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Fatty Acid Acylation of Proteins in Bioluminescent Bacteria†

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ABSTRACT: Acylation of proteins with [^{3}H]tetradecanoic acid (+ATP) has been demonstrated in extracts of different strains of luminescent bacteria. The labeled polypeptides from *Photobacterium phosphoreum* (34K and 50K) have been identified as being involved in the acyl-protein synthetase activity that is part of a purified fatty acid reductase complex responsible for synthesis of long-chain aldehydes for the bioluminescent reaction. The two polypeptides (34K and 50K) have been separated from the acyl-CoA reductase enzyme (58K) of the complex and resolved from each other, and the 50K polypeptide was further purified to >95% homogeneity. Acylation of the 50K polypeptide, alone, occurred at a low rate; however, the rate and level of acylation were greatly stimulated by the addition of either the 34K or the 58K polypeptide. Cold chase experiments demonstrated that the acylated 50K polypeptide turned over in the presence of the 58K polypeptide but not in a mixture containing only the 34K and 50K polypeptides. Furthermore, the acylated 50K polypeptide could function as the immediate substrate for the fatty acyl-CoA reductase enzyme (58K), being reduced with NADPH to aldehyde. The 34K polypeptide was acylated only when all three polypeptides (34K, 50K, and 58K) were present. Fatty acid reductase activity could be restored by mixing of only the 58K (acyl-CoA reductase) and 50K polypeptides, showing that the 50K polypeptide is responsible for fatty acid activation in the fatty acid reductase complex and raising the question of what role the 34K polypeptide plays in fatty acid utilization in the luminescent system.

Studies concerning the incorporation of fatty acids into proteins have increased over the last few years. The formation of acyl-protein intermediates during fatty acid metabolism is well documented particularly with respect to the covalent attachment of fatty acids to acyl-carrier protein (ACP) as part of the mechanism of fatty acid synthesis (Vagelos, 1973; Rock et al., 1981; Jaworski & Stumpf, 1974). Studies on fatty acyl intermediates of other proteins involved in fatty acid metabolism are not extensive; however, evidence for this type of intermediate has been obtained in a few cases (Ayling et al., 1972; Bar-Tana et al., 1973). Interest in the acylation of proteins has also been stimulated by the recent discovery that fatty acids are covalently incorporated into specific membrane proteins as posttranslational events in both animal cells and viruses (Magee & Schlesinger, 1982; Schmidt & Schlesinger, 1979). Consequently, the isolation and study of enzymes responsible for acyl-protein formation are becoming of increasing biological importance.

In *Photobacterium phosphoreum*, the reduction of long-chain fatty acids to the corresponding aldehydes required for the luminescent reaction has recently been shown to involve the formation of acylated proteins (Riendeau et al., 1982). This activity, designated as acyl-protein synthetase, was measured by the incorporation of fatty acid into material insoluble in chloroform/methanol/acetic acid in an assay similar to that developed for measuring acyl-ACP synthetase activity (Ray & Cronan, 1976). The acyl-protein synthetase activity is part of a fatty acid reductase complex which can be resolved into acyl-protein synthetase and acyl-CoA reductase activities (Riendeau et al., 1982). Although the kinetics of formation of the acyl-protein were altered after

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0006-2960/83/0422-5604$01.50/0

resolution from the acyl-CoA reductase, the relative amount of acyl-protein formed in the assay was comparable to that in the unresolved complex. Recently the acyl-CoA reductase has been purified to homogeneity and shown to consist of polypeptides with molecular weights of 58K (Rodriguez et al., 1983), whereas the polypeptide composition and purity of acyl-protein synthetase have not yet been reported.

Similar studies on acyl-protein synthetase activity in other luminescent bacteria have not yet been conducted, although fatty acid reductase activity has been detected in extracts of two other *Photobacterium* strains (Riendeau & Meighen, 1980; Ulitzur & Hastings, 1980) and in vivo experiments on fatty acid stimulation of dark mutants (Ulitzur & Hastings, 1978, 1979) have implicated the involvement of fatty acid reduction in the luminescent system of certain *Vibrio* strains. The present experiments show that high levels of acyl-protein formation can be measured in extracts of a variety of luminescent bacteria compared to *Escherichia coli* extracts, including strains from both the *Vibrio* and *Photobacterium* genera (Baumann et al., 1980). Sodium dodecyl sulfate (SDS) gel electrophoresis and fluorography showed that only a few polypeptides are labeled and that, in *Photobacterium phosphoreum* extracts, the two major labeled polypeptides (50K and 34K) are both present in the partially purified fatty acid reductase complex. These polypeptides have been resolved and their roles as well as the role of the acyl-CoA reductase investigated in terms of protein acylation and fatty acid reductase activity.

Experimental Procedures

Materials. Cellex D and Bio-Gel P-10 were purchased from Bio-Rad, as well as all the products for polyacrylamide gel electrophoresis. DEAE-Sepharose CL-6B, Blue Sepharose CL-6B, Ficoll, and Sephacryl S-300 were from Pharmacia Fine Chemicals. [^{3}H]Tetradecanoic acid (21 Ci/mmol) was prepared by New England Nuclear by catalytic reduction of *cis*-9-tetradecenoic acid with tritium gas. Phosphate buffers were prepared by mixing appropriate amounts of K_2HPO_4 and NaH_2PO_4. Fatty acids were prepared as stock solutions in 2-propanol or ethanol.

Growth and Extraction of Bacteria. Luminescent strains of bacteria were grown in complete medium containing per liter 3.7 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 2 mL of glycerol, 5.0 g of Bacto-tryptone, and 0.5 g of yeast. *Photobacterium phosphoreum* NCMB 844 and A13 were grown at 19 °C with 3% NaCl in the medium. *Photobacterium leiognathi* (ATCC 25521) and *Vibrio harveyi* B392 were grown in 1% NaCl at 27 °C. *Vibrio fischeri* (ATCC 7744) was also grown at 27 °C but in 3% NaCl and 3 g of yeast extract (instead of 0.5 g) per L. *E. coli* K12/C600 was grown at 37 °C in broth containing 10 g of Bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl per L adjusted to pH 7.5 with NaOH. Cultures (75 mL) in 250-mL Erlenmeyer flasks were inoculated to an A_{660} of 0.05 and then grown in a reciprocatory shaker. The increase in A_{660} and the in vivo luminescence of 1-mL samples of culture were followed with growth. One light unit (LU) corresponds to 6×10^9 quanta/s based on the standard of Hastings & Weber (1963). At peak luminescence (or the mid-logarithmic phase of growth for *E. coli*), a constant amount of cells (A_{660} × milliliters of culture = 40) was harvested by centrifugation at 15000*g* for 10 min and the pellet frozen at −20 °C. The cells were thawed and then lysed by sonication (3 × 20 s) in 2 mL of 1 mM phosphate, pH 7.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM mercaptoethanol. Cellular debris was removed by centrifugation at 27000*g* for 15 min and the supernatant made 50 mM in phosphate and mercaptoethanol by addition of 1 M phosphate, pH 7.0, containing 1 M mercaptoethanol.

Purification of Acyl-Protein Synthetase from Photobacterium phosphoreum. Bacteria were grown in 10 L of medium, harvested, and lysed by osmotic shock as previously described (Riendeau et al., 1982; Riendeau & Meighen, 1980). The enzyme was initially purified as part of a complex with acyl-CoA reductase by chromatography on Cellex-D and DEAE-Sepharose, ammonium sulfate precipitation, aminohexyl-Sepharose chromatography, and gel filtration on Sephacryl S-300 as described for the purification of fatty acyl-CoA reductase (Rodriguez et al., 1983). After these steps, the enzyme was dialyzed for 2 h against 1 L of 0.05 M β-mercaptoethanol in 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, with one buffer change. The dialyzed sample was then applied on a Sepharose Blue column (1.5 × 20 cm) and washed with 70 mL of the same buffer followed by 70 mL of 0.5 M NaCl in 50 mM phosphate buffer, pH 7.0, 50 mM in β-mercaptoethanol.

Elution of the 34K polypeptide was accomplished with 0.2 M NaSCN in 50 mM phosphate/50 mM β-mercaptoethanol, pH 7.0. Fractions were assayed for their capability to stimulate the acyl-protein synthetase activity of the 50K polypeptide, pooled, dialyzed against a solution of 5% glycerol, 50 mM phosphate buffer, pH 7, and 50 mM β-mercaptoethanol, and concentrated by dialysis against Ficoll. The concentrated solution was made 30% in glycerol and kept at −20 °C.

The acyl-protein synthetase (50K polypeptide) was eluted from the Blue Sepharose column with 0.5 M NaSCN in 50 mM phosphate/50 mM β-mercaptoethanol, pH 7.0. Fractions were assayed for fatty acid reductase activity by complementation with an excess of acyl-CoA reductase. The pool of activity was dialyzed against 5% glycerol in 50 mM phosphate buffer, pH 7, and 50 mM β-mercaptoethanol, applied on a DEAE-Sepharose column (1.5 × 4 cm), and eluted with a linear gradient of 0.0–0.5 M NaCl in 50 mL of the same buffer. The peak of activity, eluted in the middle of the gradient, was made 15% in glycerol and kept frozen at −20 °C. Under these conditions, the enzyme is active for at least 4–6 months.

Enzyme Assays. Luciferase was purified and assayed as previously described (Gunsalus-Miguel et al., 1972; Meighen & Bartlet, 1980). Fatty acid reductase and acyl-CoA reductase activities were measured by the luciferase-coupled assay for the long-chain aldehyde product (Riendeau et al., 1982).

The acyl-protein synthetase assay was performed by incubation of enzyme at 22 ± 2 °C in 100 μL of 50 mM phosphate buffer, pH 7, and 30 mM β-mercaptoethanol containing 5 mM ATP and 12 μM [^{3}H]tetradecanoic acid (4.2 or 21 Ci/mmol). At the end of the incubation period (usually 10 min), 75 μL of the mixture was removed, applied to 3-cm^2 filter paper (Whatman 3), and washed 3 times in 100 mL of $Cl_3CH/CH_3OH/CH_3CO_2H$ (3:6:1) for 15 min. The filter papers were dried, and the amount of radioactivity was determined by liquid scintillation counting in Econofluor (NEN). One picomole of fatty acid at 4.2 Ci/mmol corresponded to 1400 cpm in this assay system.

SDS Gel Electrophoresis and Fluorography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970). Samples were made 10% in glycerol, 1% in SDS, 0.15 M in β-mercaptoethanol, and 50 mM in tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, placed in a boiling water bath

Table I: Incorporation of [^{3}H]Tetradecanoic Acid into Proteins by Extracts of Luminescent Bacteria

strain	growth (A_{660})	in vivo luminescence (LU/mL)	protein in extract (mg/mL)	fatty acid incorporated (pmol/mg)
P. phosphoreum (NCMB 844)	3.5	3000	1.1	150
P. leiognathi (ATCC 25521)	4.8	1800	1.1	90
P. phosphoreum (A13)	1.1	825	1.5	125
V. harveyi (B392)	2.3	630	2.7	125
V. fischeri (ATCC 7744)	2.3	160	2.2	25
E. coli K12/C600	1.1		1.0	16

for 2–3 min, and run on a 10% polyacrylamide gel with a 4% stacking gel. The running buffer was 50 mM Tris, 0.4 M glycine, 0.1% SDS, and 3 mM thioglycolic acid.

The samples for fluorography were prepared by incubation with [^{3}H]tetradecanoic acid, as described above, and the reaction was stopped by adding the electrophoresis sample buffer. After electrophoresis, the gels were stained for 2 h in 0.1% Coomassie blue R-250 in 25% 2-propanol/10% acetic acid and destained overnight in 10% acetic acid/10% 2-propanol. The gel was then soaked in Enhance (NEN) and in 5% glycerol, dried, and then exposed for 1–7 days to Kodak film (XAR-5) at −70 °C. To check radioactivity recovery after electrophoresis, the protein bands were excised from the gel, incubated overnight at 37 °C in 10 mL of a 4% Protosol (NEN) solution in Econofluor, and then counted.

Densitometric measurements on gels were carried out with an LKB 2202 Ultrascan laser densitometer.

Protein Assay. Protein concentrations were measured by using the Bio-Rad dye binding assay (Bradford, 1976) with bovine serum albumin as a standard.

Results

Acylation of Proteins in Extracts of Luminescent Bacteria. Labeling of extracts of different strains of luminescent bacteria from different genera, *Vibrio and Photobacterium*, with [^{3}H]tetradecanoic acid using the acyl-protein synthetase assay (incorporation into material insoluble in chloroform/methanol/acetic acid) shows that the extent of labeling of extracts is significantly higher in luminescent strains than in *E. coli* (Table I). Analysis of the acyl-protein products by SDS gel electrophoresis and fluorography (Figure 1) demonstrates that only a few specific bands are labeled with [^{3}H]tetradecanoic acid. Tha major bands labeled in *P. phosphoreum* extracts have molecular weights of 53K, 50K, and 34K with the relative amounts of fatty acid incorporated being 7%, 48%, and 45%, respectively. The *P. phosphoreum* A13 extract also shows three bands of similar molecular weight with relative intensities of 11%, 63%, and 26%, respectively. Only two polypeptides, 47K and 37K, are heavily labeled in the other *Photobacterium* strain, *P. leiognathi*.

The patterns of labeled polypeptides in the *Vibrio* strains appear to be somewhat different than in the *phosphoreum* strains. The major polypeptides labeled in *V. harveyi* have molecular weights of about 20K. However, minor bands at around 50K as well as a weak band at 34K are also present. In *V. fischeri*, the major labeled band of ~45K is the only polypeptide that does not appear to be dependent on the presence of ATP. This strain also contains a labeled polypeptide of molecular weight 34K. In contrast to the labeling patterns in the luminescent strains, the pattern for *E. coli* is quite different, with only a single band of low molecular weight (~20K) being weakly labeled.

Purification of Acyl-Protein Synthetase. The identification of the function of the labeled polypeptides in the luminescent bacteria and their relationship to the fatty acid reductase

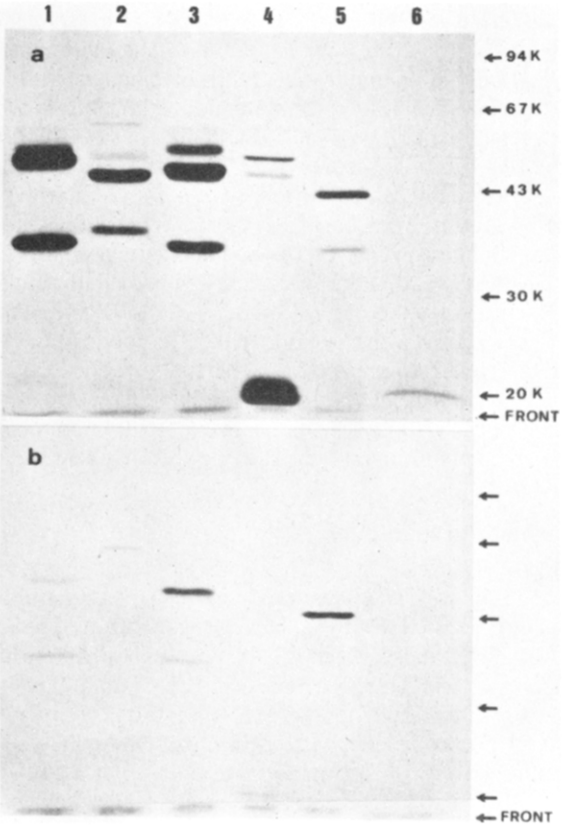

FIGURE 1: SDS gel electrophoresis and fluorography of bacterial extracts labeled with [^{3}H]tetradecanoic acid. Extracts (0.2 mL) were incubated for 10 min in a total volume of 0.4 mL of 50 mM phosphate/30 mM mercaptoethanol, pH 7.0, containing 12 μM [^{3}H]-tetradecanoic acid (21 Ci/mmol) either in the presence (a) or in the absence (b) of 5 mM ATP. The reaction was stopped by addition of the sample buffer for SDS electrophoresis and placement of the samples in a boiling water bath. SDS gel electrophoresis and fluorography (for 7 days) were carried out as described under Experimental Procedures with 25 μg of protein from each extract. Lane 1, *P. phosphoreum* (NCMB 844); lane 2, *P. leiognathi*; lane 3, *P. phosphoreum* (A13); lane 4, *V. harveyi*; lane 5, *V. fischeri*; lane 6, *E. coli*. Arrows refer to molecular weight standards: 94K, phosphorylase *b*; 67K, bovine serum albumin; 43K, ovalbumin; 30K, carbonic anhydrase; 20K, soybean trypsin inhibitor.

complex in *P. phosphoreum* is important to understand the role of protein acylation in the luminescent system. Table II shows that the ratio of acyl-protein synthetase to the fatty acid reductase activity remains constant through the steps of purification of the fatty acid reductase complex. The enzyme at this stage of purification contains two major polypeptides, 58K and 50K, as well as a band at 34K (Figure 2) in molar ratios of 1.0:0.9:0.5, based on densitometric analysis of the staining intensities of several enzyme preparations and their respective molecular weights. It should be recognized, however, that the different polypeptides may not have the same staining intensity per unit weight. Some minor polypeptides of higher molecular weight with weaker staining intensity are

Table II: Purification of Acyl-Protein Synthetase[a]

purification step	total protein (mg)	acyl-protein synthetase activity (nmol/mg)	fatty acid reductase activity (nmol min^{-1} mg^{-1})	activity ratio[b]
extract	2350	0.09	0.53	6
Cellex-D chromatography	256	0.65	6.6	10
DEAE-Sepharose concentration	204	0.72	7.2	10
$(NH_4)_2SO_4$ precipitation (30–55% saturation)	130	0.95	9.9	10
gel filtration	51	2.15	20	9
aminohexyl-Sepharose chromatography	32	2.50	25	10
Blue Sepharose chromatography	7	(2.35)[c]		
DEAE-Sepharose chromatography	5	(2.55)[c]		

[a] From 44 g wet weight of *P. phosphoreum* cells. [b] Fatty acid reductase to acyl-protein synthetase activity. [c] After complementation with an excess of acyl-CoA reductase to regenerate acyl-protein synthetase activity.

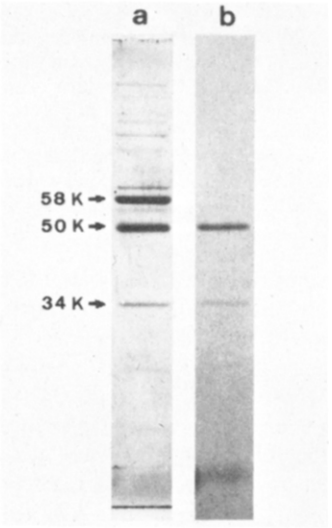

FIGURE 2: Acylation of fatty acid reductase. Fatty acid reductase purified through the gel filtration step was incubated at a final concentration of 0.28 mg/mL with 12 μM [^{3}H]tetradecanoic acid (4.2 Ci/mmol) and 5 mM ATP for 10 min and run on SDS gel electrophoresis as described under Experimental Procedures. (a) Coomassie blue staining; (b) fluorograph after 7-days exposure.

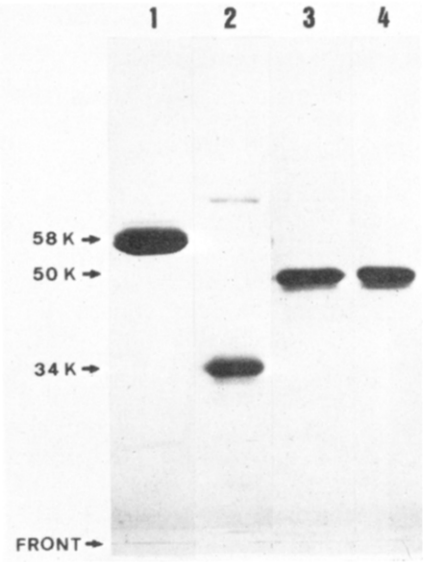

FIGURE 3: SDS gel electrophoresis of polypeptides in the fatty acid reductase complex after resolution by Blue Sepharose chromatography. (1) Hepes eluant (16 μg); (2) 0.2 M NaSCN eluant (10 μg); (3) 0.5 M NaSCN eluant (10 μg); (4) the 50K polypeptide from the 0.5 M NaSCN eluant after DEAE-Sepharose chromatography (10 μg).

also present; however, attempts to further purify the complex and remove some of these polypeptides result in dissociation of the complex and loss of fatty acid reductase activity.

Reaction of the fatty acid reductase enzyme with [^{3}H]-tetradecanoic acid in the presence of ATP shows that two polypeptides, 50K and 34K, are acylated (Figure 2). These polypeptides correspond in molecular weight to the polypeptides labeled in crude extracts of *P. phosphoreum* (see Figure 1), although the relative labeling intensity of the 34K polypeptide compared to that for the 50K polypeptide appears to be less in the purified enzyme than in the extract. The fatty acid reductase can be resolved into acyl-CoA reductase and acyl-protein synthetase activities by chromatography on Blue Sepharose, the acyl-CoA reductase being eluted in the void volume of the column (Riendeau et al., 1982). Purification of this enzyme to homogeneity has shown that it is composed of polypeptides of 58K daltons. The acyl-protein synthetase activity remains bound to the column and contains most of the other polypeptides including the 50K and 34K polypeptides. Resolution of these polypeptides has now been achieved by elution of the Blue Sepharose with 0.2 M NaSCN followed by elution with 0.5 M NaSCN. SDS gel electrophoresis (Figure 3) shows that the 34K polypeptide was eluted with 0.2 M NaSCN whereas the 50K band was eluted with 0.5 M NaSCN. Further purification of the 50K band by DEAE-Sepharose chromatography results in removal of contaminating

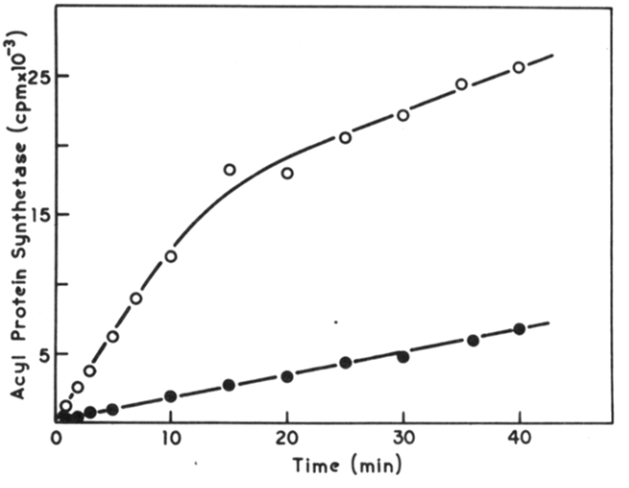

FIGURE 4: Effect of the 34K polypeptide on acyl-protein formation. The 50K polypeptide was assayed for acyl-protein synthetase activity in the absence (●) and presence (○) of the 34K polypeptide. The concentration of each polypeptide in the assay was 35 μg/mL, and the specific radioactivity of [^{3}H]tetradecanoic acid was 4.2 Ci/mmol.

polypeptides of higher molecular weight, giving a single band of apparent homogeneity of over 95%. A polypeptide in low amounts of slightly lower molecular weight which increases on storage and manipulation of the preparation is also found

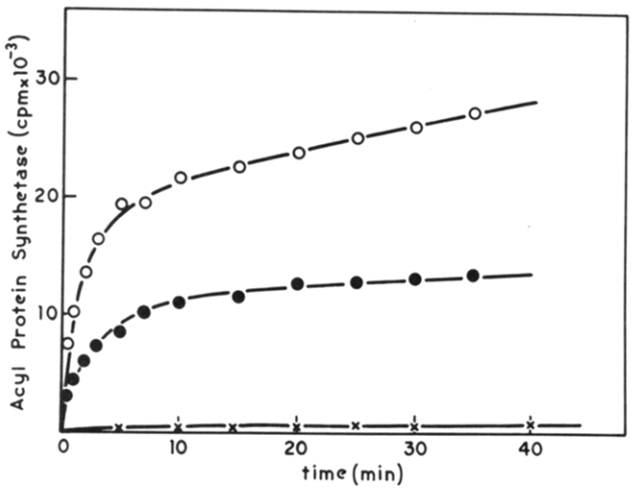

FIGURE 5: Effect of the 58K polypeptide on acyl-protein formation. Acyl-protein synthetase activity was measured under the same conditions as in Figure 4 for mixtures of the 58K and 34K polypeptides (×), the 58K and 50K polypeptides (●), and all three polypeptides (34K, 50K, and 58K) added together (○).

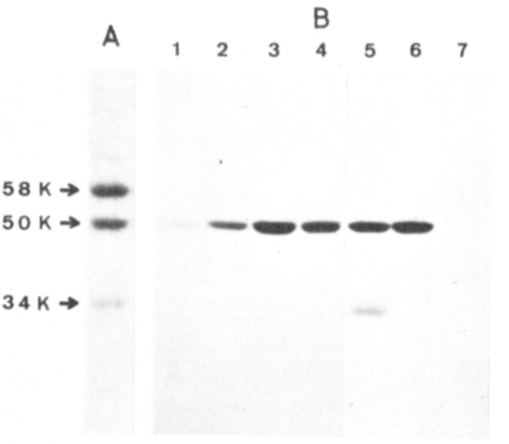

FIGURE 6: Identification of the acylated polypeptides by SDS gel electrophoresis. Mixtures of the three polypeptides in 100 μL (at 50 μg/mL each unless noted otherwise) were acylated with [^{3}H]tetradecanoic acid (21 Ci/mmol), and one-third of the sample was run on SDS gel electrophoresis as described in Figure 1. (A) Coomassie blue staining of the mixture of 58K, 50K, and 34K polypeptides. (B) Fluorogram, 1-day exposure: (1) 50K polypeptide; (2) 50K + 58K polypeptides; (3) 50K, 58K, and 34K polypeptides; (4) 50K + 34K polypeptides; (5) 50K, 58K, and 250 μg/mL 34K; (6) 50K and 250 μg/mL 34K; (7) 250 μg/mL 34K.

in most preparations (Figure 3).

Reconstitution of Acyl-Protein Synthetase Activity. The determination of which polypeptides are required for protein acylation was investigated by mixing the 34K and 50K polypeptides and measuring the extent of incorporation of fatty acid into protein as a function of time (Figure 4). A slow linear increase in the amount of [^{3}H]tetradecanoic acid insoluble in chloroform/methanol/acetic acid is observed only with the 50K polypeptide, whereas absolutely no fatty acid incorporation is observed with the 34K polypeptide alone. Mixing of the 34K and 50K polypeptides results in a large increase in the amount of acyl-protein formed with time.

The effect of the 58K polypeptide (acyl-CoA reductase) on acyl-protein formation is shown in Figure 5. Formation of acyl-protein is not observed with a mixture of the 34K and 58K polypeptides, whereas mixing of the 58K and the 50K proteins results in the rapid formation of acyl-protein, reaching a plateau within 10 min. Addition of larger amounts of 58K protein does not increase the amount of acyl-protein formed, although differences can be observed in the rate of formation of the acyl-protein at shorter incubation times (results not shown). A mixture of all three polypeptides, 34K, 50K, and 58K, also shows rapid acyl-protein formation, with the plateau level being higher than with binary mixtures of the 50K and 58K polypeptides.

SDS gel electrophoresis and fluorography of the labeled products of the reaction are shown in Figure 6. A low level of labeling of the 50K polypeptide is observed on incubation of this peptide alone, in agreement with the results in Figure 4. This result indicates that this peptide contains the acyl-protein synthetase activity. Addition of the 58K polypeptide results in a large increase in the labeling of the 50K polypeptide.

Since both the 50K and 34K polypeptides are acylated in *P. phosphoreum* extracts and in the purified fatty acid complex, it was expected that the stimulation of acyl-protein synthetase activity on addition of the 34K to the 50K polypeptide (see Figure 4) resulted from the labeling of the 34K polypeptide. Figure 6 shows that this is not the case; the addition of the 34K polypeptide to the 50K polypeptide results in a large increase in the extent of labeling of the 50K peptide. Even on addition of 5 times the amount of the 34K protein, only the 50K polypeptide is labeled with [^{3}H]tetradecanoic acid under these conditions. However, when all three polypeptides are mixed, both the 34K and 50K polypeptides are labeled, showing that the 50K and 58K peptides are required for the acylation of the 34K polypeptide.

The dependence of the degree of labeling of the 50K polypeptide on the addition of either the 34K or the 58K polypeptide was an unexpected result. Differences in the kinetics of labeling of the 50K polypeptide were observed not only in the rate and plateau levels but also in the dependence on ATP concentration. At 1000 times lower ATP levels (i.e., 5 μM), the plateau level of acyl-protein formed on mixing the 50K and 58K polypeptides started to decrease after 15 min, suggesting that the acyl-protein is turning over with continuous cleavage of ATP. A similar decrease in acyl-protein formation has been observed in the fatty acid reductase complex upon incubation at lower ATP concentrations. In contrast, when the 50K and 34K polypeptides are mixed, acyl-protein formation is identical at 5 μM or 5 mM ATP concentrations, and no decrease is seen after 40 min of incubation, which indicates that the acyl-protein is not being turned over and there is no extensive removal of the ATP (data not shown).

These results were directly confirmed by determining whether or not unlabeled tetradecanoic acid could cold chase the [^{3}H]acyl group from the 50K polypeptide (Figure 7a). After acylation with ^{3}H-labeled fatty acid of a mixture of the 50K and 58K polypeptides for 10 min, the concentration of fatty acid was increased 5-fold by the addition of either cold tetradecanoic acid or [^{3}H]tetradecanoic acid of the same specific radioactivity (control). A rapid decrease (5-fold) in the amount of ^{3}H label in the 50K polypeptide can clearly be observed (Figure 7) on addition of the cold tetradecanoic acid in comparison to the control. Consequently, incorporated ^{3}H-labeled fatty acid is being replaced by cold fatty acid, and the acyl group in the 50K polypeptide must be turned over.

In marked contrast, addition of cold tetradecanoic acid to a mixture of the 34K and 50K polypeptides labeled with ^{3}H-labeled fatty acid does not cause a decrease in the amount of ^{3}H labeled in the 50K polypeptide (Figure 7b). In comparison to the control, the only difference is a slower increase in the amount of labeled fatty acid subsequently incorporated into protein, as expected for the 5-fold decrease in specific radioactivity on addition of cold fatty acid. Consequently, in this

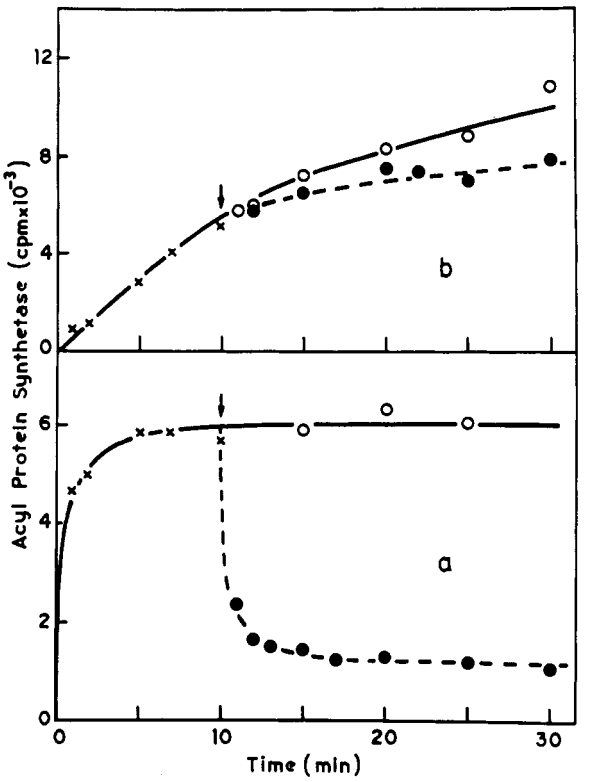

FIGURE 7: Cold chase with tetradecanoic acid of the [^{3}H]acyl group on the 50K polypeptide. The ^{3}H-acylated 50K polypeptide (50 μg/mL) was prepared by incubation with equal amounts of either the 58K polypeptide (a) or the 34K polypeptide (b) in 50 mM phosphate buffer, pH 7.0, and 30 mM β-mercaptoethanol containing 5 mM ATP and 5 μM [^{3}H]tetradecanoic acid (4.2 Ci/mmol) (×). After 10 min, unlabeled tetradecanoic fatty acid (●) or [^{3}H]tetradecanoic acid (4.2 Ci/mmol) (○) was added to give a final concentration of fatty acid of 25 μM. Aliquots were assayed for the acyl-protein product as described under Experimental Procedures.

instance, the ^{3}H-labeled fatty acid incorporated into the 50K polypeptide is not turned over.

To determine if the same site is acylated on the 50K protein after incubation with the 34K or 58K polypeptide, the 50K protein was acylated with ^{3}H-labeled fatty acid and then chased with cold fatty acid (as in Figure 7b) except that the 58K protein was added at the same time as the cold chase. As can be seen in Figure 8, the ^{3}H label on the 50K protein can be removed by the 58K protein, even though acylation of the 50K protein had originally occurred in the presence of only the 34K polypeptide. This result suggests that the same site is acylated on the 50K protein upon incubation with either the 34K or the 58K polypeptide.

Direct support for this conclusion was obtained by determining whether or not the protein-bound acyl groups could be reduced to aldehyde by the acyl-CoA reductase enzyme (58K). In this experiment, the 50K protein was acylated in the presence of the 34K polypeptide, resulting in the incorporation of 260 pmol of fatty acid as determined by the acyl-protein synthetase assay. After resolution from the substrates (ATP and free fatty acid) by gel filtration on Bio-Gel P-10, 120 pmol of the acylated protein was recovered and incubated with the 58K protein (acyl-CoA reductase) and NADPH (0.1 mM) for 4 min. The aldehyde produced was then determined by the luminescent assay. Ninety picomoles of aldehyde was obtained from the 120 pmol of acylated 50K protein, with 11 pmol of protein-bound fatty acid still remaining on the 50K protein after luminescent analysis for free aldehyde. Controls (minus NADPH and minus acylated protein) gave less than 5% of the luminescence response. These

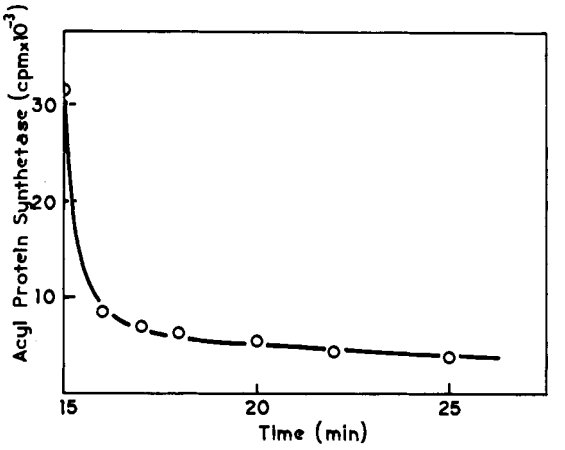

FIGURE 8: Fatty acid cold chase of acylated 50K polypeptide (formed with the 34K polypeptide) by addition of the 58K protein. The ^{3}H-acylated polypeptide was prepared by incubation of equal amounts (1.5 μM) of 50K and 34K polypeptides with 5 mM ATP and 5 μM [^{3}H]tetradecanoic acid (4.2 Ci/mmol) in 50 mM phosphate buffer, pH 7.0, and 30 mM β-mercaptoethanol. After 15 min, cold tetradecanoic acid and 58K protein were added to a final concentration of 25 and 1.5 μM, respectively. At the indicated times, 75-μL aliquots were the acyl-protein product as described under Experimental Procedures.

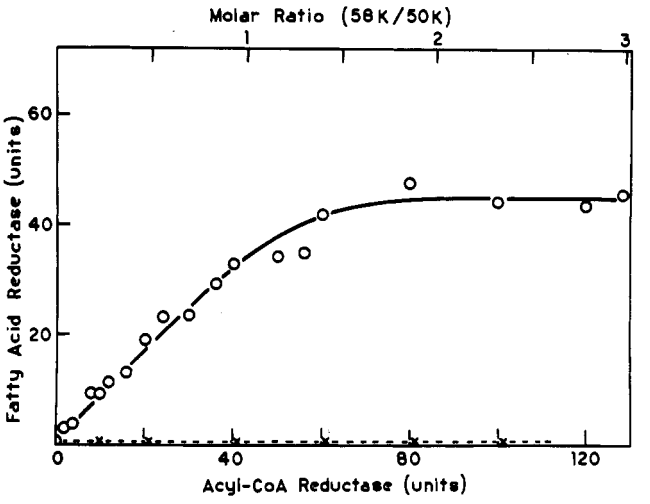

FIGURE 9: Reconstitution of fatty acid reductase activity. Fixed amounts (5 μg) of either the 34K (×) or the 50K (○) polypeptide were analyzed for fatty acid reductase activity after titration with increasing amounts of 58K polypeptide (acyl-CoA reductase) and incubation at 22 °C with 1 mM ATP, 5 μM tetradecanoic acid, and 0.1 mM NADPH in 1 mL of 50 mM phosphate buffer, pH 7.0, 20 mM β-mercaptoethanol, and 10 mM $MgSO_4$ for 4 min. One unit of fatty acid reductase or acyl-CoA reductase activity equals 1 pmol/min of aldehyde produced.

results show that the acylated 50K protein can be reduced in the fatty acid reductase reaction and can serve as the immediate substrate for the acyl-CoA reductase component (58K) in the complex.

Reconstitution of the Fatty Acid Reductase Activity. Since the partially purified fatty acid reductase complex contains all three polypeptides, 34K, 50K, and 58K, it was of interest to know if the fatty acid reductase activity could be regenerated by mixing the 58K polypeptide (acyl-CoA reductase) with either the 50K or the 34K polypeptide (Figure 9). Absolutely no activity was obtained on titration of the 34K polypeptide with acyl-CoA reductase (58K). In contrast, titration of the 50K polypeptide with the 58K polypeptide effectively regenerated the fatty acid reductase to a plateau level of activity (~45 units). From the molar ratio of the 58K polypeptide

to the 50K polypeptide, it appears that a 1:1 molar complex is being formed between the two polypeptides and is in good agreement with the densitometric measurements of staining intensity of the SDS gel of the 50K and 58K polypeptides in the partially purified complex (Figure 2). Although addition of an excess of the 34K polypeptide to the mixture of 50K and 58K polypeptides caused a small stimulation of fatty acid reductase activity (25%) (data not shown), the results clearly indicate that only the 50K and 58K polypeptides are required for the fatty acid reductase reaction.

Discussion

The conversion of ^{3}H-labeled fatty acid into a form insoluble in chloroform/methanol/acetic acid by extracts from luminescent bacteria provides strong evidence that the fatty acid is covalently linked to a protein. This conclusion was supported in the present experiments by the demonstration that the fatty acid was bound to specific polypeptides after electrophoresis in the presence of reducing agents and sodium dodecyl sulfate. Relatively stable bonds must be formed between the fatty acid and the protein since between 50% and 100% of the applied radioactivity in the acylated proteins could be recovered from the gels after electrophoresis. Thus the ^{3}H-labeled polypeptides on the gel represented the majority (or perhaps all) of the acylated proteins precipitated by the organic solvent. Although the high stability of the acyl-polypeptides under the electrophoresis conditions (pH 8.8) might suggest that an oxygen ester is formed (Stadtman, 1957), the thio ester of acyl-ACP has also been demonstrated to be stable under similar conditions (Rock et al., 1981). Characterization of the labeled peptides will be necessary to determine the chemical nature of the covalent bond formed in the different acyl-proteins.

Extracts from five different strains of bioluminescent bacteria exhibited an ATP-dependent incorporation of [^{3}H]tetradecanoic acid into protein. In contrast, very little incorporation was observed in *E. coli* extracts, and the only acylated polypeptide migrates on SDS gel electrophoresis at a molecular weight of 20K, the anomalous position expected for acyl-ACP (Rock & Cronan, 1979). Thus, the acylation of the higher molecular weight proteins in the extracts of the bioluminescent bacteria may be characteristic of the bioluminescent system and possibly even serve as a fingerprint for a particular bacterial strain. In the *Photobacterium* strains, two major sets of bands with molecular weights of 47K–50K and 33K–37K were detected. These polypeptides were found to be involved in the acyl-protein synthetase activity in the fatty acid reductase complex of the *P. phosphoreum* NCMB 844 strain. Although the acyl-CoA reductase component of this complex (58K polypeptide) might also be acylated, evidence for the formation of this intermediate has not yet been obtained, indicating that either it is not present or the acyl bond with the 58K polypeptide is labile under the assay conditions. In the reconstituted system, the relative acylation of 50K and 34K polypeptides differs from the extracts, suggesting that the acyl-protein synthetase activity was not fully restored and/or the crude extract contains other elements that may be involved in fatty acid acylation of proteins.

After removal of the 58K and 34K polypeptides, the 50K polypeptide could still be labeled at a low rate with [^{3}H]tetradecanoic acid in the presence of ATP, indicating that this polypeptide is responsible for fatty acid activation. The unusual dependence of a functional property (acylation) of this protein on the addition of one protein or another (34K or 58K) raises a number of interesting questions about the nature of the protein interactions, mechanism, and regulation of the protein acylation in this system. The results are consistent with the interaction of both the 34K and 58K polypeptides with the 50K protein leading to the stabilization of its active conformation and an increased rate of fatty acid acylation. This conclusion is supported in the case of the 50K and 58K polypeptides by the regeneration of fatty acid reductase activity on mixing of the two polypeptides. Furthermore, the initial plateau level of acyl-protein formed in this case decreased on longer incubation times when 10^3-fold lower ATP concentrations (5 μM) were used. Since less than 25% of the 50K polypeptide ($\sim$0.5 μM total) is labeled at the plateau level, the acyl group in the 50K protein must be turnover over in the presence of the 58K polypeptide. In contrast, protein acylation in a mixture of the 34K and 50K polypeptides was unaffected by decreasing the ATP concentration, indicating that the acylated 50K polypeptide was not turned over under these conditions. Cold chase experiments confirmed these conclusions by showing that the [^{3}H]acyl group on the 50K polypeptide was displaced by cold tetradecanoic acid only in the presence of the 58K polypeptide. Consequently, although both the 34K and 58K polypeptides stimulate the acylation of the 50K protein, only the 58K polypeptide can cause the removal of the acyl groups directly from the 50K polypeptide. The cleavage of the acyl group appears to reflect the acyltransferase activity of the 58K polypeptide that occurs in the absence of NADPH (Rodriguez et al., 1983). In the presence of NADPH and 58K protein, the acyl group is reduced to aldehyde. These results agree with the regeneration of the fatty acid reductase on mixing of these two polypeptides and indicate that the acylated 50K protein is the immediate substrate for the acyl-CoA reductase enzyme (58K polypeptide). It is not yet known whether it is necessary for the acyl group to be transferred to the 58K protein before reduction can take place.

Experiments to determine the possible role of the 34K polypeptide in the fatty acid reductase complex and in fatty acid metabolism are currently in progress. The apparent copurification of this polypeptide with the 50K and 58K polypeptides and its effect on acylation of the 50K polypeptide do suggest that this polypeptide may be part of this enzyme complex. A role in the mechanism or regulation of the fatty acid reductase reaction cannot be eliminated since although it is not necessary for this reaction, the 34K polypeptide has been shown to cause a small stimulation in this activity. This stimulation may arise by stabilization of the active conformation of the 50K polypeptide as suggested above, for its effect on protein acylation. One clue to its role may reside in its ability to be acylated with fatty acid only on incubation with the 50K and 58K polypeptides, and experiments are being designed to investigate this possibility.

Acknowledgments

We thank Rose Szittner for valuable assistance in the purification of the enzymes, Dr. P. Baumann, University of California, Davis, for providing a number of the luminescent strains used in these studies, and Dr. John Lee, University of Georgia, for strain A13.

Registry No. Acyl-protein synthetase, 82657-98-5; fatty acid reductase, 75718-33-1; acyl-CoA reductase, 50936-56-6.

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Relative Roles of Cyclopropane-Containing and Cis-Unsaturated Fatty Acids in Determining Membrane Properties of *Acholeplasma laidlawii*: A Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: Dihydrosterculic acid (*cis*-9,10-methyleneoctadecanoic acid, 19:cp,cΔ^9), specifically deuterated at several positions along the chain, has been incorporated biosynthetically into the membrane lipids of *Acholeplasma laidlawii* B. A detailed study of acyl chain order was carried out with deuterium nuclear magnetic resonance. The transition from the gel to the liquid-crystalline phase was determined to occur from −15 to 0 °C, a range somewhat narrower than, but with a midpoint similar to, that found for membranes enriched in oleic acid. The acyl chains of 19:cp,cΔ^9-containing membranes are less mobile in the gel state than those of oleic acid containing membranes. Above 0 °C, the lipids are in the liquid-crystalline phase and give rise to powder spectra characteristic of axially symmetric motion. The C^2H_2 segments near the cyclopropane ring gave rise to a quadrupolar powder pattern indicative of inequivalence of the two deuterons. The orientational fluctuations of the fatty acid chain segments in the membrane lipids are described in terms of deuterium order parameters. The overall ordering is greater everywhere than that in the case of oleoyl chains and features a maximum at the cyclopropyl moiety, in sharp contrast to the plateau found with saturated chains. Detailed analysis of the data for the cyclopropane ring indicates that the C-9–C-10 bond is inclined at 89° relative to the director of motional averaging, in sharp contrast to the 3° estimated for oleic acid in the same membranes. The effect of incorporation of cholesterol at 35 mol % lipid was examined. This had little effect on the breadth of the gel to liquid-crystal transition but did result in a gel state with lipid that is rotationally more rigid.

Biological membranes consist primarily of a lipid matrix in which other membrane components are organized. The lipid molecules may contain saturated, unsaturated, branched-chain, and cyclopropane fatty acids. Lipids containing saturated and/or unsaturated fatty acyl chains have been extensively studied while those containing other classes of fatty acids have received little attention. Fatty acids containing cyclopropyl rings are found in a variety of organisms and are common components of the membrane lipids of microorganisms (Christie, 1970). Although fatty acids containing cyclopropane rings have been postulated as replacements for unsaturated fatty acids (Christie, 1970; Cronan & Vagelos, 1972), their precise biochemical role remains obscure. An antioxidant role (Law et al., 1963) does not appear to be tenable since both anaerobes and aerobes possess these acids. Since the rate of cyclopropane ring biosynthesis is not influenced by the level of *S*-adenosylmethionine (SAM)[1] (Cronan et al., 1974), the formation of the cyclopropane ring is an unlikely mechanism

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[1] Abbreviations: SAM, *S*-adenosylmethionine; NMR, nuclear magnetic resonance; 19:cp,cΔ^9, *cis*-9,10-methyleneoctadecanoic acid; 18:1cΔ^9, *cis*-9-octadecenoic acid; PDSPC, 1-palmitoyl-2-dihydrosterculoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

0006-2960/83/0422-5611$01.50/0