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Lysophosphatidylethanolamine Is a Substrate for the Short-Chain Alcohol Dehydrogenase SocA from *Myxococcus xanthus*▽†

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Short-chain alcohol dehydrogenases (SCADHs) synthesize a variety of intercellular signals and other chemically diverse products. It is difficult to predict the substrate of a SCADH on the basis of amino acid sequence homology, as the substrates are not known for most SCADHs. In *Myxococcus xanthus*, the SCADH CsgA is responsible for C signaling during fruiting body development, although the mechanism is unclear. Overexpression of the SCADH SocA compensates for the lack of CsgA and restores development and C signaling in *csgA* mutants. The potential of SocA in generating the C signal enzymatically was explored by developing a dehydrogenase assay-based screen to purify the SocA substrate(s). A SocA substrate was extracted from *M. xanthus* cells with acidified ethyl acetate and sequentially purified by solid-phase extraction on silica gel and by reverse-phase high-performance liquid chromatography. The fraction with the highest SocA dehydrogenase activity contained the lysophospholipid 1-acyl 2-hydroxy-*sn*-glycerophosphoethanolamine (lyso-PE) as indicated by the fragment ions and a phosphatidylethanolamine-specific neutral loss scan following liquid chromatography coupled to mass spectrometry. The abundant lysophospholipid with the mass *m*/*z* 450 (molecular ion [M-H]$^-$) had a monounsaturated acyl chain with 16 carbons. SocA oxidizes lyso-PE containing either saturated or unsaturated fatty acids but exhibits poor activity on L-α-glycerophosphorylethanolamine, suggesting that an acyl chain is important for activity. Of the five different head groups, only ethanolamine showed appreciable activity. The apparent K_m and V_{max} for lyso-PE 18:1 were 116 μM and 875 μmol min^{-1} mg^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) was 1×10^8 M^{-1} s^{-1}. The proposed product, 1-acyloxy-3-(2-aminoethylphosphatyl) acetone was unstable, and the fragmented products were unable to rescue *csgA* mutant development. The active fraction from thin-layer chromatography also contained an unidentified SocA substrate that had morphogenic properties.

Myxococcus xanthus serves as a prokaryotic paradigm to study social behavior and multicellular development. Nutrient limitation initiates a developmental program in which thousands of cells glide toward aggregation centers and build multicellular fruiting bodies. Within a fruiting body, the rod-shaped vegetative cells differentiate into dormant myxospores. Development is governed by a series of extracellular signals (24). The protein that initiates C signaling belongs to the short-chain alcohol dehydrogenase (SCADH) family (12).

The C signal is a concentration-dependent developmental timer that controls spatial and temporal gene expression with the emergence of the morphologically distinct developmental events rippling, fruiting body morphogenesis, and sporulation (8, 10, 15). The *csgA* gene, which is responsible for the manifestations of C signaling, encodes a 25-kDa protein that undergoes N-terminal proteolytic processing into a 17-kDa form (9, 16). Two strikingly different models have emerged to explain the molecular basis of C signaling. Kim and Kaiser proposed that the 17-kDa form is the C signal and acts as a short-range hormone (9, 16). Lee et al. (12) proposed that the full-length form of CsgA is an enzyme that generates the C signal. Although several interesting experiments have been performed to address questions in both directions, neither model provides conclusive evidence for the nature of the C signal.

Genetic evidence that the 25-kDa form is an enzyme has come from isolation of an unusual class of suppressor mutants. Overexpression of SocA, a putative SCADH, in a *socC* mutant restored development by restoring C signaling in *csgA* mutants (13, 14). The *socC csgA* double mutant restored *csgA* mutant development in cell mixtures, suggesting intercellular transfer of the C signal. The 28% amino acid identity between CsgA and SocA was confined to largely internal regions of the protein known to be required for catalytic activity (14). Mutational analysis of CsgA showed that conserved amino acids known to be essential for catalytic activity in other SCADHs are also essential for C signaling (12). These data raise the possibility that SocA can utilize the same substrate as CsgA to generate the C signal (Fig. 1). Many SCADH enzymes play critical roles in eukaryotic signaling, producing products such as steroids, prostaglandins, and retinoids (20).

The enzyme model suffers from lack of knowledge about the SocA and CsgA substrates. The lack of biochemical characterization of most SCADHs makes it difficult to predict the substrate on the basis of amino acid sequence. CsgA and SocA have more homology with SCADHs utilizing lipid substrates than with enzymes utilizing hydrophilic substrates (12, 14). Beyond that, there are few clues as to the nature of their substrates. Furthermore, it has not been possible to overexpress CsgA in another host. The potential role of SocA in

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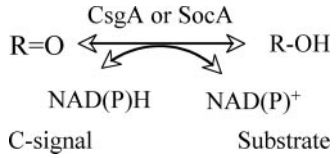

FIG. 1. Model for the roles of CsgA and SocA in production of the C signal. CsgA and SocA are SCADHs, which oxidize alcohol groups by reduction of $NAD(P)^+$. Mutation of the *csgA* gene results in loss of C signaling, while overexpression of SocA restores C signaling to *csgA* mutants. The model proposes that the C signal is the enzymatic product of CsgA. The model further proposes that SocA has substrate overlap with CsgA and can generate the C signal in lieu of CsgA. The substrate for neither enzyme is known.

synthesizing the C signal made it an ideal candidate to attempt to identify the SocA substrate(s). Using a dehydrogenase assay-based screening method to purify the SocA substrate, we show that SocA performs a novel biochemical reaction in oxidizing 1-acyl 2-hydroxy-*sn*-glycerophosphoethanolamine (lyso-PE) and provide evidence for a second, unidentified substrate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* DK1622 (3) and LS523 (25) were used as the wild type and *csgA* mutant, respectively. Cells were grown at 32°C in CYE broth (10 g $liter^{-1}$ Casitone, 5 g $liter^{-1}$ yeast extract, 4 mM $MgSO_4$, 10 mM *N*-morpholinopropanesulfonic acid [MOPS] [pH 7.6]) or on CYE agar (CYE plus 15 g $liter^{-1}$ Difco agar). *Escherichia coli* BL21Star(DE3), used for *socA* expression, was grown in Luria-Bertani broth (10 g $liter^{-1}$ tryptone, 5 g $liter^{-1}$ yeast extract, and 5 g $liter^{-1}$ sodium chloride) supplemented with 100 μg ml^{-1} ampicillin.

Expression and purification of SocA. The *socA* gene was amplified by the PCR using forward primer 5′-CACCATGATGGCGTTCAGTGGCAAG-3′ and reverse primer 5′-AGCCGAAAAGCCCCATCGAC-3′ (Gibco BRL Life Technologies, Gaithersburg, MD). The PCR fragment was cloned into different pET/D-TOPO expression vectors (Invitrogen Corporation, Carlsbad, CA) for generation of clones expressing SocA with different fusion tags. pLEE118 was made using pET102/D-TOPO and produces a SocA fusion protein with N-terminal thioredoxin and a C-terminal V5 epitope followed by six histidines (tSocAh). pLEE112 was made using pET100/D-TOPO and produces a SocA fusion protein with an N-terminal Xpress epitope preceding a six-histidine tag (hSocA). The expression host BL21Star(DE3) is a derivative of *E. coli* B that is deficient in the Lon and OmpT proteases (7, 17).

Both versions of SocA were purified in a similar manner. Cells were lysed by three passes through a SIM-AMINCO French pressure cell (Spectronic Unicam, Rochester, NY) at 10,000 lb/in^2 and separated into soluble and insoluble fractions by centrifugation at 21,000 × *g* for 1 h. Soluble protein was subjected to affinity chromatography on a nickel column (Amersham Biosciences, Piscataway, NJ). The loaded column was washed with 100 mM imidazole, and SocA was eluted with 300 mM imidazole. The protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis\ and stained using Silver Stain Plus reagents (Bio-Rad Laboratories, Hercules, CA). Protein concentration was determined using the bicinchoninic acid assay (Pierce Chemicals Co., Rockford, IL) with bovine serum albumin as a standard. Attempts to express CsgA using the three pET/D-TOPO systems and other bacterial and yeast expression systems were uniformly unsuccessful.

Dehydrogenase assay. The ability of recombinant SocA to oxidize partially pure cellular extracts or synthetic substrates at 25°C was monitored at 340 nm using a spectrophotometer (DU 640B; Beckman Instruments Inc., Fullerton, CA). The reaction mixture contained 100 mM sodium phosphate buffer, pH 6.8, 1 mM NAD^+, and various amounts of substrate and protein. The cellular extracts were solubilized in 100% methanol, and the final concentration of methanol in the reaction mixture was maintained at less than 2.5%. The change in absorbance due to the production of NADH was monitored for at least 5 min or until the reaction plateau. The specific activity was calculated from the initial rates by using a molar extinction coefficient of 6,220 M^{-1} cm^{-1} for NADH. Specific activity is defined as micromoles of NADH formed per minute per milligram of protein. Specific activity was routinely determined from three independent protein preparations and is shown as mean ± standard deviation. In the absence of SocA, the spectrophotometer did not detect NADH production (<2 nmol).

Extraction and purification of SocA substrates. *M. xanthus* DK1622 cells grown in 2.85 liters CYE to stationary phase were harvested by centrifugation at 10,000 × *g* for 10 min. The cell pellet was resuspended in 105 ml MOPS buffer (10 mM MOPS, pH 7.2, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 50 mM NaCl). Following two freeze-thaw cycles (−20°C and room temperature for 4 h each), the cell suspension was subjected to sonication using 10 30-second pulses at 40 μW (W380; Heat Systems Ultrasonics). Three extraction methods were compared: extraction with ethyl acetate containing 0.02% glacial acetic acid (acidified ethyl acetate), extraction with ethyl acetate, and extraction with methanol-chloroform.

For extraction with acidified ethyl acetate, a 35-ml cell lysate was extracted with 25 ml acidified ethyl acetate at 37°C for 30 min followed by centrifugation at room temperature for 45 min at 10,000 × *g* to separate the phases. The aqueous phase was reextracted with 25 ml acidified ethyl acetate, and the pooled ethyl acetate fractions were dried under N_2. The dried extract was dissolved in 1 ml methanol. Extraction with ethyl acetate was the same except for the omission of glacial acetic acid.

Extraction with methanol-chloroform was performed by the method of Bligh and Dyer as modified by Kearns and Shimkets (5). A 35-ml cell lysate was extracted with 15 ml methanol:chloroform (2:1) at 37°C for 1 h followed by centrifugation at room temperature for 5 min at 10,000 × *g* to separate the aqueous phase from the solvent phase. The pellet was reextracted with 5 ml methanol:chloroform:water (2:1:0.8, vol/vol/vol), vortexed, and centrifuged again. The chloroform phase was removed, dried under N_2, and dissolved in 1 ml methanol.

The amount of substrate in each sample was determined using the dehydrogenase assay. The absorbance at 340 nm at the reaction plateau was used to calculate the amount of substrate converted, assuming a 1:1 molar ratio with the amount of NADH produced.

The crude extract was fractionated on a 500 mg Supelclean LCSi silica gel Sep-Pak column (Supelco, Bellefonte, PA). The column was conditioned with 4 ml hexane: methyltertiary butylether (MTBE) (96:4) followed by 6 ml hexane. The sample was loaded in hexane:MTBE (200:3). The column was then washed with solvents of increasing polarity in the following order: 10 ml hexane:MTBE (200:3), 10 ml hexane:MTBE (96:4), 10 ml hexane:acetic acid (100:0.2), 10 ml hexane:MTBE: acetic acid (100:2:0.2), 10 ml MTBE:acetic acid (100:0.2), 6 ml MTBE:methanol: ammonium acetate (25:4:1), 10 ml MTBE:methanol:ammonium acetate (10:4:1), 10 ml MTBE:methanol:ammonium acetate (5:4:1), and 10 ml MTBE:methanol:ammonium acetate (5:8:2) (1). The solvents were evaporated under N_2, and the dry extract was resuspended in 500 μl methanol. Twenty microliters of each of these fractions was used in the dehydrogenase assay to quantify the amount of substrate recovered.

The active fraction from LCSi was further purified on a C_{18} column (3.9 × 150 mm; Waters, Milford, MA) by reverse-phase high-performance liquid chromatography on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a photodiode array detector. Diode array detector spectral data were monitored from 200 to 600 nm. Elution was carried out using an alkaline solvent system of water and methanol with a gradient from 55% to 95% methanol in 30 min hold for 12 min followed by reequilibration of the column. Each fraction from the C_{18} column was assayed for dehydrogenase activity, and the peak corresponding to the active fractions was further analyzed by electrospray ionization tandem mass spectrometry (ESI-MS-MS) (MDS SCIEX API365, Concord, Ontario, Canada). The fractions with SocA substrate(s) showed abundant peaks in the mass range *m/z* 300 to 500 atomic mass units (amu). Most signals appeared with even mass numbers, suggesting the occurrence of an odd number of nitrogen molecules. A quaternary nitrogen is part of the head group of phosphatidylethanolamine (PE), the most abundant phospholipid in *M. xanthus* (6). As PE is not predicted to be a substrate for an alcohol dehydrogenase, the active fraction from LCSi was screened for lyso-PE species using a neutral loss of *m/z* 141 in the positive MS-MS mode. This represents a very selective way to detect the PE (27) and revealed abundant ions at *m/z* 452, 474, and 490, which correspond to the $[M+H]^+$ of lyso-PE 16:1 and its adducts with sodium and potassium, respectively. Screening in the mass range *m/z* 400 to 900 amu in the negative mode, a peak at *m/z* 450 amu was found that corresponds to the expected $[M-H]^-$ ion of lyso-PE 16:1. At this point it is not clear whether one or more of the other abundant mass peaks found in the negative-mode scan represents another SocA substrate, since the negative mode reveals only a portion of the compounds that are present.

Preparation of lysophospholipid substrates. Synthetic lipids were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All other substrates and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Lysophospholipid substrates with an unsaturated fatty acid were solubilized in 100% methanol and diluted in the reaction mixture to a final methanol concentration of 5%.

Lysophospholipids with a saturated fatty acid were solubilized in chloroform:methanol:water (65:25:4) and added to the dehydrogenase assay mixture, and the chloroform phase that separates from the aqueous assay medium was removed before addition of the enzyme. Mixed micelles of the lysophospholipids and Triton X-100 were prepared by equilibrating 2.5 mM lysophospholipid with 5 mM Triton X-100 in water at room temperature overnight.

1-*O*-[11-(*Z*)-Hexadecanoyl]-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (lyso-PE 16:1 ω5c) was generated by treating 1,2-*O*-bis[11-(*Z*)-hexadecanoyl]-*sn*-glycero-3-phosphoethanolamine (PE-16:1 ω5c/16:1 ω5c) (courtesy of A. Venot and G. J. Boons) with phospholipase A2. Six hundred micrograms of PE-16:1 ω5c/16:1 ω5c was incubated with 20 μg phospholipase A2 from *Naja mossambica mossambica* (Sigma Chemical Co., St. Louis, MO) in the presence of 10 mM $CaCl_2$ and 200 mM Tris, pH 8.0, at 37°C for 2 h. The lipid was extracted with chloroform:methanol:acetic acid (50:50:1) and purified by thin-layer chromatography (TLC) on silica gel 60 (20 × 20 cm; layer thickness, 250 μm; EM Science, Gibbstown, NJ) with chloroform:methanol:water (65:25:4) as the mobile phase. The same solvent was used for extraction of crude and synthetic preparations of lyso-PE from silica gel for subsequent use in the dehydrogenase assay.

Extraction and quantification of lysophosphatidylethanolamine. *M. xanthus* cells in CYE were harvested at a density of 5×10^8 ml^{-1} by centrifugation at 10,000 × *g* for 10 min, resuspended in fresh CYE, and spread on TPM agar (10 mM Tris, pH 7.6, 1 mM KH_2PO_4, and 10 mM $MgSO_4$, 1.5% [Difco] agar) at a final density of 2.8×10^6 cells cm^{-2}. After 24 h at 32°C, the cells were removed from the agar and used as the source of developmental lysophospholipids. Vegetative *M. xanthus* cells grown in CYE to a density of 5×10^8 cells ml^{-1} were used as the source of vegetative lysophospholipids. The lysophospholipids were extracted in chloroform:methanol (5) and subjected to ESI-MS. Synthetic lyso-PE 16:0 and lyso-PE 18:1 were used as standards for quantification of the saturated and unsaturated lyso-PE, respectively.

Thin-layer chromatography. Oxidized lyso-PE 16:0 was examined by thin-layer chromatography on silica gel 60 (20 × 20 cm; layer thickness, 250 μm; EM Science, Gibbstown, NJ) using chloroform:methanol:water (65:25:4) as the mobile phase. The TLC plates were stained with iodine vapors to observe molecules containing fatty acids. The TLC plates were stained with ninhydrin (0.5% ninhydrin in 1-butanol containing 3% acetic acid) and incubated at 100°C for 3 min to observe molecules containing an amine group.

Extracts from the LCSi column were further purified by thin-layer chromatography under the conditions described above. Fifteen different fractions (1 cm tall × 3.5 cm wide), extending from the origin to the solvent front, were extracted from the silica gel with 500 μl methanol. The silica gel and methanol mixture was vortexed for 15 seconds and then subjected to centrifugation at 14,000 × *g* for 1 min to separate the supernatant, which was used as the source of substrate in the enzyme and bioassays.

SocA oxidation products. The products of SocA-mediated lyso-PE oxidation were extracted with chloroform and concentrated by evaporation under N_2. A 10-ml aqueous enzyme assay mixture was vortexed with 2 ml chloroform for 1 min at 25°C and centrifuged at 10,000 × *g* for 1 min to separate the chloroform phase from the aqueous phase. The chloroform phase was dried under N_2, and the extract was resuspended in 100 μl methanol.

For nuclear magnetic resonance (NMR) spectroscopy, oxidized and reduced lyso-PE 16:0 was dissolved in deuterated chloroform:methanol (2:1). One-dimensional proton and two-dimensional gradient correlation spectroscopy spectra were recorded on a Varian Inova 500 MHz instrument at 25°C.

For gas chromatography-mass spectrometric (GC/MS) analysis, oxidized lyso-PE 16:0 was pertrimethylsilylated by treatment with Tri-Sil (Pierce Chemical Co, Rockford, IL) at 80°C for 20 min. Trimethylsilyalate (73 amu) reacts with the hydroxyl groups on the analyte, enabling identification of the molecules on the basis of a shift in the molecular mass. The analysis was performed by chemical ionization-GC-MS using an Agilent 6890N GC (DB-1, 30-m tall, 0.25-mm inner diameter; Supelco, Bellefonte, PA) interfaced with a 5973 mass-selective detector with ammonia as ionizing gas.

For matrix-assisted laser desorption ionization mass spectrometry, oxidized lyso-PE 16:0 was added to sinapinic acid matrix prepared in acetronitrile:water (50:50) with 0.1% trifluoroacetic acid and dried before analysis. A Bruker Autoflex mass spectrometer with a nitrogen laser was used to acquire the data. The analysis was also repeated in the presence of cesium chloride, which forms adducts with ionized molecules, shifting the molecular mass of the analyte by 132 amu.

Bioassay for rescue of *csgA* development. A qualitative biological assay was employed to examine the potential of partially pure extracts and the synthetic lipids to rescue *csgA* mutant development. The partially pure extract was oxidized with tSocAh and extracted with methanol:chloroform (2:1). Various amounts (250 nmol, 100 nmol, 50 nmol, 25 nmol, 10 nmol, and 1 nmol) of extracts were dried on individual filter paper discs (Whatman no. 1 filter paper; 0.5-mm diameter).

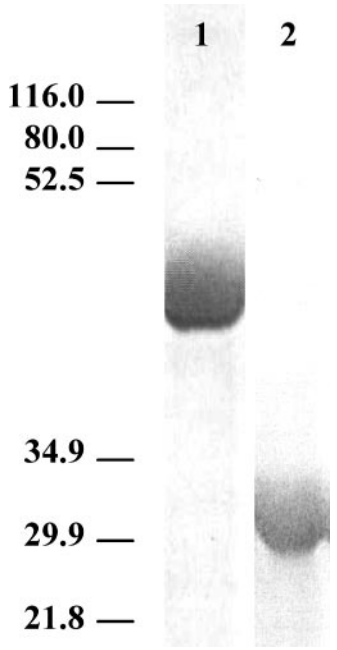

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel showing tSocAh (lane 1), and hSocA (lane 2) purified on a nickel column and visualized with silver stain. Each lane contains 30 μg protein. The numbers to the left of the gel show the molecular masses of protein standards (in kilodaltons).

csgA cells were grown to mid-log phase in CYE and harvested by centrifugation at 10,000 × *g* for 10 min at room temperature. The cell pellet was resuspended in fresh CYE to a final density of 5×10^9 ml^{-1}. Development was induced by spreading 200 μl of the cell suspension on TPM agar at 32°C. Whatman filter paper discs containing various amounts of the oxidized and reduced extracts were placed on the *csgA* cell lawn 6 h later. Rescue of developmental aggregation and sporulation was monitored over the next 5 days with a dissecting microscope.

Lyso-PE 16:0 and lyso-PE 16:1 were oxidized by SocA, and various amounts (100 μg, 50 μg, 25 μg, 10 μg, 5 μg, and 1 μg) of the oxidized and reduced forms of lyso-PE 16:0 and lyso-PE 16:1 were tested for their ability to rescue the developmental defects of *csgA* mutants.

RESULTS

The objective of this work was to identify an enzymatic product for SocA and determine whether it behaves like the C signal to restore *csgA* mutant development. The work proceeded in several stages. First, SocA was expressed in *E. coli* and purified. Second, extracts of *M. xanthus* cells were sequentially purified, and a dehydrogenase assay-based screening method was used to monitor for enrichment of the SocA substrate, which was subsequently identified by mass spectrometry. Third, the SocA enzymatic parameters were examined using highly pure, synthetic substrates. Finally, the enzymatic products were defined and examined for morphogenic activity.

Expression and purification of SocA. SocA was produced in *E. coli* with either an N-terminal (hSocA) or C-terminal (tSocAh) histidine (six-His) fusion. tSocAh and hSocA were purified on a nickel affinity column 8-fold and 5-fold, respectively, to apparent homogeneity (Fig. 2). The yields of tSocAh and hSocA were 24 mg liter^{-1} and 9 mg liter^{-1}, respectively. Although both tSocAh and hSocA had comparable specific

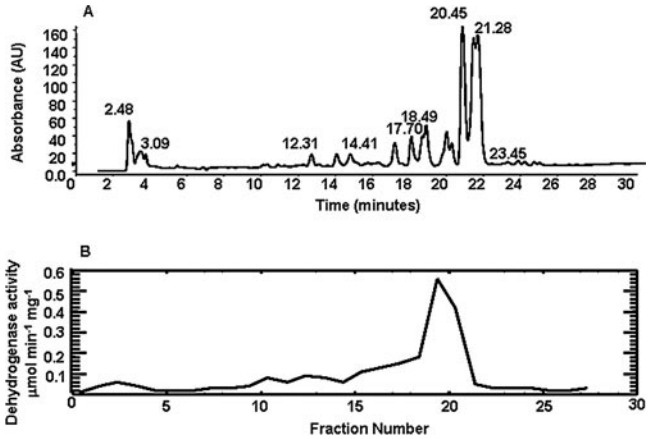

FIG. 3. Separation of fractions from LCSi on a C_{18} reverse-phase column. A. Total wavelength chromatogram (TWC) of the diode array detector. B. Enzyme activity profile of fractions using the SocA dehydrogenase assay. (Note that retention time is not the same as fraction number. For example, fraction number 19 included retention time 18 to 19 min. Abundant peaks in TWC eluted after the fractions containing major SocA dehydrogenase activity.)

activities, tSocAh was used in all enzyme studies due to the higher protein yield.

Purification of SocA substrate. Preliminary experiments indicated that SocA reduced NAD^+ when the *M. xanthus* membrane fraction from either vegetative or developing cells was used as the substrate. No activity was detected when the cytosolic fraction from vegetative cells was used as a source of substrate. Subsequently, vegetative *M. xanthus* cells were subjected to three different organic extractions to determine the most efficient extraction method. The amount of substrate in the solvent was quantified using a spectrophotometric assay in which NAD^+ is reduced to NADH by tSocAh, with the assumption that one molecule of substrate is oxidized for each molecule of NAD^+ reduced. The acidified ethyl acetate extraction gave the highest yield, 4.1 μmol 10^{10} $cells^{-1}$. Extraction with ethyl acetate yielded 3.2 μmol 10^{10} $cells^{-1}$ (78% of that of the acidified ethyl acetate), and a modified Bligh and Dyer extraction yielded 1.1 mol 10^{10} $cells^{-1}$ (26%). Acidified ethyl acetate was used for all subsequent extractions.

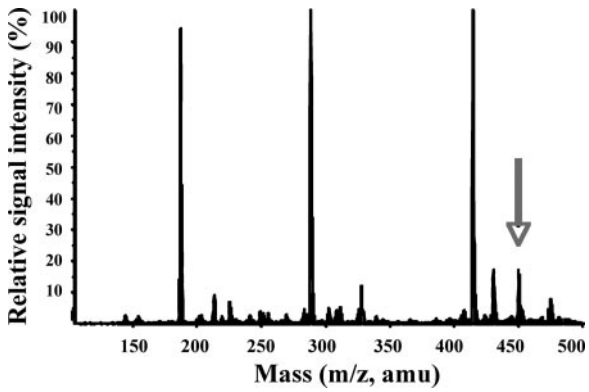

FIG. 4. Mass spectrum between 140 and 500 amu of the combined fractions 19 to 21. The intensity of individual masses is plotted against the respective *m/z*. (The *m/z* 450.7 corresponds to the mass of lyso-PE 16:1 in the negative mode.)

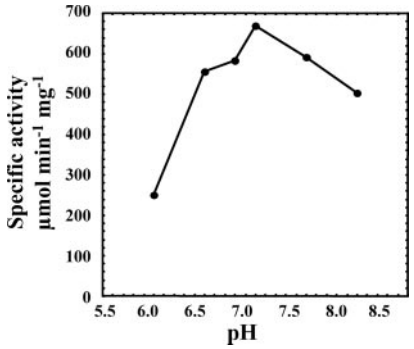

FIG. 5. Effect of pH on lyso-PE 16:0 oxidation by SocA. Enzyme was assayed with 100 μM lyso-PE 16:0 in sodium phosphate buffer.

The acidified ethyl acetate extract was applied to a solid-phase LCSi silica gel column and eluted stepwise with solvents of increasing polarity. The substrate, identified with the SocA dehydrogenase assay, eluted with MTBE:acetic acid (100:0.2) in a single fraction of intermediate polarity, with 83% recovery of the original activity (data not shown). The active fraction from LCSi was concentrated and purified by reverse-phase high-performance liquid chromatography on a C_{18} column. The highest SocA substrate activity was detected with fractions that eluted at 18 to 19 min from the liquid chromatography column (Fig. 3B). This broad SocA activity peak eluted just before the two major diode array detector (DAD) spectral peaks at 19 to 21 min (Fig. 3A). One possible explanation for the broad substrate peak is the presence of multiple SocA substrates. Attempts to devise another purification step with solid-phase separation on StrataX (Phenomenx, Torrance, CA) did not improve the purification.

SocA is a lysophosphatidylethanolamine dehydrogenase. Various modes enabled by tandem mass spectrometry (electrospray ionization and atmospheric chemical ionization in the positive and negative modes; neutral loss scan and product ion scan) were used to determine the molecular masses of compounds in the active fractions. The highest signal intensity was found in the range of *m/z* 140 to 500 atomic mass units. Comparison with a database of hydrophobic compounds of known mass (Fig. 4) revealed a correlation with 1-acyl 2-hydroxy-*sn*-glycerophosphoethanolamine containing a C16:1 fatty acid (lyso-PE 16:1; *m/z* 450.7 amu). The mass peak *m/z* 450.7 (negative mode) was positively identified as lyso-PE from a neutral loss scan for *m/z* 141 in the positive mode, yielding the $[M+H]^+$ ion, and from a product ion scan in the negative mode, yielding the fragment ions indicative of the PE head group plus a 16:1 acyl chain (data not shown) (18, 27). Previously it was found that C16:1 ω5c is by far the most abundant monounsaturated fatty acid in *M. xanthus* (6).

Since lyso-PE 16:1 ω5c is not commercially available, biochemical analysis began with synthetic lyso-PE 16:0, which has a saturated fatty acid of identical length. Lipid vesicles were prepared by addition of chloroform:methanol:water (65:25:4)-solubilized lipid to the aqueous assay mixture. The specific activities of tSocAh and hSocA with 100 μM lyso-PE 16:0 were 558 ± 69 and 461 ± 96 μmol min^{-1} mg^{-1}, respectively.

TABLE 1. Acyl chain specificity of SocA[a]

Substrate	Sp act (μmol min^{-1} mg^{-1}) (mean ± SD)	% Converted in 10 min
L-α-Glycerophosphorylethanolamine	10 ± 9	8
1-Acyl 2-hydroxy-PE 14:0	247 ± 61	39
1-Acyl 2-hydroxy-PE 16:0	558 ± 69	70
1-Acyl 2-hydroxy-PE 16:1 ω5c	345 ± 239	38
1-Acyl 2-hydroxy-PE 18:0	428 ± 164	40
1-Acyl 2-hydroxy-PE 18:1 ω9c	299 ± 69	32

[a] Enzyme assays were carried out in the presence of 1 mM NAD^+, 100 μM substrate, and 100 μM sodium phosphate buffer, pH 6.8. The standard deviation of the mean was calculated from three independent experiments.

TABLE 2. Phospholipid head group specificity of SocA[a]

Substrate[b]	Sp act of lyso-PE 16:0 (μmol min^{-1} mg^{-1}) (mean ± SD) in:	
	Methanolic lipid[c]	Mixed micelles[d]
PE (1-acyl 2-hydroxy-PE 16:0)	558 ± 69	106 ± 43
PC (1-acyl 2-hydroxy-PC 16:0)	9 ± 4	23 ± 7.5
PS (1-acyl 2-hydroxy-PS 18:1)	<0.01	<0.01
PG (1-acyl 2-hydroxy-PG 16:0)	<0.01	<0.01
PA (1-acyl 2-hydroxy-PA 16:0)	5 ± 8	<0.01

[a] Enzyme assays were carried out in the presence of 1 mM NAD^+, 100 μM substrate, and 100 μM sodium phosphate buffer, pH 6.8. The standard deviation of the mean was calculated from three independent assays.

[b] PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid.

[c] Specific activity determined using lipid solubilized in chloroform and methanol and added to the reaction mixture.

[d] Specific activity determined with mixed micelles of the lysophospholipids and Triton X-100.

SocA oxidized the substrate over a pH range from pH 6.0 to 8.0 with maximal activity near pH 7.0 with 100 μM lyso-PE 16:0 (Fig. 5) and 50 μM lyso-PE 16:0 (not shown). NAD^+ binding SCADH have an aspartate in β2 that repels the negatively charged phosphate group on $NADP^+$ to confer NAD^+ specificity (4). SocA lacks this aspartate and is predicted to utilize both coenzymes with comparable efficiencies. Indeed, SocA reduced NAD^+ (558 ± 69 μmol min^{-1} mg^{-1}) and $NADP^+$ (652 ± 59 μmol min^{-1} mg^{-1}) at comparable rates.

Substrate specificity and kinetics. The fatty acid specificity was examined with lyso-PE species with different acyl chain lengths and degrees of saturation (Table 1). Although SocA oxidizes lyso-PE 16:0 with the highest specific activity, lyso-PE substrates with different acyl chain lengths were oxidized at similar rates. A *sn*-1 fatty acid appears to be essential, as SocA was unable to oxidize L-α-glycerophosphorylethanolamine. As the acyl chain in 1-hydroxy 2-acyl lysophospholipids rapidly migrates to *sn*-1 (21), 1-hydroxy 2-acyl lyso-PE was the only form of the molecule available for examination.

Although SocA oxidizes lyso-PE 16:0 with a greater specific activity, initial reaction rates were analyzed with lyso-PE 18:1, which is the only commercially available lyso-PE that is soluble in a water-miscible solvent (e.g., methanol). Lyso-PE 16:1 is not commercially available. Hence, using lyso-PE 18:1 permitted kinetic analysis in a more straightforward manner. Initial rates were fitted to the Michaelis-Menten equation using nonlinear regression (Fig. 6). The apparent K_m and V_{max} were 116 μM and 875 μmol min^{-1} mg^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) was 1×10^8 M^{-1} s^{-1}. The range of concentrations tested was limited by the solubility of lyso-PE 18:1, as methanol inhibited protein activity at higher concentrations

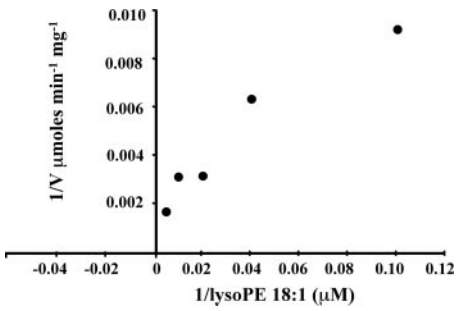

FIG. 6. Double-reciprocal plot of the initial velocity of the enzymatic reaction versus lyso-PE 18:1 concentration.

and the detection limits of the spectrophotometer did not permit readings at lower substrate concentrations.

Only 60 to 70% of the substrate was converted after 10 min when the enzyme velocity plateaus. One interpretation of this result is that the lipid is in a heterogeneous mixture of states not all of which are substrates. The biophysical structures adopted by aqueous suspensions of lyso-PE have not been extensively examined, but under these conditions, the lipid may be present in both micellar and unilamellar forms. In order to develop a uniform method of lipid presentation, mixed micelles were created with lyso-PE and Triton X-100 (11). The specific activity of micellar lyso-PE 16:0 was fivefold lower than that observed with methanolic lipid (Table 2), and the substrate conversion was only 20% in 10 min. The lower specific activity may be due to inhibition by the detergent. Thus, while micellar lyso-PE appears to be a substrate, the reason only a fraction of the substrate is oxidized remains unknown.

SocA head group specificity was examined with Triton X-100 mixed micelles from glycerophospholipids with choline, serine, glycerol, phosphatidic acid, or ethanolamine head groups. In all but one case, synthetic substrates with the C16:0 acyl chain were available. SocA preferentially oxidized lysophospholipids with the ethanolamine head group (Table 2). Assays performed with detergent-free lysophospholipids solubilized in organic solvents also indicated a strong preference for lyso-PE (Table 2).

Lyso-PE 16:0 oxidation products. The oxidation product was purified and concentrated by chloroform extraction of the enzyme reaction. While the predicted product of lyso-PE oxidation is 1-acyloxy-3-(2-aminoethylphosphatyl) acetone, the NMR spectrum of the oxidation product indicated the absence of the phosphoethanolamine moiety. The dihydroxyacetone core of this molecule can rearrange via keto-enol tautomerism to glyceraldehyde, with the concomitant loss of both the phosphoethanolamine and acyl groups. The oxidized molecule could also undergo acid hydrolysis at the ester linkages, resulting in formation of glycerophosphorylethanolamine and a free fatty acid or monoacylglycerol phosphate and ethanolamine. NMR spectroscopy and mass spectrometry indicated that oxidized lyso-PE 16:0 underwent substantial degradation but could not

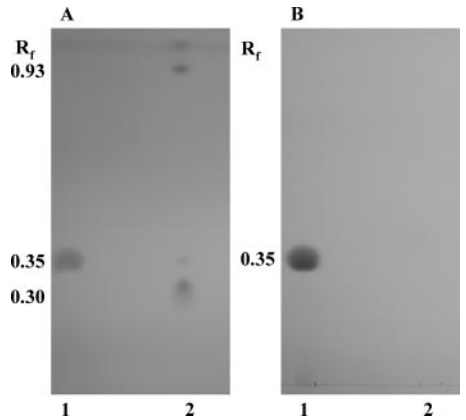

FIG. 7. Thin-layer chromatography of lyso-PE 16:0 (lane 1) and the enzymatically oxidized lyso-PE 16:0 (lane 2) stained with iodine (A) and ninhydrin (B). The reduced form (lane 1 in panels A and B) stains with both iodine and ninhydrin, while the oxidized form (lane 2 of panels A and B) stains only with iodine. The corresponding R_f value is indicated on the left side of the respective panel.

unambiguously identify the products (data not shown). TLC analysis of oxidized lyso-PE 16:0 showed two iodine-stained spots with R_f values of 0.30 and 0.93, indicating hydrolysis of the product (Fig. 7A). The R_f value of 0.93 corresponds to the free fatty acid and monopalmitoylglycerol (data not shown). The identity of the iodine-stained spot with a R_f value of 0.30 remains unknown. Ninhydrin staining revealed that neither product contained the ethanolamine group, which probably remained in the aqueous portion of the chloroform-extracted enzyme mixture (Fig. 7B).

An oxidation product of lyso-PE 16:0 was reduced by SocA using NADH (the reverse reaction of the dehydrogenase assay with NAD^+), with a specific activity of 733 ± 247 μmol min^{-1} mg^{-1}. The possible substrates include 1-acylglyceraldehyde, 1-acyl-2-keto glycerol, and glyceraldehyde. SocA did not reduce glyceraldehyde (data not shown), which again revealed the importance of the acyl chain.

SocA acts on multiple substrates. Lyso-PE species were quantified during growth and development of wild-type *M. xanthus* cells (Table 3). Vegetative cells contain about 10^5 molecules $cell^{-1}$, which increase about 10-fold during development. With the exception of lyso-PE 15:1, all the species of lyso-PE increased during development. The levels of lyso-PE 16:1 and lyso-PE 18:0 (iso-methyl 17:0; in *M. xanthus*, this is the only saturated acyl chain with 18 carbons) show striking increases of 40-fold and 300-fold, respectively.

Quantification of the substrate after partial purification on LCSi revealed about 5×10^7 substrate molecules per cell, about 100-fold higher than the entire pool of lyso-PE molecules present per cell, providing evidence for a second SocA substrate. To determine whether the bulk of the alcohol dehydrogenase activity was correlated with lyso-PE, the LCSi fraction was further separated by TLC under conditions where all lyso-PE species have the same R_f value regardless of the length and saturation of the acyl chain. The TLC plate was harvested in 1-cm-wide bands, and each fraction was tested for its ability to be oxidized by SocA. A minor amount of SocA substrate was detected with the fraction in which lyso-PE is normally found (R_f of 0.35 using a lyso-PE 16:0 standard). Large amounts of the SocA substrate(s) were found in three fractions near the solvent front (R_fs of 1.00; 0.93, and 0.80), confirming the presence of a substrate(s) with higher abundance than lyso-PE. Preliminary attempts to identify the molecule(s) with morphogenetic properties by liquid chromatography-MS-MS analysis were not successful.

Bioassay for morphogenic activity. As SocA overproduction compensates for the signaling defect in *csgA* mutants (13, 14), we examined whether the oxidation products of lyso-PE could be a cell signal in *M. xanthus*. A bioassay in which *csgA* cells were allowed to develop in the presence of lyso-PE 16:0 or its oxidation products was used. Unfortunately, the predicted lyso-PE oxidation product, 1-acyloxy-3-(2-aminoethylphosphatyl) acetone, is so unstable it does not persist long enough for study to determine whether it rescues *csgA* mutant development. Neither the substrates nor the hydrolysis products of oxidized lyso-PE were able to rescue development of a *csgA* mutant (data not shown).

Because the nature of the acyl chain might be important, synthetic lyso-PE 16:1 ω5c was generated and oxidized with SocA. This species was selected because the C16:1 ω5c fatty acid was previously found to be the most abundant unsaturated fatty acid in *M. xanthus* and because PE containing 16:1 ω5c is a chemoattractant (6). The SocA oxidation products were unable to rescue *csgA* development.

The same bioassay was used to determine whether the LCSi fraction was able to rescue *csgA* development. The LCSi fraction oxidized by SocA stimulated aggregation in *csgA* mutants, and some of the aggregates matured into large, spore-filled fruiting bodies after 5 days (data not shown). The TLC fractions assayed for dehydrogenase activity were examined for their ability to rescue CsgA development. The biological activity was located at the solvent front in the fraction with a R_f of 1.00, which accounted for about 58% of the total substrate. The amount of substrate was quantified with the dehydrogenase assay, and assuming 100% oxidation, 250 nmol of this fraction stimulated aggregation of *csgA* mutants along the circumference of the filter paper disc (Fig. 8). The significance of these results awaits the identification of the active molecule(s) in this fraction.

DISCUSSION

The SCADH family is functionally versatile with proteins from three EC classes, oxidoreductases (EC 1.1), dehydratases

TABLE 3. Amounts of lyso-PE species in vegetative and developing cells[a]

Cell type	Amt (molecules/cell) of lyso-PE species associated with the following fatty acids:				
	15:0	15:1	16:0	16:1	18:0
Vegetative cells	5.8×10^4	2.8×10^2	4.4×10^4	9.6×10^2	6.0×10^1
Developmental cells	3.0×10^5	2.6×10^2	1.3×10^5	4.0×10^4	2.0×10^4

[a] Cells were extracted with chloroform:methanol (2:1) and then subjected to ESI-MS-MS. The lyso-PE species were quantified using calibration curves derived from known amounts of lyso-PE 16:0 for the saturated species and lyso-PE 18:1 for the unsaturated species.

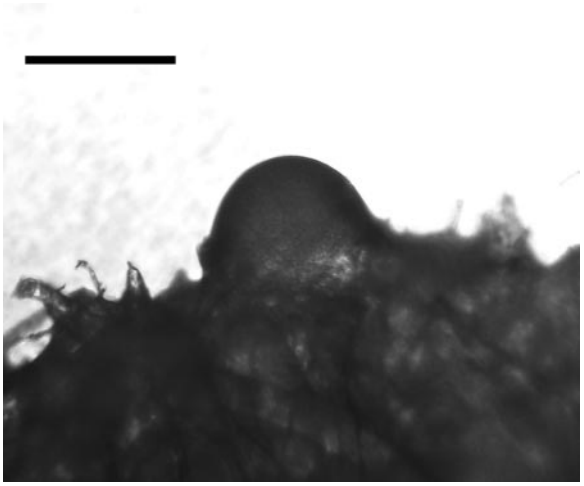

FIG. 8. Rescue of fruiting body development by a TLC-purified extract oxidized with SocA. A single fruiting body is shown along the edge of the filter paper disc. Bar, 200 μm.

(EC 4.1), and epimerases (EC 5.1) (19). Homology modeling suggests that both CsgA and SocA belong to the oxidoreductase EC group and are presumed to catalyze the interconversion of secondary alcohols and ketones using NAD(P)(H) sequestered in the N-terminal Rossmann fold (Fig. 1). SocA, the first lyso-PE dehydrogenase to be described, acts on lysophospholipid substrates with fatty acids of different chain length and saturation (Table 1), showing significant preference for the ethanolamine head group (Table 2). The efficiency of catalysis is $9 \times 10^7\ M^{-1}\ s^{-1}$ and is comparable to other well-characterized enzymes. The *sn*-2 hydroxyl group is likely oxidized to a keto group, but the product is unstable and fragments, hindering unambiguous structural identification and bioassay.

Lysophospholipids are produced in three ways. First, lysophosphatidic acid is a biochemical intermediate in the synthesis of PE, the major phospholipid in *M. xanthus* and *E. coli*. Second, lysophospholipids are generated by phospholipase A, which hydrolyzes the fatty acid at either *sn*-1 or *sn*-2 as part of a stress response to membrane damage in *E. coli* (26). Finally, phospholipids are used as substrates for acyltransferases, which transfer an acyl chain from a phospholipid to a wide variety of molecules. In cases involving removal of the *sn*-1 fatty acid from a phospholipid, the *sn*-2 acyl chain migrates to *sn*-1 (21). Lysophospholipids can be reacylated by the PlsC acyltransferase, which adds a fatty acid to the *sn*-2 position to produce a mature phospholipid. In this work, lyso-PE was measured during growth and development of *Myxococcus* and found to be quite low, suggesting that lysophospholipids are either catabolized or efficiently reacylated.

Lysophospholipids serve as signaling molecules in mammals and mediate differentiation of cells of the immune system as well as those involved in neurogenesis, angiogenesis, or carcinogenesis. Lysophospholipids exert their influence through specific G-protein-coupled receptors (2), which then transmit the signal to proteins involved in second messenger generation (23). While their role as mediators of signal transduction has been studied extensively in eukaryotes, a similar role for lysophospholipids as bacterial signals has not been reported. Given a potential role for SocA in intercellular signaling during development of *M. xanthus*, we examined whether SocA mediates lyso-PE signaling in *M. xanthus*. Extracellular complementation of either reduced and enzymatically oxidized lyso-PE 16:0 and lyso-PE 16:1 did not rescue the developmental defects of the *csgA* mutant. The conclusions of the experiment are somewhat limited because of the instability of the oxidized form. SocA-oxidized lyso-PE likely undergoes keto-enol tautomerization with subsequent hydrolysis into products like 1-acyl glyceraldehyde, 1-acyl 2-keto glycerol, free fatty acid, and/or glyceraldehyde. Since there was no developmental rescue of a *csgA* mutant following extracellular complementation with oxidized lyso-PE, it is unlikely that the fragmented lipid products play a role in multicellular morphogenesis. Whether the short-lived keto derivative of lyso-PE is a signal could not be directly examined due to its fragmentation during purification following enzymatic oxidation. It is also possible that the keto form is used in the synthesis of another compound. It resembles 1-acyl dihydroxyacetone phosphate, an intermediate in the synthesis of some important eukaryotic lipids, including plasmalogens.

A single SCADH can act on structurally diverse biomolecules. For example, human type II hydroxyacyl-coenzyme A dehydrogenase/amyloid-β binding alcohol dehydrogenase is an oxidoreductase that acts on 3-hydroxyacyl-coenzyme A derivatives, hydroxysteroids, alcohols, and β-hydroxybutyrate (22). In this work we provide evidence for a second, more abundant SocA substrate in purified *M. xanthus* extracts. The identity of the abundant substrate is currently unknown but is worth pursuing because the fraction containing this substrate also appears to have morphogenetic activity. Matrix-assisted laser desorption ionization–time of flight/time of flight and/or NMR analysis may help to reveal the unidentified substrate(s), although both require higher concentrations of the target molecules.

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