolipid biosynthesis is a function of chloroplasts (18–20), and SQD1 has previously been 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 with 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 was indicated in Fig. 7 or generated inside the plastid remains unclear.

**Sulfite Is the Sulfur Donor**—Of all of the possible sulfur donors tested, none was more active than sulfite. Compounds that could spontaneously give rise to sulfite in aqueous solu-
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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. DAG, diacylglycerol.

Table I

SQD1 assay with excess sulfite and added salt

| Added salt | Concentration mm | SQD1 activity pmol min⁻¹ |
|------------|------------------|--------------------------|
| No salt    |                  |                          |
| Na₂SO₃     | 1                | 6.6 ± 1.7                |
| Na₂SO₄     | 10               | 3.8 ± 0.2                |
| NaH₂PO₄    | 100              | 21.5 ± 1.9               |
| NaCl       | 100              | 27.3 ± 0.7               |

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 T145A mutant of SQD1, which was virtually inactive while retaining a native structure. Although clearly measurable, the reaction of the wild-type protein is already slow, with 0.1 turnovers/min. 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 with the recombinant SQD1 protein and seem to work properly in vivo. Second, an allosteric factor is missing that 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 Tyr₁₈₂ and/or His¹⁸³. The amino acid Tyr¹₈₂ 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, Tyr⁹⁴, has an estimated pKₐ of 6.08 (36). If Tyr¹₈₂ of SQD1 had a similar pKₐ, SQD1 would lose activity as the pH decreased. Moreover, His 183 is considered to be the general base in the dehydration step that requires the removal of the C-5' proton from glucose (16). Unless perturbed by the local environment, His¹⁸³ should have a pKₐ of ~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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