Purification and Properties of Acyl Coenzyme A Thioesterase II from *Rhodopseudomonas sphaeroides*†

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ABSTRACT: A high molecular weight acyl coenzyme A (acyl-CoA) thioesterase, designated thioesterase II, has been purified 5300-fold from photoheterotrophically grown cells of *Rhodopseudomonas sphaeroides*. In contrast to *R. sphaeroides* acyl-CoA thioesterase I [Boyce, S. G., & Lueking, D. R. (1984) *Biochemistry 23*, 141–147], thioesterase II has a native molecular mass (M_r) of 120000, is capable of hydrolyzing saturated and unsaturated acyl-CoA substrates with acyl chain lengths ranging from C_4 to C_{18}, and is completely insensitive to the serine esterase inhibitor diisopropyl fluorophosphate. Palmitoyl-CoA and stearoyl-CoA are the preferred (lowest K_m) saturated acyl-CoA substrates and vaccenoyl-CoA is the preferred unsaturated substrate. However, comparable V_{max} values were obtained with a variety of acyl-CoA substrates. Unlike a similar thioesterase present in cells of *Escherichia coli* [Bonner, W. M., & Bloch, K. (1972) *J. Biol. Chem. 247*, 3123–3133], *R. sphaeroides* thioesterase II displays a high ratio of decanoyl-CoA to palmitoyl-CoA activities and exhibits little ability to hydrolyze 3-hydroxyacyl-CoA substrates. Only 3-hydroxydodecanoyl-CoA supported a measureable rate of enzyme activity. With the purification of thioesterase II, the enzymes responsible for >90% of the acyl-CoA thioesterase activity present in cell-free extracts of *R. sphaeroides* have now been identified.

The in vivo functions of the long-chain acyl-CoA[1] thioesterases possessed by procaryotic organisms are unknown. *Escherichia coli* contains two distinct acyl-CoA thioesterases (Kass et al., 1967; Barnes et al., 1970; Barnes & Wakil, 1968; Bonner & Bloch, 1972); however, attempts to ascribe precise roles to these enzymes have been hampered by the lack of physiologic and genetic information, as well as uncertainties concerning the involvement of acyl-CoA substrates in bacterial lipid metabolism (Boyce & Lueking, 1984; Rock & Cronan, 1982). As was previously discussed by Boyce and Lueking (1984), under conditions where cellular fatty acids are being produced de novo, a need for acyl-CoA intermediates, or an acyl-CoA thioesterase, in bacterial lipid metabolism cannot be clearly identified.

In an attempt to obtain definitive information concerning the physiological roles of the long-chain acyl-CoA thioesterases in bacterial lipid metabolism, we have initiated a systematic study of these enzymes in cells of the non-sulfur purple photosynthetic bacteria *Rhodopseudomonas sphaeroides*. This organism has been widely employed for studies on the mode and regulation of bacterial membrane assembly (Kaplan, 1978; Kaplan et al., 1983) and phospholipid metabolism (Cain et al., 1983, 1984; Cooper & Lueking, 1984; Donohue et al., 1982) and displays properties that are uniquely suited to our proposed studies. In this regard, the obligate requirement displayed by cells of *R. sphaeroides* for acyl-ACP substrates as acyl donors for cellular phospholipid synthesis is prominent among these properties (Lueking & Goldfine, 1975; Cooper & Lueking, 1984).

Recently, Boyce and Lueking (1984) reported the purification of a long-chain acyl-CoA thioesterase, designated thioesterase I, from phototrophically grown cells of *R. sphaeroides*. During this study, an additional acyl-CoA thioesterase activity was identified; however, the purification of the enzyme(s) responsible for this activity was not attempted. Thus, as a prerequisite for future physiological and genetic studies on the bacterial acyl-CoA thioesterases, this study describes the purification and properties of a second major thioesterase, designated thioesterase II, from phototrophically grown cells of *R. sphaeroides*.

Materials and Methods

Chemicals. Sephadex G-200 and Sepharose 6B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Streptomycin sulfate, ammonium sulfate, DEAE-cellulose, phenyl- and octyl-Sepharoses, diisopropyl fluorophosphate (DFP), coenzyme A (CoA), butanoyl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, myristoyl-CoA, myristoleoyl-CoA, palmitoyl-CoA, stearoyl-CoA, palmitoleoyl-CoA, oleoyl-CoA, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. Ethylene glycol and anhydrous diethyl ether were obtained from MCB Manufacturing Chemists, Inc., Cincinnati, OH. All polyacrylamide gel electrophoresis materials were obtained from Bio-Rad, Richmond, CA. Ethyl bromoacetate, zinc, octyl aldehyde, decyl aldehyde, dodecyl aldehyde, and tetradecyl aldehyde were the best available from Aldrich Chemical Co., Milwaukee, WI. All other chemicals were of reagent grade or better.

Quantitation of acyl-CoA esters (including saturated, unsaturated, and 3-hydroxyacyl derivatives) was conducted by monitoring the absorbance of adenine plus the thio ester bond at 232 nm (ϵ_{232} = 9.4 mM^{-1} cm^{-1}) (Stadtman, 1957). The

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[1] Abbreviations: CoA, coenzyme A; ACP, acyl carrier protein; DFP, diisopropyl fluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane.

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3-hydroxy fatty acids were synthesized by the Reformatsky reaction as described by Smith and Waldron (1980). All the 3-hydroxy fatty acids were recrystallized from acetone. Purity was monitored with O-acetylated, methyl ester derivatives of the 3-hydroxy fatty acids by gas chromatographic analysis with a Hewlett-Packard 5790A series gas chromatograph equipped with a Supelco 10% DEGS-PS column. Infrared spectroscopic analysis of the free 3-hydroxy fatty acids was conducted with a Perkin-Elmer Model 221 infrared spectrometer. This analysis showed, for all the 3-hydroxy fatty acids synthesized, a sharp absorbance at a frequency of 3500 cm^{-1} characteristic of the –OH stretch. Also present were absorbance frequencies characteristic of fatty acids. Nuclear magnetic resonance was conducted with a Varion EM-390 NMR spectrometer, and samples were solubilized in $CDCl_3$ for analysis. NMR spectroscopic studies, conducted on all the synthesized fatty acids except 3-hydroxypalmitic acid (because of its $CDCl_3$ insolubility), revealed peaks accounting for the proton shifts of all the proton groups of the 3-hydroxy fatty acid structure. The peak shapes and areas were consistent with the structure of the 3-hydroxy fatty acid analyzed.

Thiol esterification of the 3-hydroxy fatty acids was carried out by the enzymatic procedure of Merrill et al. (1982) using rat fatty acid-CoA ligase immobilized on Matrix Gel Red A. This is the first use of this procedure for the synthesis of 3-hydroxyacyl-CoA esters and further broadens the range of substrates known to be utilized by this enzyme. After purification over octyl-Sepharose CL-4B, the acyl-CoA derivatives were chromatographed on silica gel H plates in the solvent system of Jackowski and Rock (1981). The mobilities obtained (R_f 0.5) are consistent with the products being acyl-CoA.

Organism, Medium, and Growth Conditions. *R. sphaeroides* M29-5 (Leu^-, Met^-), derived from wild-type strain 2.4.7, was obtained from Samuel Kaplan, University of Illinois, Urbana, IL. Growth was conducted in succinic acid minimal medium (Sistrom, 1962), as modified by Lueking et al. (1978), supplemented with 0.2% (w/v) casamino acids. Incubations were conducted photoheterotrophically at 32 °C, either in completely filled 18-mL tubes, 1-L flat-walled vessels or under an atmosphere of a 95% N_2–CO_2 mixture. Culture growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a No. 66 red filter. Preliminary studies indicated that the yield of thioesterase activity per gram wet weight of cells was independent of the growth phase. Therefore, preparative quantities of cells were harvested in the stationary phase of growth. *R. sphaeroides* stock cultures were maintained in 30% (v/v) glycerol at –20 °C and thoroughly adapted to the casamino acid supplemented succinic acid minimal media prior to use.

Thioesterase Assays. Thioesterase activity was routinely assayed spectrophotometrically at room temperature by monitoring the increase in absorbance at 412 nm resulting from the generation of free CoASH in the presence of DTNB (Ellman, 1959). Activities were calculated with an ϵ_{412} of 13.6 mM^{-1} cm^{-1} for the 2-nitrobenzoate anion (Ellman, 1959). A typical reaction mixture contained 0.1 mM DTNB, 0.025 mM acyl-CoA substrate, 50 mM Tris-HCl, pH 8.0, and 1–2 units of thioesterase activity in a final volume of 1 mL. A unit of enzyme activity is defined as the amount of enzyme required to hydrolyze 1 nmol of acyl-CoA/min. Reactions were initiated by the addition of enzyme, and enzyme activity was linear with time (1–5 min) and protein concentration (1–25 μg). No activity was obtained if either the substrate or enzyme was omitted from the reaction mixture.

Enzyme activities for pH optima studies was conducted by following the decrease in absorbance at 232 nm resulting from the hydrolysis of the acyl thiol ester bond and was calculated with a ϵ_{232} of 4.0 mM^{-1} cm^{-1} (Bonner & Bloch, 1972). Enzyme activities determined by the two described assay procedures agreed within 10%.

Protein Determinations. Protein was determined by the method of Lowry et al. (1951) and by the method of McKnight (1976) with bovine serum albumin as standard.

Continuous-Flow Electrophoresis. Continuous-flow electrophoresis (preparative elution electrophoresis) was conducted by a modification of a procedure previously described (Boyce & Lueking, 1984). A Hoeffer SE 600 vertical slab gel apparatus equipped with a Bio-Rad Laboratories continuous-flow adapter kit was used. The acrylamide–bis(acrylamide) (40:1 w/w) concentrations for each gel were as follows: the plug gel (4 cm) was 20% (w/v), the resolving gel (3 cm) was 7.5% (w/v), and the stacking gel (1 cm) was 3.0% (w/v). Each gel also contained 0.375 M Tris-HCl, 0.10% (v/v) *N,N,N,N*-tetramethylethylenediamine, and 0.46% (w/v) ammonium persulfate. The elution chamber was established by layering 50% (w/v) sucrose over the plug gel and then overlaying the resolving gel. Preelectrophoresis was conducted for 10 min (30-mA constant current, 10 °C) after layering of the tracking dye solution [0.001% (w/v) bromophenol blue in 5% (v/v) glycerol] on the stacking gel. The elution buffer was 20 mM potassium phosphate, pH 7.0, and was pumped through the elution chamber at 60 mL/h during dye and protein elution. After preelectrophoresis, a 1.0-mg sample from the activity pooled from the phenyl-Sepharose column was layered onto the stacking gel and electrophoresis was continued until all activity had eluted.

Analytical Polyacrylamide Gel Electrophoresis. Both native and denaturing $NaDodSO_4$–polyacrylamide gel electrophoreses (PAGE) were routinely used to monitor sample purity. $NaDodSO_4$–PAGE was conducted as described by Laemmli and Favre (1973) with Bio-Rad low molecular weight standards.

Native gels were prepared the same as the $NaDodSO_4$ gels except $NaDodSO_4$ was omitted and the samples were suspended in 0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 0.001% bromophenol blue and were not heated prior to sample addition. Native gels were run at 30-mA constant current. A Hoeffer SE600 vertical slab gel apparatus was used for all electrophoresis procedures. The gels were stained either with 0.1% Coomassie Brilliant Blue in 10% (v/v) acetic acid–10% (v/v) methanol or by the silver staining procedure of Merril et al. (1982), or by both procedures. Coomassie blue stained gels were destained in a solution of 10% (v/v) acetic acid–10% (v/v) methanol.

RESULTS

Resolution of Thioesterase I and II Activities. The cellular localization and the initial steps of the purification of *R. sphaeroides* acyl-CoA thioesterase II were conducted as previously described by Boyce and Lueking (1984) for the purification of thioesterase I from this organism. Unless otherwise noted, all procedures were conducted at 4 °C. The resolution of thioesterase I and II activities present in the resolubilized 30–80% $(NH_4)_2SO_4$ fraction was accomplished by gel-filtration chromatography on a 5 × 90 cm (effective) column of Sephadex G-200 equilibrated in 20 mM KH_2PO_4 (pH 7.0) buffer. As is shown in Figure 1, this procedure resolves two peaks of palmitoyl-CoA thioesterase activity and one peak of decanoyl-CoA thioesterase activity, which coelutes with the high molecular weight palmitoyl-CoA thioesterase.

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

step	total protein (mg)	total C_{10} units (nmol/min)	sp act. (nmol of C_{10} min^{-1} mg^{-1})	ratio of C_{10} to C_{16} sp act.	C_{10} x-fold purification	yield (%) of C_{10} (C_{16})
crude supernatant	10610	8.25×10^4	7.8	0.97	1	100 (100)
high-speed supernatant	4980	7.93×10^4	15.9	0.98	2.04	96 (96)
ammonium sulfate and streptomycin sulfate	2280	6.03×10^4	26.5	0.93	3.40	73 (77)
Sephadex G-200	1417	5.68×10^4	40.1	1.62	5.14	69 (42)
EtOH–ether precipitation	488.8	3.72×10^4	76.1	1.74	9.76	45 (25)
DEAE-cellulose	327.1	3.06×10^4	93.6	1.73	12.0	37 (21)
phenyl-Sepharose	6.90	1.98×10^4	2870	1.79	367.9	24 (13)
continuous-flow electrophoresis[b]	0.102	4.24×10^3	41570	1.24	5330	5 (4)

[a] Purification from 100 g of a frozen paste of *R. sphaeroides* M29-5. [b] Based upon results obtained when 14% of the phenyl-Sepharose fraction was subjected to continuous-elution electrophoresis.

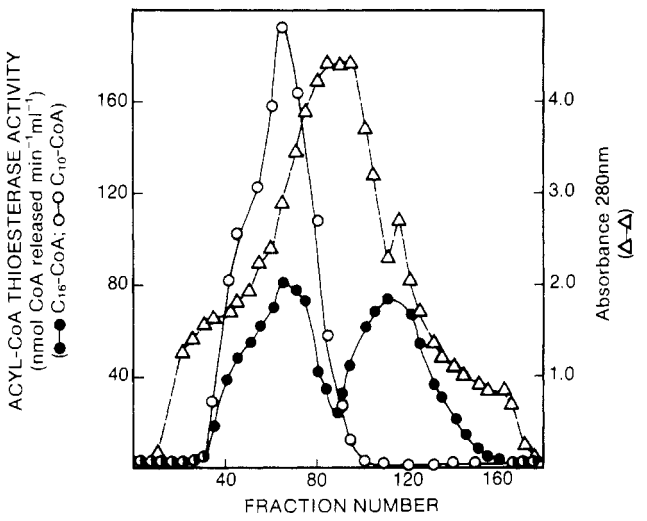

FIGURE 1: Resolution of thioesterase I and II activities by Sephadex G-200 column chromatography. The resolubilized 30–80% ammonium sulfate fraction (2.28 g of protein) was applied to a 5 × 90 cm column of Sephadex G-200 equilibrated in 20 mM potassium phosphate buffer (pH 7.0). Column fractions (10 mL) were collected (33 mL/h) and assayed for thioesterase II activity with decanoyl-CoA as substrate and for both thioesterase I and II activities with palmitoyl-CoA as substrate (Materials and Methods). Peak I (fractions 32–90) contained all of the decanoyl-CoA thioesterase activity and 46% of the recovered palmitoyl-CoA thioesterase activity. Peak II (fractions 91–160) contained 54% of the palmitoyl-CoA thioesterase activity recovered from the column.

The low molecular weight thioesterase has been purified by Boyce and Lueking (1984). The fractions (32–90) containing the high molecular weight thioesterase, designated thioesterase II, were pooled and adjusted to 80% (w/v) $(NH_4)_2SO_4$, and the resulting precipitate was collected by centrifugation 7000*g*, 20 min) and resolubilized in a minimal volume of phosphate buffer. This material contained 94 and 46%, respectively, of the recovered decanoyl- and palmitoyl-CoA thioesterase activity and 37% of the recovered protein (30 mg of protein/mL) and displayed an overall purification of 5.1-fold on the basis of the specific activity observed with decanoyl-CoA as substrate (Table I). However, the preparation still contained a significant quantity of pigmented material, and this material was removed by extraction of the preparation with 10 volumes of absolute ethanol–diethyl ether (1:1 v/v) that had been chilled to −20 °C. Following rapid mixing with the extraction solvent, the precipitated protein and residual $(NH_4)_2SO_4$ were immediately collected by centrifugation (7000*g*, 10 min), the resulting pellets were resuspended in 500 mL of KH_2PO_4 (pH 7.0) buffer, and the mixture was allowed to stir overnight at 4 °C. The insoluble material that remained following this treatment was removed by centrifugation (7000*g*, 20 min) and the clarified supernatant was removed and monitored for thioesterase II activity. This procedure (Table I) resulted in a 2-fold increase in decanoyl-CoA thioesterase activity with a 65% recovery of enzyme activity. It should be noted that observed recoveries of enzyme activity ranged between 60 and 90% with maximum recovery being critically dependent upon the temperature (<20 °C) of the extraction solvent and the use of a suitable excess (1 mL/mg of protein) of phosphate buffer during protein resolubilization.

Ion-Exchange Chromatography. The material recovered following the organic solvent treatment (489 mg of protein) was adjusted to a conductivity of 3.5 $m\Omega^{-1}$, and this solution was applied to a 2.5 × 25 cm column of DEAE-cellulose that had been equilibrated in 0.02 M KH_2PO_4 (pH 7.0) buffer. Development of the column with a linear gradient of NaCl (0–1 M) resulted in the coelution of decanoyl- and palmitoyl-CoA thioesterase activities between 0.09 and 0.22 M (4–12 $m\Omega^{-1}$) NaCl. This procedure yielded an 82% recovery of decanoyl-CoA thioesterase activity and an overall purification of enzyme activity of 12-fold (Table I).

Phenyl-Sepharose Column Chromatography. Acyl-CoA thioesterase II readily bound to octyl- and phenyl-substituted Sepharoses, enzyme activity could be recovered by elution of these resins with ethylene glycol, and maximum yield and purification of enzyme activity were obtained when the enzyme was chromatographed on a column of Phenyl-Sepharose developed with a linear gradient of ethylene glycol. The thioesterase II activity contained in the pooled fractions following ion-exchange chromatography was directly applied to a 1.5 × 10 cm column of Phenyl-Sepharose equilibrated in phosphate buffer at room temperature. Under these conditions, the bulk of the protein (66%) and all of the thioesterase II activity were bound to the column; however, >90% of the nonenzyme protein was readily removed from the column by washing with 2–4 column volumes of low ionic strength (0.02 M) phosphate buffer (pH 7.0). The remaining protein, including all the recoverable thioesterase II activity, was then eluted with a linear gradient (0–100%) of ethylene glycol. It should be noted that the binding of thioesterase II to Phenyl-Sepharose, as was reported for the binding of *R. sphaeroides* thioesterase I to octyl-Sepharose (Boyce & Lueking, 1984), does not require the presence of salt. However, desalting of the material applied to these resins may be required if quantitative absorption of enzyme activity is not achieved. Thioesterase II activity was recovered from the phenyl-Sepharose column as one distinct peak of activity, reflecting the coincident elution of decanoyl- and palmitoyl-CoA thioesterase activities. This peak of activity contained 65% of the units of enzyme activity applied to the column and displayed a specific activity of 2.8 μmol of decanoyl-CoA hydrolyzed min^{-1} (mg of protein)$^{-1}$. This procedure resulted in a 30-fold purification of enzyme activity, and the recovered material displayed essentially the same ratio (1.79) of deca-

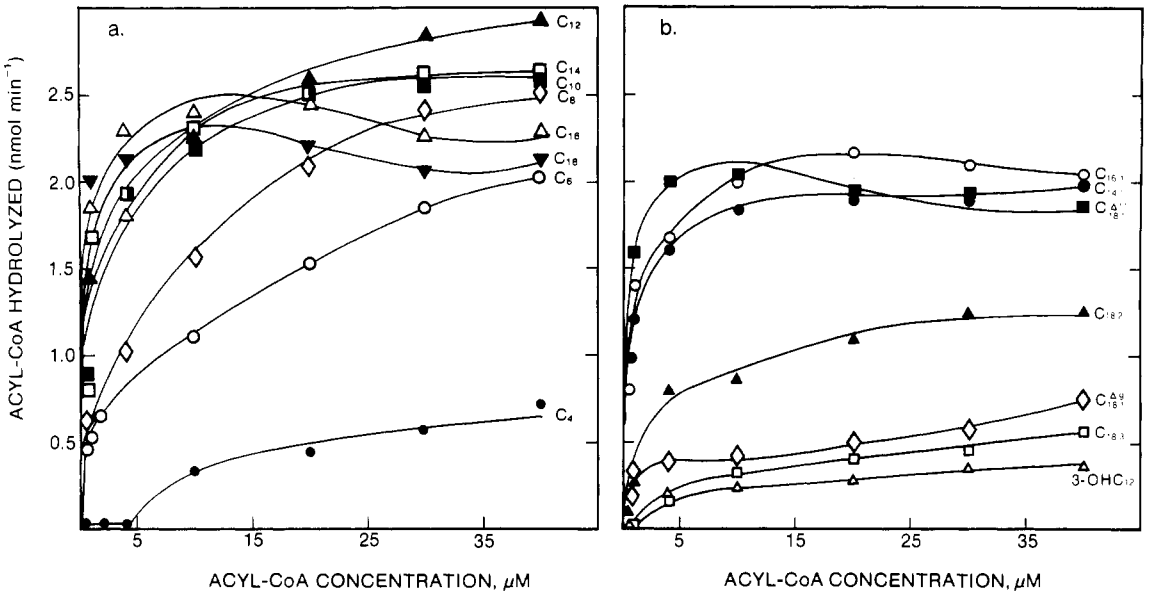

FIGURE 2: Substrate saturation curves for thioesterase II employing saturated, unsaturated, and 3-hydroxyacyl-CoA substrates. Reaction mixtures contained 0.1 mM DTNB, 50 mM Tris-HCl (pH 8.0), 1.26 μg of protein (phenyl-Sepharose pooled material), and the indicated concentration of acyl-CoA substrate in a final volume of 1 mL (Material and Methods). Acyl-CoA substrate concentrations were determined spectrophotometrically as described under Materials and Methods.

noyl-CoA to palmitoyl-CoA thioesterase activities as was observed with the material recovered from the three previous purification procedures (Table I).

Continuous-Elution Electrophoresis. Attempts to further purify thioesterase II with hydroxylapatite, CM-cellulose, QAE-cellulose, chromatogofocusing, ultrafiltration, and Sephadex S-200 and Sepharose 4B gel filtration chromatography, in addition to various differential organic precipitation procedures, were unsuccessful. Either thioesterase II activity did not appreciably bind to the resins (CM-cellulose and hydroxylapatite), or chromatography on these resins resulted in a significant loss of enzyme activity. Ultrafiltration was ineffective either as a purification procedure or as a concentration procedure, since considerable (50%) losses of thioesterase activity occurred during filtration, possibly as a result of irreversible binding of the protein to the cellulose membranes. However, preliminary studies revealed that thioesterase activity could be recovered from native polyacrylamide gels, which suggested the possible use of electrophoresis as a purification procedure. Also, continuous elution electrophoresis has been shown by Boyce and Lueking (1984) to be a useful technique for the purification of thioesterase I from *R. sphaeroides*.

The procedure for continuous elution electrophoresis and the native polyacrylamide gel system employed are outlined under Materials and Methods. Analysis of the fractions obtained by this procedure again showed the coelution of decanoyl- and palmitoyl-CoA thioesterase activities with a recovery of approximately 20% of the units of enzyme activity applied to the gel. This procedure resulted in an individual purification of 15-fold and an overall purification of enzyme activity of 5300-fold (Table I).

Properties of R. sphaeroides Thioesterase II. The material obtained following continuous-elution electrophoresis was shown to contain only one major protein band when analyzed by native polyacrylamide gel electrophoresis followed by protein visualization by silver staining (Materials and Methods). The native molecular mass (M_r) of thioesterase II was determined to be 120 000 by gel-filtration chromatography on a calibrated column of Sepharose 6B, and this value is in good agreement with the molecular weight (122 000) reported for the large acyl-CoA thioesterase from cells of *E. coli* (Bonner & Bloch, 1972). Unfortunately, attempts to evaluate protein quaternary structure have been unsuccessful. As was observed with *R. sphaeroides* thioesterase I (Boyce & Lueking, 1984), thioesterase II stains poorly, especially following gel electrophoresis under denaturing conditions.

Maximal enzyme activity was observed at pH values between 8.3 and 8.5 with either decanoyl- or palmitoyl-CoA as substrate. Further, the enzyme was completely insensitive to inhibition by DFP (2 mM, 50 min), was stimulated 50% by BSA at 10 μg/mL, and was stabilized by either glycerol or ethylene glycol. Enzyme activity displayed a $t_{1/2}$ of 1 year when stored at −20 °C in the presence of 20% (v/v) glycerol or ethylene glycol. At 4 °C, the enzyme was stable for several months ($t_{1/2}$ = 60 days) when stored in the presence of 50–70% (v/v) ethylene glycol, which was normally present following phenyl-Sepharose chromatography. Upon removal of ethylene glycol, or following continuous-elution electrophoresis, enzyme activity was lost rapidly ($t_{1/2}$ = 15 days, 4 °C). Under no condition was enzyme activity stable for more than a day when stored at room temperature.

Enzyme Kinetic Studies. The results presented in Figure 2 show that *R. sphaeroides* thioesterase II is capable of hydrolyzing a variety of saturated and unsaturated acyl-CoA substrates. Measurable activities were observed with acyl chain lengths from C_4 to C_{18} with relatively constant V_{max} values being observed with saturated acyl-CoA substrates possessing acyl chain lengths from C_8 to C_{18} (Table II). These results are in marked contrast to those reported for *R. sphaeroides* thioesterase I (Boyce & Lueking, 1984), which was unable to hydrolyze acyl-CoA substrates with acyl chain lengths of C_{10} or less and displayed an increase in activity (V_{max} value) as the chain length of the saturated acyl-CoA substrate was increased from C_{12} to C_{18}. As is shown in Table II, the preferred saturated acyl-CoA substrates were palmitoyl- and stearoyl-CoA with apparent K_m values of 0.5 and 0.4 μM, respectively. Regarding unsaturated acyl-CoA substrates, comparable K_m and V_{max} values were observed with vaccenoyl- and myristoleoyl-CoA as substrates (Figure 2a, Table II) with the enzyme displaying a slight preference for vaccenoyl-CoA. Further, thioesterase II was found to be highly discriminatory

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

acyl-CoA substrate	K_m (μM)	V_{max} (nmol min^{-1})
saturated acyl group		
butanoyl-CoA	13.2	0.8
hexanoyl-CoA	5.5	1.9
octanoyl-CoA	7.0	2.8
decanoyl-CoA	2.7	2.8
dodecanoyl-CoA	1.8	2.8
myristoyl-CoA	1.7	2.7
palmitoyl-CoA	0.5	2.4
stearoyl-CoA	0.4	2.3
unsaturated acyl group		
myristoleoyl-CoA	1.1	2.0
palmitoleoyl-CoA	1.3	2.3
oleoyl-CoA	2.6	0.6
vaccenoyl-CoA	1.1	2.4
linoleoyl-CoA	9.9	1.9
linolenoyl-CoA	10.9	0.7
3-hydroxyacyl group[b]		
3-hydroxydodecanoyl-CoA	3.2	0.3

[a] K_m and V_{max} values were determined by least-squares analysis of double-reciprocal plots of the data presented in Figure 4. [b] 3-Hydroxydecanoyl-CoA, 3-hydroxymyristoyl-CoA, and 3-hydroxypalmitoyl-CoA were inactive as substrates when tested over a concentration range of 0.5–40 μM.

regarding the position and degree of acyl group unsaturation. Apparent K_m values determined with linoleoyl- and linolenyl-CoA as substrates were 10-fold higher than those observed with vaccenoyl-CoA (Table II) and substitution of oleoyl-CoA ($C_{18:1}$) for vaccenoyl-CoA ($C_{18:1}$) resulted in a 2.5-fold increase in the apparent K_m and a 4-fold decrease in the value for V_{max} (Table II). Finally, in marked contrast to the high molecular weight thioesterase from cells of *E. coli*, *R. sphaeroides* acyl-CoA thioesterase II displayed little ability to hydrolyze 3-hydroxyacyl-CoA substrates. No enzymatic activity was observed with either 3-hydroxymyristoyl-CoA, 3-hydroxydecanoyl-, or 3-hydroxypalmitoyl-CoA as substrate, and the enzyme only poorly hydrolyzed 3-hydroxydodecanoyl-CoA. As is shown in Table II, the K_m (3.2 μM) determined for 3-hydroxydodecanoyl-CoA was higher than any of the K_m values determined for the saturated and monounsaturated acyl-CoA substrates possessing acyl chain lengths > C_8, and the V_{max} observed with this substrate was the lowest value observed with any of the acyl-CoA substrates examined. It should be mentioned that the chemically synthesized 3-hydroxyacyl-CoA substrates utilized in the present study were readily hydrolyzed by a crude preparation of *E. coli* thioesterase II.

Discussion

As has recently been discussed by Boyce and Lueking (1984) and Rock and Cronan (1985), the in vivo functions of the bacterial acyl-CoA thioesterases are unknown. However, the remarkable similarities in properties (i.e., size, DFP sensitivity, substrate specificity) displayed by the thioesterases from *R. sphaeroides* and the presumptive analogous enzymes possessed by *E. coli*, an organism that is only distantly related to *R. sphaeroides* (Chory & Kaplan, 1982), suggest that the activities of these enzymes play funadmental roles in acyl-CoA metabolism in Gram-negative organisms. On the basis of the stringent requirement for acyl-ACP substrates for phospholipid synthesis exhibited by *R. sphaeroides* (Cooper & Lueking, 1984), Boyce and Lueking (1984) proposed that *R. sphaeroides* thioesterase I participated in the interconversion of acyl-CoA and acyl-ACP substrates, thus allowing *R. sphaeroides* to directly utilize exogenously supplied long-chain fatty acids for phospholipid synthesis. However, a similar role for *R. sphaeroides* thioesterase II in exogenous fatty acid utilization seems unlikely. Proposals attempting to ascribe an in vivo role to thioesterase II must accommodate the broad chain length specificity displayed by this enzyme. Further, the virtual inability of *R. sphaeroides* thioesterase II to hydrolyze 3-hydroxyacyl-CoA substrates, together with the recent demonstration by Anderson et al. (1985) that the production of the lipid A component of *E. coli* lipopolysaccharide requires hydroxyacyl thio ester derivatives of ACP, rather than CoA, as acyl donor substrates, ostensibly precludes a general role for thioesterase II (Barnes & Wakil, 1969) in providing hydroxyacyl substrates for the production of lipid A in Gram-negative organisms. In this regard, the basis for the marked difference in the abilities of the high molecular weight thioesterases from *E. coli* and *R. sphaeroides* to hydrolyze 3-hydroxyacyl-CoA substrates is unknown. In any event, the purification and characterization of the dominant acyl-CoA thioesterases possessed by phototrophically growing *R. sphaeroides* has provided the requisite information for future physiological and genetic studies on the roles of these enzymes in an organism where the functions of acyl-CoA and acyl-ACP substrates in cellular lipid metabolism are less equivocal than is currently the case with *E. coli*.

Registry No. Butanoyl-CoA, 2140-48-9; hexanoyl-CoA, 5060-32-2; octanoyl-CoA, 1264-52-4; decanoyl-CoA, 1264-57-9; dodecanoyl-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, 40757-73-1; linoleoyl-CoA, 6709-57-5; linolenoyl-CoA, 13673-87-5; 3-hydroxydodecanoyl-CoA, 6245-70-1; acyl coenzyme A thioesterase II, 69553-49-7.

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Isolation and Properties of N^{ϵ}-Hydroxylysine:Acetyl Coenzyme A N^{ϵ}-Transacetylase from *Escherichia coli* pABN11†

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ABSTRACT: The enzyme N^{ϵ}-hydroxylysine acetylase has been isolated from *Escherichia coli* 294 carrying recombinant plasmid ABN11. Activity of the enzyme was followed by measurement of the rate of appearance of 2-nitro-5-thiobenzoate, the product of cleavage of 5,5′-dithiobis(2-nitrobenzoate) by free coenzyme A released from its acetyl derivative. The enzyme bound firmly to Reactive Blue 2–Sepharose CL-6B and was eluated with 1.5 M KCl. The protein gave a single band, corresponding to a M_r of 33 000, on polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In contrast, gel filtration of the native enzyme gave a M_r of 150 000–200 000. A sequence analysis of the DNA at the junction of the first and second genes in the aerobactin operon, considered in conjunction with the N-terminal amino acid sequence of the isolated protein, enabled the conclusion that the acetylase is specified by the second gene in the complex. The enzyme transfers the acetyl moiety from acetyl coenzyme A to a variety of hydroxylamines, with N^{ϵ}-hydroxylysine as the preferred substrate. In agreement with the results found by affinity chromatography, Coomassie Blue was observed to act as a potent inhibitor.

Siderophores, defined as virtually iron(III)-specific ligands, occur in nearly all aerobic and facultative anaerobic microbial species where they have been shown by both genetic and biochemical techniques to be involved in a high-affinity iron assimilation process (Neilands, 1984). Iron is a virulence factor for microorganisms pathogenic to man and animals (Weinberg, 1984), and the capacity to synthesize a specific siderophore, aerobactin, has been correlated with the invasive character displayed by certain clinical isolates of *Escherichia coli* (Warner et al., 1981). In plants, synthesis of siderophores by root-colonizing bacteria is thought to have a significant effect on growth (Schroth & Hancock, 1981). Iron, although usually a nutritionally essential and beneficial element, is at the same time responsible for the generation of oxidizing free radicals (Willson, 1984). For all of these reasons, siderophore-mediated assimilation of iron and the regulation of this process have assumed considerable interest in biology.

Substantial progress has been made in understanding the molecular genetics of a particular siderophore system commonly found in *E. coli* and related enteric bacteria. Thus, the aerobactin biosynthetic and transport complex encoded on plasmid ColV-K30 of hospital strains of *E. coli* has been cloned (Bindereif & Neilands, 1983), its main promoter has been identified (Bindereif & Neilands, 1985), and the general organization of the operon has been established (de Lorenzo et al., 1986). The genetic determinants of a regulatory protein, Fur (*Fe uptake regulation*), which controls at the the transcriptional level all siderophore-mediated iron uptake systems in *E. coli*, has been mapped (Bagg & Neilands, 1985), cloned (Hantke, 1984), and sequenced (Schaffer et al., 1985). Thus, the molecular mechanism of iron assimilation, and its regulation, may soon be known in some detail in bacteria, and it now remains to extend these studies to a eukaryotic species.

In contrast to bacteria, only limited techniques are currently available for gene cloning in fungi other than *Saccharomyces cerevisiae*, which, paradoxically, has not been shown to form a siderophore. One possibility is to isolate from a fungal species a pure biosynthetic or transport protein for a siderophore. The immunological properties of this protein or knowledge of its amino acid sequence can then be used to probe for its gene.

The enzyme performing the acetylation step in the synthesis of N^{δ}-acetyl-N^{δ}-hydroxyornithine, the iron(III)-binding amino acid common to ferrichrome and to many other hydroxamate-type siderophores of fungal origin, has been partially purified from the basidiomycete *Ustilago sphaerogena* (Ong & Emery, 1972). The acetylase synthesizing N^{ϵ}-acetyl-N^{ϵ}-hydroxylysine, the analogous next higher amino acid present

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