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Purification and Characterization of a Long-Chain Acyl Coenzyme A Thioesterase from *Rhodopseudomonas sphaeroides*†

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ABSTRACT: A long-chain acyl coenzyme A thioesterase has been purified over 10000-fold (38% yield) from photoheterotrophically grown cells of the facultative phototrophic organism *Rhodopseudomonas sphaeroides*. This enzyme, designated thioesterase I, has a native molecular mass (M_r) of 22400 as estimated by gel filtration and apparently consists of two subunits of M_r 12500 each as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The enzyme will only hydrolyze acyl thio esters of coenzyme A (CoA) and displays a strict specificity for acyl coenzyme A substrates where the acyl moiety is $\geq C_{12}$. Palmitoyl coenzyme A and stearoyl coenzyme A are the preferred saturated acyl-CoA substrates while vaccenoyl coenzyme A is the preferred unsaturated substrate. The purified enzyme has a pH optimum of 8.0, is stabilized by 25% (w/v) glycerol, and is inhibited (90%) by treatment with 10 μM diisopropyl fluorophosphate. The physical and biochemical properties of the enzyme resemble those reported for the *Escherichia coli* low molecular weight thioesterase, and it is proposed that the *R. sphaeroides* thioesterase participates in the cellular mechanism for the direct utilization of exogenously supplied fatty acids for membrane phospholipid biosynthesis.

The cytosolic fraction of cells of *Escherichia coli* contains two distinct acyl-CoA[1] thioesterases, designated thioesterases I and II,[2] which are easily distinguished by size, sensitivity to inhibition by DFP, and substrate specificity (Kass et al., 1967; Barnes et al., 1970; Barnes & Wakil, 1968; Bonner & Bloch, 1972). The functions of these enzymes in the lipid metabolism of *E. coli* are unknown. In bacteria such as *E. coli*, which only possess a type II fatty acid synthetase (Bloch & Vance, 1977), the intermediates and products of fatty acid synthesis occur covalently attached to ACP (Vagelos, 1971; Prescott & Vagelos, 1972; Bloch & Vance, 1977), and the freely dissociable acyl-ACP products of de novo fatty acid synthesis are known to efficiently serve as immediate substrates for the bacterial *sn*-glycerol-3-phosphate acyltransferase, the enzyme that catalyzes the first committed step in bacterial phospholipid synthesis (Ailhaud & Vagelos, 1966; van den Bosch & Vagelos, 1970; Goldfine et al., 1967; Goldfine & Ailhaud, 1971; Lueking & Goldfine, 1975a; Cronan, 1978; Rock & Cronan, 1982). Thus, under conditions where cellular fatty acids are being produced de novo, a need for an acyl-CoA thioesterase, as well as for the acyl-CoA substrates themselves, is not readily apparent and not surprisingly, an intracellular pool of these intermediates has not been detected in *E. coli* (Klein et al., 1971; Frerman & Bennett, 1973; Rock & Jackowski, 1982).

The ability of bacteria to directly utilize exogenously supplied fatty acids for phospholipid synthesis suggests a specific function for acyl-CoA thioesterases in the lipid metabolism of procaryotes. Studies conducted with *E. coli* have shown that fatty acid transport is accompanied by an activation of the fatty acid to its acyl-CoA form (Overath et al., 1969; Klein et al., 1971; Frerman & Bennett, 1973). Thus, if one assumes that the direct utilization of exogenous fatty acids for phospholipid synthesis requires the entry of the fatty acids into an intracellular acyl-ACP pool, a conversion of acyl-CoA to acyl-ACP must occur and could result from the combined actions of an acyl-CoA thioesterase and the more recently identified acyl-ACP synthetase (Ray & Cronan, 1976; Spencer et al., 1978; Rock & Cronan, 1979). Whether such a process actually occurs in *E. coli*, however, is unclear, since in vitro studies have shown that this organism's *sn*-glycerol-3-phosphate acyltransferase can independently utilize both acyl-CoA and acyl-ACP substrates (van den Bosch & Vagelos, 1970;

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[1] Abbreviations: ACP, acyl carrier protein (reduced form); acyl-ACP, acylated acyl carrier protein; BSA, bovine serum albumin; CoA, coenzyme A; acyl-CoA, acyl coenzyme A; cmc, critical micellar concentration; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate; $NaDodSO_4$, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

[2] For the reasons previously discussed by Spencer et al. (1978), we have chosen to utilize the nomenclature of Barnes et al. (1970) for the designations of the *Escherichia coli* acyl-CoA thioesterases.

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Lueking & Goldfine, 1975a; Rock et al., 1981). The direct utilization of exogenous fatty acids by the facultative phototrophic bacterium *Rhodopseudomonas sphaeroides*, however, must occur by a mechanism similar to that proposed because this organism's *sn*-glycerol-3-phosphate acyltransferase has an obligate requirement for acyl-ACP substrates as acyl donors for *sn*-glycerol 3-phosphate acylation (Lueking & Goldfine, 1975b). In this regard, the present study describes the purification and characterization of an acyl-CoA thioesterase from phototrophically grown cells of *R. sphaeroides* that displays properties that are consistent with its participation in the envisioned mechanism for the direct utilization of exogenous fatty acids for phospholipid synthesis.

Experimental Procedures

Chemicals. Sephadex G-50, G-100, and G-200 were purchased from Pharmacia Fine Chemicals. Diisopropyl fluorophosphate, DEAE-Sephadex A-25, octyl-Sepharose, DTNB, butanoyl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, lauroyl-CoA, myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, myristoleoyl-CoA, palmitoleoyl-CoA, oleoyl-CoA, vaccenic acid, linoleic acid, linolenic acid, and CoA were obtained from Sigma Chemical Co. Acyl-CoA thio ester derivatives of vaccenic, linoleic, and linolenic acids were prepared by the enzymatic procedure of Merrill et al. (1982) with resin-bound rat liver fatty acyl-CoA ligase. These substrates were shown to be >95% pure by the chromotographic procedure of Shapiro & Prescott (1978) and exhibited A_{232}/A_{260} and A_{250}/A_{260} ratios of 0.58 and 0.81, respectively. 2-Propanol was purchased from Fisher Scientific Co., and chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad. All other chemicals were of reagent grade or better.

Organism, Medium, and Growth Conditions. R. sphaeroides strain M29-5 (Leu^-, Met^-), derived from strain 2.4.7, was obtained from Samuel Kaplan, University of Illinois, Urbana, IL. Growth was conducted in a succinic acid minimal medium (Lueking et al., 1978) supplemented with 0.2% (w/v) casamino acids. Stock cultures were maintained at −20 °C in this same medium adjusted to 10% (w/v) glycerol. Incubations were conducted (32 °C) photoheterotrophically (anaerobic–light) in 1–20-L vessels under an atmosphere of 95% N_2–5% CO_2. Continuous illumination of the cultures was provided by banks of 60-W Lumiline lamps (Sylvania). Culture growth was monitored turbidimetrically by employing a Klett-Summerson colorimeter equipped with a No. 66 filter. Since control studies showed that the yield of thioesterase activity per gram wet weight of cells was identical for cells harvested in the logarithmic and stationary phases of growth, stationary-phase cells were employed for the purification of preparative quantities of enzyme.

Thioesterase Assays. Thioesterase activity was routinely assayed spectrophotometrically by monitoring the increase in absorbance at 412 nm resulting from the generation of free CoASH in the presence of DTNB (Ellman, 1959). Enzyme activities were calculated by employing an E_{412} of 13.6 mM^{-1} cm^{-1} for the 2-nitro-5-thiobenzoate anion. A typical incubation mixture contained 50 mM Tris-HCl (pH 8.0), 30 μM acyl-CoA substrate, 0.10 mM DTNB, and 1–2 units of enzyme in a final volume of 1 mL. All assays were conducted at 25 °C, and initial reaction rates were linear with respect to both time (4 min) and the concentration of extract protein. No activity was observed in control incubations lacking either extract protein or acyl-CoA substrate. A unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 nmol of acyl-CoA/min. Alternatively, to determined pH optima, thioesterase activity was assayed by monitoring the decrease in absorbance at 232 nm resulting from the hydrolysis of the acyl-CoA thio ester bond. In this instance, enzyme activity was calculated by employing an E_{232} of 4.0 mM^{-1} cm^{-1} (Bonner & Bloch, 1972). Enzyme activities determined by employing the two assay procedures agreed within 10%.

Protein Assays. Protein determinations were conducted by the method of Lowry et al. (1951) and by the method of McKnight (1976) with BSA as standard.

Gel Electrophoresis. The final step of enzyme purification consisted of preparative elution electrophoresis of the enzyme activity recovered following gel chromatography on a column of Sephadex G-50. Electrophoresis was conducted by employing a Hoefer SE 600 vertical slab gel apparatus equipped for continuous elution with a Bio-Rad Laboratories preparative elution adapter kit. Native polyacrylamide gels (3 mm) were constructed in the following manner: [plug gel (4 cm)] 15% (w/v) acrylamide [all gels contained an acrylamide to bis(acrylamide) ratio of 40:1], 0.375 M Tris-HCl (pH 8.8), 0.15% (v/v) TEMED, 0.025% (w/v) $(NH_4)_2S_2O_8$; [elution chamber (0.4 cm)] 50% (w/v) sucrose; [resolving gel (5.0 cm)] 15% (w/v) acrylamide, 0.375 M Tris-HCl (pH 8.8), 25% (w/v) glycerol, 0.13% (v/v) TEMED, 0.02% (w/v) $(NH_4)_2S_2O_8$; [stacking gel (1.0 cm)] 7% (w/v) acrylamide, 0.313 M Tris-HCl (pH 6.8), 5% (w/v) glycerol, 0.11% (v/v) TEMED, 0.10% (w/v) $(NH_4)_2S_2O_8$. A solution of 25 mM Trizma base and 0.192 M glycine was employed as electrode buffer. Prior to sample addition, a solution of bromophenol blue (0.001%, w/v) tracking dye in 10% (w/v) glycerol was layered onto the stacking gel, and the system was preelectrophoresed for 2 h (30-mA constant current) at 10 °C to remove unreacted radicals and ultraviolet-absorbing material (Gordon, 1974; Chrambach et al., 1976) and to allow visual inspection of gel performance. During this period, the elution buffer [10 mM NaH_2PO_4, pH 7.0, containing 25% (w/v) glycerol] was pumped through the elution chamber at 15 mL/h. After the tracking dye had migrated 2.5 cm into the resolving gel, the thioesterase preparation obtained after Sephadex G-50 chromatography was adjusted to 25% (w/v) glycerol and layered upon the stacking gel. Electrophoresis was continued under these same conditions, and when the tracking dye had reached the elution chamber, the flow rate of the elution buffer was increased to 55 mL/h and fractions were collected.

The purity and molecular weight of thioesterase I was examined under denaturing conditions on analytical 15% $NaDodSO_4$–polyacrylamide gels exactly as described by Laemmli & Favre (1973). Gels were stained with Coomassie brilliant blue R-250 as described by Laemmli & Favre (1973) or with a 0.1% (w/v) solution of Coomassie blue in 50% (v/v) methanol–10% (v/v) acetic acid (Rock & Cronan, 1981). Gels stained with Coomassie blue were subsequently examined by staining them with a photochemical silver stain (Merril et al., 1982). The examination of protein preparations by nondenaturing (native) analytical polyacrylamide gels (15%) was performed as described above except that $NaDodSO_4$ was omitted and samples were applied to gels without heating.

Native Molecular Weight Determination. Thioesterase I native molecular weight was estimated by gel filtration with employment of a Sephadex G-100 (1.5 × 58 cm) column calibrated with bovine serum albumin (66 200), ovalbumin (45 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) as described by Andrews (1965).

Results

Cellular Localization of Acyl-CoA Thioesterase Activity. Cells of *R. sphaeroides* (200 g wet wt) were resuspended in 500 mL of 0.2 M KH_2PO_4 (pH 7.0) buffer, and the cells were

Table I: Purification of Thioesterase I from *R. sphaeroides*[a]

step	total act. units ($\times 10^{-4}$ nmol/min)	total protein (mg)	sp act. (nmol min^{-1} mg^{-1})	purification (x-fold)	units yield (%)
crude supernatant	12.00	19340	6.2	(1.0)	(100.0)
high-speed supernatant	11.90	7550	15.8	2.5	99.0
streptomycin sulfate–ammonium sulfate	8.90	7492	11.9	1.8	74.0
Sephadex G-200	4.70	2340	20.0	3.2	39.0
DEAE-Sephadex	3.30	497	66.0	10.7	28.0
octyl-Sepharose	2.91	5.1	5686	917	24.0
Sephadex G-50	2.72	1.4	19092	3079	23.0
preparative elution electrophoresis[b]	2.24	0.34	63555	10267	18.7

[a] A purification from 200 g of a frozen paste of *R. sphaeroides* M29-5 is given. [b] Based on results obtained when 25% of the G-50 fraction was subjected to preparative elution electrophoresis. Determined with vaccenoyl-CoA as substrate.

disrupted by two passages through an Aminco French pressure cell at 16 000 lb/in.^{2}. The crude extract was centrifuged at 10000*g* for 30 min, and the resulting supernatant was monitored for total thioesterase activity (Table I). This crude supernatant was then further subjected to three successive centrifugations at 106000*g* (1 h), and the resulting pellets were resuspended in phosphate buffer and washed by centrifugation to recover entrapped soluble material. The combined high-speed supernatants possessed 99% of the total acyl-CoA thioesterase activity originally present in the crude cellular extract (Table I) and displayed a specific activity of 15.8 nmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$.

Purification of Thioesterase I from R. sphaeroides. (*Step 1*) *Streptomycin Sulfate and Ammonium Sulfate Fractionation.* The soluble protein fraction obtained as described above was freed of residual nucleic acids by treatment with streptomycin sulfate as described by Majerus et al. (1969). A 30–80% $(NH_4)_2SO_4$ fraction was then prepared, and this material was resolubilized (40 mg of protein/mL) in 20 mM KH_2PO_4 (pH 7.0) buffer. Unless otherwise noted, all purification procedures were performed at 4 °C. Although treatment of the soluble protein fraction with $(NH_4)_2SO_4$ typically resulted in a loss (15–25%) of thioesterase activity and a decrease in the specific activity of the preparation (Table I), this treatment was necessary in order to remove residual pigmented membranous material that interferred with subsequent purification procedures.

(*Step 2*) *Sephadex G-200 Chromatography.* The 30–80% $(NH_4)_2SO_4$ protein fraction was applied to a column of Sephadex G-200 that had been preequilibrated in 20 mM KH_2PO_4 (pH 7.0) buffer, and the column was eluted with this same buffer. As is shown (Figure 1), gel filtration resulted in the resolution of two distinct peaks of palmitoyl-CoA thioesterase activity. Peak 1, eluting immediately after the column void volume, contained 42% of the protein applied to the column and 43% of the recovered thioesterase activity and displayed a specific activity with palmitoyl-CoA as substrate of 11 nmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$. The second peak possessed 31% of the applied protein and 57% of the recovered thioesterase activity and exhibited a specific activity of 20 nmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$ (Table I).

(*Step 3*) *DEAE-Sephadex A-25 Chromatography.* Although the levels of the two thioesterase activities resolved by gel chromatography were comparable, the thioesterase activity comprising peak 2 had a strict requirement for long-chain ($\geq C_{12}$) acyl-CoA substrates, whereas peak 1 thioesterase activity (Figure 1) was more active with decanoyl-CoA rather than palmitoyl-CoA as substrate (T. Seay and D. R. Lueking, unpublished observation). Thus, the substrate preference for

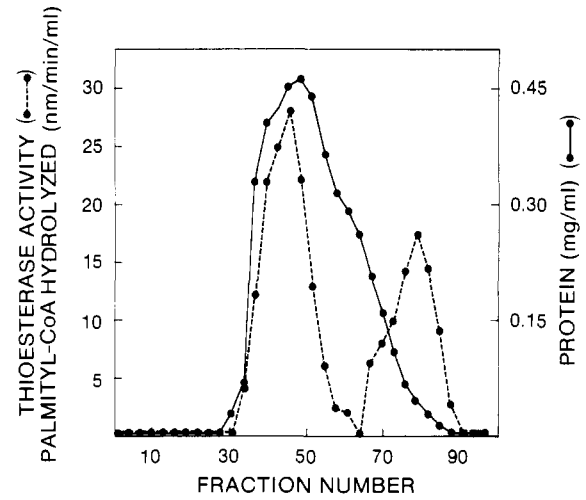

FIGURE 1: Resolution of thioesterase activity by gel-filtration chromatography. Application of the ammonium sulfate fraction (95 mg of protein) onto a column of Sephadex G-200 (2.5 × 100 cm; 12 mL/h) resulted in complete separation of two thioesterase activities. Peak 1 (fractions 34–63) possessed 55% of the recoverable thioesterase activity with the remaining 45% of the total activity localized in peak 2 (fractions 65–88). Fractions (4.0 mL) were assayed for thioesterase activity by utilizing palmitoyl-CoA as substrate (Experimental Procedures).

the peak 2 thioesterase activity was more compatible with the results reported by Campbell & Lueking (1983), which showed that only long-chain fatty acids were capable of relieving a cerulenin-mediated inhibition of growth of *R. sphaeroides*.

To continue the purification of the *R. sphaeroides* peak 2 thioesterase activity, fractions 64–90 (Figure 1) were pooled and applied to a column of DEAE-Sephadex A-25 equilibrated in 20 mM KH_2PO_4 (pH 7.0) buffer, and the column was eluted with a linear gradient of NaCl (0–1.0 M) in this same buffer. This procedure (Figure 2) further resolved two peaks of palmitoyl-CoA thioesterase activity. One activity, eluting at NaCl concentrations between 0.03 and 0.12 M, comprised from 5 to 30% of the recoverable thioestease activity, depending upon the preparation, and this material was not further analyzed. A second activity, representing 70–95% of the recoverable thioesterase activity (Figure 2), was eluted between 0.22 and 0.72 M NaCl, and this dominant thioesterase activity was desalted and concentrated by ultrafiltration in an Amicon cell equipped with a YM-5 membrane.

(*Step 4*) *Octyl-Sepharose Column Chromatography.* The concentrated, desalted material obtained following ion-exchange chromatography was applied to a column (1 × 15 cm) of octyl-Sepharose CL-4B that had been equilibrated with 20 mM KH_2PO_4 buffer (pH 7.0) at 25 °C, and the column was washed with this same buffer until no A_{280}-absorbing material

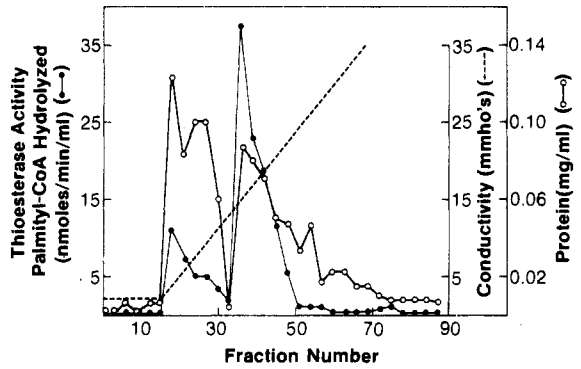

FIGURE 2: Purification of peak 2 (G-200) thioesterase activity by DEAE ion-exchange chromatography. Peak 2 (Figure 1) protein (10 mg) was applied to a column (1.5 × 10 cm) of DEAE-Sephadex A-25 equilibrated in 20 mM KH_2PO_4 (pH 7.0) buffer, and the column was eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer. Fractions (1.25 mL) were examined for thioesterase activity with palmitoyl-CoA as substrate and NaCl concentration by employing a Radiometer-Copenhagen conductivity meter. The thioesterase activity eluting with low salt (0.03–0.12 M NaCl; 3–9 mmhos, values uncorrected for 0.8-cm interelectrode distance) possessed 30% of the recoverable units of activity while the remaining activity (70%) eluted at a higher salt concentration (0.22–0.72 M NaCl; 12–30 mmhos).

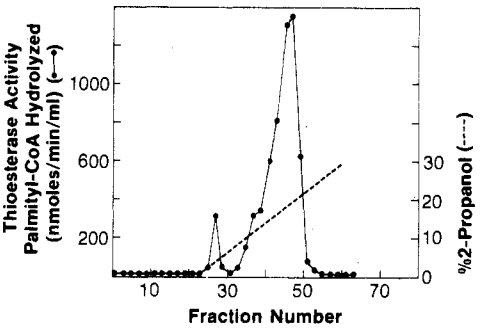

FIGURE 3: Octyl-Sepharose column chromatography. The material (peak 2) resulting from ion-exchange chromatography (Figure 2) was loaded onto a column of octyl-Sepharose CL-4B (1 × 15 cm) equilibrated in 20 mM KH_2PO_4 (pH 7.0) at 25 °C. After the protein was loaded, the column was washed with phosphate buffer until no material absorbing at 280 nm was in the column eluate. A flow rate of 20 mL/h was employed for column packing, sample addition, and elution. The 2-propanol gradient was formed with a Mariotte flask, constructed entirely with glass and Teflon fittings, as a mixing chamber. Undiluted 2-propanol was allowed to mix with 150 mL of 20 mM KH_2PO_4 (pH 7.0) in the mixing chamber. All solutions were thoroughly degassed immediately prior to use. The concentration of 2-propanol through the gradient was determined by monitoring the refractive index of individual fractions. Refractive index (η) is a linear function of 2-propanol concentration from 0 (=1.3335) to 30% (=1.3604) (w/v) (Rock & Garwin, 1978). Fractions (1.0 mL) were assayed for thioesterase activity directly, the removal of 2-propanol not being necessary to localize activity, by employing palmitoyl-CoA as substrate.

was detectable in the eluate. Under these conditions, only 5% of the applied protein, but all of the thioesterase activity, was bound to the resin. Pilot studies showed that thioesterase activity was insensitive to relatively high concentrations of 2-propanol, and by elution of the column with a linear gradient of 2-propanol (0–30%, w/v) in 20 mM phosphate buffer (pH 7.0) (Figure 3), 90–95% of the enzyme activity applied to the column could be recovered in two distinct peaks of activity. One activity, eluting at 2-propanol concentrations between 4 and 8% (w/v), represented 5–10% of the recovered thioesterase activity and had a specific activity of 900 nmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$. The second peak of activity (Figure 3), which eluted at 2-propanol concentrations between 15 and 22% (w/v), contained 90–95% of the recovered activity and displayed a specific activity of 5.6 μmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$. Thus, this procedure resulted in an 85-fold purification of enzyme activity (Table I). The column fractions containing the major peak of thioesterase activity (fractions 35–50) were then pooled and freed of 2-propanol by ultrafiltration and washing with phosphate buffer until the refractive index of the filtrate was the same as that of the buffer.

Although hydrophobic interactions are normally promoted by increased ionic strength (Hofstee, 1973; Hjertén, 1973), the binding of the thioesterase to octyl-Sepharose does not require the presence of salt. In fact, failure to desalt the material obtained following ion-exchange chromatography markedly enhances the binding of lesser hydrophobic proteins upon application of this material to the resin, and the column rapidly becomes saturated. As a consequence, thioesterase activity is not quantitatively absorbed to the resin.

(Step 5) Sephadex G-50 Chromatography. The octyl-Sepharose column fractions (35–50; Figure 3) containing the major thioesterase activity were pooled and concentrated as described above and were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Experimental Procedures). Overloaded gels (300 μg of protein; data not shown) revealed a spectrum of poorly staining (Coomassie blue) low molecular weight proteins and one prominent protein (~80% of the staining material; M_r 22 000) that was tentatively identified as the acyl-CoA thioesterase on the basis of its relative abundance. However, following chromatography of this material on a column of Sephadex G-50, the pooled fractions containing 90% of the applied enzyme activity were found to be markedly deficient in the relative level of the M_r 22 000 polypeptide (data not shown). As is shown in Table I, this procedure yielded a 3.3-fold purification of enzyme activity, and the recovered material had a specific activity of 19.09 μmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$.

(Step 6) Preparative Elution Electrophoresis. Analysis of the material obtained by Sephadex G-50 chromatography by analytical native gel electrophoresis (Experimental Procedures) indicated that further purification of enzyme activity could be achieved by subjecting the material to preparative elution electrophoresis. The native polyacrylamide gel system utilized for preparative elution electrophoresis is described under Experimental Procedures. Fractions collected during preparative elution electrophoresis were monitored for the presence of thioesterase activity, and those fractions exhibiting significant activity were pooled and concentrated by ultrafiltration. This material, designated thioesterase I, contained 81% of the enzyme activity applied to the gel and displayed a specific activity (Table I) of 63.5 μmol of palmitoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$. The procedure outlined in Table I resulted in a >10 000-fold purification of *R. sphaeroides* thioesterase I with a yield of 19% of the total cellular thioesterase activity. If one assumes that the thioesterase I activity represents 50% of the total cellular thioesterase activity (Figure 1), the described purification procedure (Table I) results in a 38% yield of this specific enzyme.

Properties of R. sphaeroides Thioesterase I. Analysis of purified preparations of thioesterase I by native and $NaDodSO_4$–polyacrylamide gel electrophoresis revealed the presence of one major protein band with an apparent molecular mass (M_r) of 12 500 and a trace level of a protein of M_r 30 000. In contrast, analysis of the enzyme by gel filtration on a calibrated column of Sephadex G-100 gave a native molecular weight of 22 400. Thus, these data suggest that the enzyme exists as a dimer. Interestingly, the *E. coli* thioesterase I was also determined to have a molecular weight of 22 000 (Barnes &

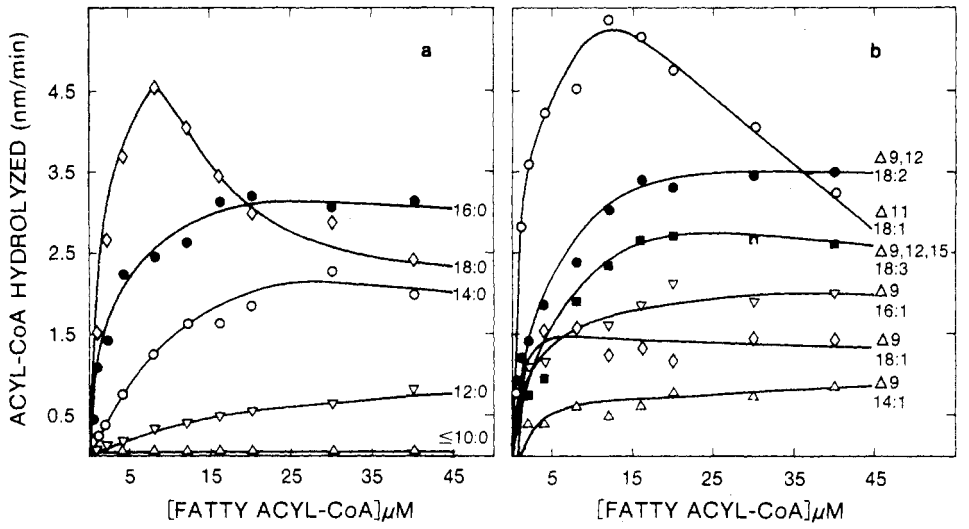

FIGURE 4: Substrate saturation curves for thioesterase I employing saturated and unsaturated fatty acyl-CoA substrates. Reaction mixtures contained 25 μmol of Tris-HCl (pH 8.0), 50 nmol of DTNB, 95 ng of protein, and the CoA thio ester of the fatty acid indicated to give the concentration shown in a 0.5-mL final volume. Reactions were initiated by addition of enzyme. Acyl-CoA substrate concentrations were determined spectrophotometrically by examining total adenine plus thio ester bond concentration [E_{232} = 9400 M^{-1} cm^{-1} (Stadtman, 1957)].

Wakil, 1968), upon the basis of sedimentation velocity and gel-filtration studies; however, the analysis of this enzyme in denaturing polyacrylamide gels was not conducted.

Enzyme activity was optimal at pH 8.0 and was directly proportional to the concentration of enzyme protein (10–400 ng/mL). Enzyme activity was rapidly lost upon dilution, but this problem could be circumvented by conducting dilutions in the presence of 25% (w/v) glycerol and by initiating enzyme assays with enzyme rather than with the acyl-CoA substrates. The presence of the acyl-CoA substrate presumably stabilizes enzyme activity upon dilution of the enzyme with the components of the assay mixture. BSA (5–80 μg/mL) had no stabilizing effect on the *R. sphaeroides* enzyme, and attempts to stabilize enzyme activity with dimethyl sulfoxide (2–30%, v/v) resulted in significant inhibition (5–95%) of enzyme activity. In addition, enzyme activity was 100-fold more sensitive to inhibition by DFP than was observed with *E. coli* thioesterase I with a >90% inhibition observed at 10 μM DFP (40 min, 25 °C). The enzyme was stable indefinitely when stored at −20 °C in the presence of 25% (w/v) glycerol but displayed a half-life of 90 days when stored at 4 °C under identical conditions.

Substrate Specificity. The data presented in Figure 4 show the dependence of thioesterase activity upon the concentration, acyl chain length, and degree of unsaturation of a variety of acyl-CoA substrates. The enzyme displayed a high specificity for long-chain ($\geq C_{12}$) acyl-CoA thio esters and was unable to hydrolyze enzymatically synthesized palmitoyl and vaccenoyl thio esters of *E. coli* ACP at a measurable rate (data not shown). The enzyme was completely inactive upon butanoyl-, hexanoyl-, octanoyl-, and decanoyl-CoA thio esters at all substrate concentrations examined (0.50–40 μM) (Figure 4a). Among the saturated acyl thio ester substrates evaluated, enzyme activity (V_{max}, Table II) increased as the length (C_{12} to C_{18}) of the acyl chain increased, and at low substrate concentrations (<7.5 μM), maximal activity was observed with stearoyl-CoA as substrate. However, stearoyl-CoA severely inhibited enzyme activity at concentrations >7.5 μM, and at saturating (20–40 μM) acyl-CoA concentrations, palmitoyl-CoA was the preferred saturated substrate (Figure 4a). A summary of the enzyme kinetic parameters (K_m and V_{max} values) observed with the different acyl-CoA substrates is presented in Table II.

Table II: Summary of Kinetic Constants for *R. sphaeroides* Thioesterase I[a]

fatty acyl-CoA substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
saturated		
≤decanoyl-CoA[b]	0.00	0.00
lauroyl-CoA	44.68	19.91
myristoyl-CoA	8.67	24.03
palmitoyl-CoA	1.86	31.60
stearoyl-CoA	3.50	72.70
unsaturated		
myristoleoyl-CoA	4.60	9.26
palmitoleoyl-CoA	3.62	22.84
oleoyl-CoA	1.62	22.40
vaccenoyl-CoA	0.84	54.68
linoleoyl-CoA	1.40	35.83
linolenoyl-CoA	7.86	36.63

[a] The values for K_m and V_{max} were calculated by least-squares analysis of double-reciprocal plots of the data presented in parts a and b of Figure 4. [b] No measurable enzymatic activity was observed with butanoyl-, hexanoyl-, octanoyl-, or decanoyl-CoA as substrates when they were examined at concentrations of 0.5–40 μM.

Vaccenic acid comprises 90% of the total fatty acids possessed by phototrophically growing cells of *R. sphaeroides* (Campbell & Leuking, 1983; Kenyon, 1978), and the CoA thio ester of this fatty acid was the preferred (lowest K_m) unsaturated acyl-CoA substrate for the thioesterase (Figure 4b; Table II). As was previously observed with stearoyl-CoA as substrate (Figure 4a), however, the presence of relatively low (>12 μM) concentrations of vaccenoyl-CoA resulted in significant inhibition of enzyme activity.

As is shown in Table II, an increase in the degree of unsaturation of the acyl-CoA substrate resulted in a decrease in enzyme activity and an increase in the value for the apparent K_m. The K_m values determined with linoleoyl-CoA (2.93 μM) and linolenoyl -CoA (7.86 μM) as substrates were 3.5- and 9.4-fold higher, respectively, than the K_m value (0.84 μM) determined with vaccenoyl-CoA as substrate. Additionally, the position of the double bond in the monounsaturated fatty acyl-CoA substrates also affected the rate of substrate hydrolysis by thioesterase I (Table II). A decrease in enzyme activity (V_{max}) and an increase in the apparent K_m were observed when the double bond of the acyl chain was positioned

nine carbon atoms from the methyl end (oleoyl-CoA, $C_{18:1}^{\Delta 9}$) rather than seven carbons from the methyl end (vaccenoyl-CoA, $C_{18:1}^{\Delta 11}$).

Comments on Purification of R. sphaeroides Thioesterase. Whether the peak 1 thioesterase activity resolved during ion-exchange chromatography (fractions 15–29; Figure 2) represents a distinct thioesterase activity or is a modified form of the dominant thioesterase activity resolved by this procedure is unknown. Due to the virtually complete separation of the high and low molecular weight thioesterase activities achieved by gel filtration (Figure 1), it seems unlikely that the minor enzyme activity resolved by ion-exchange chromatography represents contaminating activity from the peak 1 material shown in Figure 1. Furthermore, the nature of the minor thioesterase activity recovered from the octyl-Sepharose column (Figure 3) is also unknown. Hofstee (1973) observed apparent inhomogeneities in electrophoretically pure BSA when chromatographed on a hydrophobic matrix developed with ethylene glycol gradients and suggested that the inhomogeneities were due to the presence of different BSA conformers that were bound to the column with differing affinities. Whether the minor activity eluted from octyl-Sepharose (Figure 3) is a distinct thioesterase or is a physically and/or chemically modified form of the dominant thioesterase activity is presently under investigation.

Discussion

This paper describes the purification and characterization of a long-chain acyl-CoA thioesterase from phototrophically grown cells of *R. sphaeroides*. The properties of this enzyme were found to be remarkably similar to the properties displayed by the *E. coli* thioesterase I (Barnes & Wakil, 1968), suggesting that these two enzymes are functionally analogous. Both enzymes have a native molecular mass (M_r) of approximately 22000, exhibit a strict requirement for acyl-CoA substrates with acyl chain lengths from C_{12} to C_{18}, and display a marked preference (lowest K_m) for vaccenoyl-CoA as substrate. Neither enzyme is able to hydrolyze fatty acyl thio esters of ACP at a physiologically significant rate, and as is typical of many esterases, both enzymes are sensitive to inhibition by the serine esterase inhibitor DFP. Finally, the *E. coli* thioesterase I also readily and quantitatively binds to octyl-substituted Sepharose under conditions of low ionic strength, and the activity may be recovered from this resin, with an appreciable purification, by elution with buffered solutions of 2-propanol (S. G. Boyce and D. R. Lueking, unpublished observations). Thus, as was observed with the *R. sphaeroides* enzyme, the *E. coli* thioesterase I appears to be considerably hydrophobic.

Substrate saturation of the *R. sphaeroides* enzyme generally occurred at acyl-CoA concentrations at, or below, 20 μM with all acyl-CoA substrates examined (Figure 4). A notable exception was that of lauroyl-CoA (Figure 4a), which was a poor substrate for the *R. sphaeroides* thioesterase (K_m = 45 μM) and, interestingly, has a cmc of about 3 mM (Powell et al., 1981). With respect to palmitoyl-, oleoyl-, and stearoyl-CoA, enzyme saturation occurred considerably below the newly determined cmc values (30–60 μM) for these molecules (Powell et al., 1981). This is also true as regards the inhibition of enzyme activity observed with vaccenoyl-CoA[3] and stearoyl-CoA, indicating that the observed inhibition is not causally related to micelle formation and, as was discussed by Powell et al. (1981), may reflect a physiologically important allosteric interaction between the fatty acyl-CoA and the enzyme. In this regard, Tippett & Neet (1982a,b) recently reported that long-chain fatty acyl-CoA mediated a specific, allosteric inhibition of rat liver glucokinase activity when utilized at concentrations considerably below the known cmc.

As was mentioned previously, the in vivo functions of the bacterial fatty acyl-CoA thioesterases are unknown. Moreover, it is also realized that, in the absence of supporting genetic evidence, proposals attempting to ascribe specific cellular roles to these enzymes are at best speculative. Nevertheless, upon consideration of the mode of phospholipid biosynthesis in *R. sphaeroides*, as opposed to *E. coli*, a cellular basis for this organism's possession of an acyl-CoA thioesterase can be clearly identified. Specifically, the activity of the *R. sphaeroides* thioesterase I, together with the activity of an acyl-ACP synthetase, would provide the endogenous acyl-ACP substrates required for the direct utilization of exogenous fatty acids for phospholipid synthesis. Although the *E. coli* thioesterase I could presumably fulfill a similar assimilatory role, the recognition of such a role is presently precluded by the uncertainties concerning the precise route by which *E. coli* directly assimilates exogenous fatty acids (Rock & Cronan, 1982).

Although *R. sphaeroides* was previously reported to be unable to directly utilize exogenous fatty acids for cellular phospholipid synthesis (Wood et al., 1965), Campbell & Lueking (1983) recently demonstrated that a variety of long-chain fatty acids could significantly stimulate cell growth and phospholipid synthesis in cultures of *R. sphaeroides* grown in the presence of growth-limiting concentrations of cerulenin, a specific inhibitor of bacterial fatty acid synthesis (D'Agnolo et al., 1973). It was further shown (Campbell & Lueking, 1983) that the exogenously supplied fatty acids were directly incorporated into cellular phospholipids, and this finding, together with the information presented above, suggested that *R. sphaeroides* possessed an acyl-CoA thioesterase whose substrate specificity encompassed the spectrum of fatty acids shown to be growth stimulatory and/or assimilated. The substrate specificities displayed by the *R. sphaeroides* thioesterase I are supportive of this proposal and are compatible with presently available information on the lipid metabolism of *R. sphaeroides*. Campbell & Lueking (1983) have reported that exogenously supplied monounsaturated and polyunsaturated C_{18} fatty acids are the most effective fatty acids in relieving a growth inhibition of *R. sphaeroides* caused by cerulenin and, as shown in the present study, the CoA thio ester derivatives of these fatty acids were readily hydrolyzed by the *R. sphaeroides* thioesterase. In contrast, the enzyme also hydrolyzed CoA thio esters of C_{14} to C_{18} saturated fatty acids, even though these fatty acids are unable to mitigate the inhibitory effect of cerulenin on the growth of *R. sphaeroides*. It should be emphasized, however, that saturated (C_{12} to C_{18}) fatty acids comprise <10% of the total fatty acids possessed by phototrophically grown *R. sphaeroides* (Kenyon, 1978; Campbell & Lueking, 1983), and as was previously discussed by Campbell and Lueking (1983), it is presently unclear whether the ineffectiveness of these fatty acids in ameliorating the inhibitory effect of cerulenin reflects the assimilatory capability or physiological requirements of *R. sphaeroides*. In any event, *R. sphaeroides* provides an attractive system for the investigation of the in vivo roles of the acyl-CoA thioesterases in bacterial lipid metabolism, and further studies employing this organism are certainly warranted.

[3] Although Powell et al. (1981) did not directly determine the cmc for vaccenoyl-CoA ($C_{18:1}^{\Delta 11}$), the estimated cmc for oleoyl-CoA ($C_{18:1}^{\Delta 9}$) is presumed to be representative, since the cmc values for $C_{16:0}$, $C_{18:0}$, and $C_{18:1}^{\Delta 9}$ acyl-CoA were the same.

Registry No. Long-chain acyl-CoA thioesterase, 9025-87-0; la-

uroyl-CoA, 6244-92-4; myristoyl-CoA, 3130-72-1; palmitoyl-CoA, 1763-10-6; stearoyl-CoA, 362-66-3; myristoleoyl-CoA, 87935-97-5; palmitoleoyl-CoA, 18198-76-0; oleoyl-CoA, 1716-06-9; vaccenoyl-CoA, 13673-89-7; linoleoyl-CoA, 6709-57-5; linolenoyl-CoA, 13673-87-5.

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