Acyl-acyl carrier protein as a source of fatty acids for bacterial bioluminescence

(tetradecanoic acid/acyltransferase/Vibrio harveyi/Photobacterium phosphoreum)

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ABSTRACT Pulse-chase experiments with [3H]tetradecanoic acid and ATP showed that the bioluminescence-related 32-kDa acyltransferase from Vibrio harveyi can specifically catalyze the decylation of a [3H]-labeled 18-kDa protein observed in extracts of this bacterium. The 18-kDa protein has been partially purified and its chemical and physical properties strongly indicate that it is the fatty acyl-acyl carrier protein (acyl-ACP). Both this V. harveyi [3H]acylprotein and [3H]palmitoyl-ACP from Escherichia coli were substrates in vitro for either the V. harveyi 32-kDa acyltransferase or the analogous enzyme ("34K") from Photobacterium phosphoreum. TLC analysis indicated that the hexane-soluble product of the reaction is fatty acid. Phosphate ions and, to a lesser extent, organic alcohols stimulated the rate of acyl protein cleavage. No significant cleavage of either E. coli or V. harveyi tetradecanoyl-ACP was observed in extracts of these bacteria unless the 32-kDa or 34K acyltransferase was present. Since these enzymes are believed to be responsible for the supply of fatty acids for reduction to form the aldehyde substrate of luciferase, the above results suggest that long-chain acyl-ACP is the source of fatty acids for bioluminescence.

Bacterial luciferase catalyzes the oxidation of a long-chain aliphatic aldehyde and FMNH₂ by oxygen, resulting in the emission of light (1, 2). Several lines of evidence have indicated that three proteins are involved in the synthesis of the aldehyde substrate via the reduction of fatty acids. A fatty acid reductase complex has been isolated from Photobacterium phosphoreum (3–6) and consists of a fatty acyl-protein synthetase subunit (50K), an acyl-50K (or acyl-CoA) reductase subunit (58K), and an acyltransferase component (34K) that copurifies with the complex and can alter the enzymatic properties of the other subunits. Although fatty acid reductase activity has not been detected in extracts of Vibrio harveyi, another commonly studied luminescent strain, the isolation of a "dark" mutant (M17) whose emission is stimulated by exogenous fatty acid (7) has provided additional evidence for such a mechanism. Recently, fatty acid labeling studies have revealed that three polypeptides (32 kDa, 42 kDa, and 57 kDa) are involved in aldehyde synthesis in V. harveyi (8). Likewise, the cloning in Escherichia coli of the luminescence operon from V. fischeri has indicated that three proteins of comparable size probably perform an analogous function in that strain (9).

Although experiments with V. harveyi have shown that tetradecanoic acid produced as a result of the luciferase reaction can be recycled for aldehyde synthesis (10), it is clear that a de novo source of fatty acids for bioluminescence must also exist. Recently, Wall et al. (8) suggested that the 32-kDa polypeptide is responsible for the supply of fatty acids to the V. harveyi luminescence system, since it was not detected in the fatty-acid-stimulatable M17 mutant. This protein has since been purified to homogeneity; it possesses acyl-CoA hydrolase activity and can transfer acyl groups to various low molecular weight alcohol and thiol acceptors (11). On the basis of its enzymatic similarities to the 34K acyltransferase from P. phosphoreum, it was proposed that these enzymes play analogous roles in generating fatty acids for bioluminescence (6, 8).

Here we report that long chain acyl-acyl carrier protein (acyl-ACP) is a substrate in vitro for the bioluminescence-related acyltransferases from V. harveyi and P. phosphoreum. Since acyl-ACP is the end product of fatty acid synthesis in E. coli as well as the immediate donor of acyl groups for phospholipid synthesis (12, 13), this finding may have important implications regarding the regulation of bioluminescence within the context of overall bacterial metabolism.

MATERIALS AND METHODS

Materials. ATP, sn-glycerol 3-phosphate, 2-mercaptoethanol, and the reagents and molecular weight standards for PAGE were purchased from Sigma. DEAE-Sepharose CL-6B and Ultrogel AcA 44 were obtained from Pharmacia and LKB, respectively. β-[3H]Alanine, Econofluor and EN3HANCE were obtained from New England Nuclear and silica gel N-HR/UV254 plates were from Fisher. Organic solvents were reagent grade and were used without further purification. Phosphate buffers were prepared by mixing NaH₂PO₄ and K₂HPO₄ in the appropriate ratio.

[3H]Tetradecanoic acid (34 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham and was further purified by TLC before use. [3H]Tetradecanoyl-CoA was prepared (4) and was used to enzymatically synthesize the TLC standards [3H]tetradecanoyl-1-glycerol and [3H]tetradecanoyl-S-mercaptoethanol (6, 11). The acyltransferases from P. phosphoreum (34K) and V. harveyi (32 kDa) were prepared as described (6, 11). E. coli ACP was obtained from Sigma and E. coli [3H]palmitoyl-ACP was generously provided by E. Do (New England Nuclear).

Cell Growth and Lysis. The bioluminescent bacterial strains used in this study were V. harveyi B392 wild type and the dark mutants M17 (7) and AFM (a dark mutant derived from V. harveyi by mutagenesis with acriflavin). These mutants were kindly provided by J. W. Hastings (Harvard University). V. harveyi strains were grown at 27°C in 50 ml of complex medium (14) containing 1% (wt/vol) NaCl and cells were harvested by centrifugation when the OD₆00 of the cultures reached 2. Lysis was achieved by sonication (3 × 20 sec) in 2 ml of 50 mM phosphate, pH 7/20 mM 2-mercaptoethanol, and a cell-free supernatant was obtained by centrifugation.

Abbreviation: ACP, acyl carrier protein.

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†To avoid confusion regarding proteins from different bacterial strains, we have retained the original nomenclature for the subunits of P. phosphoreum fatty acid reductase (34K, 50K, and 58K).
Biochemistry: trifugation, washed
tifugation (17,000 g, 25 min). E. coli RR1 was grown at 30°C in Luria broth, harvested, and lysed in the manner outlined above.

To label V. harveyi ACP in vivo, the AFM mutant was grown in 1% NaCl/complex medium (2 ml) containing 0.3 μM β[3H]alanine (87 Ci/mmol). Cells were harvested by centrifugation, washed with medium, and lysed in 100 μl of 1 M phosphate, pH 7/1 mM 2-mercaptoethanol.

**Purification of [3H]Acyl-Protein from V. harveyi.** Isolation of the labeled 18-kDa protein from V. harveyi AFM cells (500-ml culture) was carried out using a modification of the isopropanol extraction procedure (15) designed for the purification of E. coli ACP. A concentrated cell-free extract (50 mg of protein) was diluted to 5 ml with final concentrations of 5 mM ATP, 10 mM MgSO4, 50 mM phosphate (pH 7), 10 mM 2-mercaptoethanol, and labeling was initiated by the addition of [3H]tetradecanoic acid (30 Ci/mmol) to 10 μM. After incubation at room temperature for 15 min, the mixture was chilled to 0°C and an equal volume of cold isopropanol was slowly added with gentle mixing. The cloudy suspension was centrifuged (10,000 g, 10 min) and the supernatant was applied directly to a DEAE-Sepharose CL-6B column (2-ml volume) equilibrated with 50 mM phosphate (pH 6). The column was successively eluted with 50 mM phosphate containing 0.0 M, 0.25 M, and finally 0.5 M LiCl (1-ml fractions were collected). The 0.5 M LiCl wash (>5 × 107 cpm in 2 ml) was applied to an Ultrogel ACA 44 gel filtration column (44 × 1.5 cm) in 50 mM phosphate (pH 6); a single radioactive peak corresponding to apparent molecular mass 30 kDa was observed. The appropriate fractions were pooled, concentrated on a small DEAE-Sepharose column, dialyzed vs. 50 mM phosphate (pH 6), and stored frozen at −20°C.

**[3H]Acyl-Protein Cleavage Assay.** The cleavage of [3H]acyl-protein to form a hexane-soluble labeled product was monitored essentially as described for acyl-CoA cleavage (4). Incubation of enzyme and substrate was carried out at room temperature (22 ± 2°C) in a total volume of 100 μl. After the reaction was stopped by addition of 10 μl of glacial acetic acid, the mixture was extracted twice with 1 ml of hexane and the combined hexane washes were added directly to 10 ml of Econnfluor for liquid scintillation counting.

**GeL Electrophoresis.** The details of in vitro labeling with [3H]tetradecanoic acid and the preparation of samples for NaDodSO4/PAGE have been outlined elsewhere (8, 11). NaDodSO4/PAGE was performed by the method of Laemmli (16), using 5% and 12% acrylamide stacking and resolving gels, respectively. Gels were stained with 0.25% Coomassie brilliant blue R-250, destained, soaked in ENHANCE and in 5% glycerol, dried under vacuum, and finally fluorographed by exposure to Kodak XAR-5 film at −70°C (11).

**Thin-Layer Chromatography.** The hexane-extractable products of [3H]acyl-protein cleavage were analyzed by TLC and fluorography (4). Hexane extracts of the reaction mixture were concentrated under N2 to a volume of about 10 μl and applied to silica gel plates. The chromatogram was developed in benzene/diethyl ether/acetic acid (90/10/2), dried, sprayed with ENHANCE, and fluorographed as described above.

**Protein Assay.** Protein concentrations were determined by the Bio-Rad dye-binding assay (17) with bovine serum albumin as a standard.

**RESULTS AND DISCUSSION**

As previously shown (8), several polypeptides, including the bioluminescence-related 32-kDa, 42-kDa, and 57-kDa proteins involved in the reduction of fatty acids to aldehydes, can be acylated in vitro when extracts of V. harveyi are incubated with [3H]tetradecanoic acid and ATP (Fig. 1A). In extracts of the fatty acid-stimulatable V. harveyi mutant M17, no label associated with the 32-kDa acyltransferase is observed (Fig. 1B), whereas in extracts of the mutant AFM, a strain that expresses bioluminescence to a very low degree, all three acylated bands are missing (Fig. 1C). To establish enzymatic character and possible interrelationships between fatty acylated proteins in V. harveyi, labeled extracts were “chased” with an excess of unlabeled tetradecanoic acid prior to the preparation of the sample for NaDodSO4/PAGE.

As illustrated in Fig. 1, fatty acid label in the 42-kDa and 57-kDa polypeptides could not be chased with unlabeled tetradecanoic acid in vitro, either in wild-type V. harveyi or in the M17 mutant. Since these proteins are not detected in certain V. harveyi mutants that require exogenous aldehyde for luminescence, they are believed to correspond to the P. phosphoreum fatty acid reductase subunits (50K and 58K) directly responsible for the reduction of fatty acids to aldehydes (8). Thus, the apparent lack of acyl turnover noted above suggests that one or both of these proteins are catalytically inactive in V. harveyi extracts under these conditions, a conclusion in agreement with the inability to detect in vitro fatty acid reductase activity in this species.

In contrast, the fatty acid label associated with the 32-kDa acyltransferase and with the heavily labeled bands centered around 18 kDa was decreased by the chase, but only in the wild-type strain (Fig. 1A–C). This observation suggests some correlation between acyl turnover in the 18-kDa bands and the presence of a functional 32-kDa enzyme. This was confirmed by adding the pure 32-kDa acyltransferase to extracts of the M17 (Fig. 1D) or the AFM (Fig. 1E) mutant prior to the labeling experiment: in both cases the observed turnover of the acyl label in the 18-kDa bands was reconstituted.
Although the identities of the acylated 18-kDa protein(s) have not beenestablished, they may represent acyl-ACP thioesters, on the basis of their apparent size on NaDodSO4/PAGE (8). These bands are present in all nonluminous mutants of V. harveyi, as well as in cells harvested prior to the induction of luminescence in mid-exponential growth (8), and thus do not appear to be bioluminescence-specific. Moreover, a band at similar molecular mass is the major protein labeled during de novo fatty acid synthesis, when V. harveyi extracts are supplied with [14C]malonyl-CoA, acetyl-CoA, and NADPH (unpublished data). Under these conditions, label is also associated with the 32-kDa enzyme in wild-type but not in M17 extracts. These results, therefore, provide initial evidence that the V. harveyi 32-kDa acyltransferase, whose in vivo role is related to the supply of fatty acids to the bioluminescence system, can cleave acyl-ACP thioesters. That the 32-kDa acyltransferase-mediated acyl-turnover effect can be reconstituted in AFM as well as in M17 extracts indicates that other bioluminescence components (for example, the 42-kDa and 57-kDa proteins) are not required, although they may modulate the activity of the acyltransferase.

A similar pulse-chase labeling experiment was conducted with extracts of E. coli, in which a band at about 18 kDa represents the only significantly acylated polypeptide (5). Here, a stronger case can be made for the identification of this band as acyl-ACP, since ACP and its acylating enzyme, acyl-ACP synthetase, have been characterized from E. coli (15, 18). As anticipated, the fatty acid label associated with this protein did not turn over in extracts of E. coli alone (Fig. 2A). However, upon addition of either the V. harveyi 32-kDa acyltransferase (Fig. 2B) or the analogous P. phosphoreum 34K enzyme (Fig. 2C), the extent of labeling of the 18-kDa band was greatly decreased, even during the pulse period, while fatty acyl groups appeared transiently with the added acyltransferases. It can be concluded from this observation that both the P. phosphoreum and V. harveyi acyltransferases catalyze the cleavage of E. coli acyl-ACP thioesters, a result not too surprising since the structure of ACP has been shown to be remarkably conserved in a variety of organisms (19).

With the assumption that the V. harveyi acylated 18-kDa bands are in fact acyl-ACP thioesters, the labeled protein was isolated from cell extracts that had been incubated with [3H]tetradecanoic acid and ATP. The purification scheme used, modified from that originally developed for E. coli ACP (15), consisted of (i) the removal of most cellular proteins by precipitation in 50% isopropanol, (ii) the application of the isopropanol supernatant to DEAE-Sepharose at pH 6 and subsequent elution with 0.5 M LiCl, and (iii) gel filtration. The V. harveyi AFM mutant was chosen as the source since the [3H]acyl-containing 18-kDa bands represent the vast majority of labeled protein in this strain (see Fig. 1C) and the acyl group is stable (due to the lack of 32-kDa enzyme). Virtually all of the [3H]acyl-protein purified in the manner expected for acyl-ACP, providing further evidence that it is acyl-ACP or at least a protein of remarkably similar acidic character and stability to organic solvents. It should be noted that only fractional acylation of the protein was achieved under the conditions employed and that most of the acyl-protein preparation obtained is likely free ACP as well as other contaminating proteins. Indeed, as ACP stains poorly with Coomassie blue (20), no clear indication of specific protein bands corresponding to the acyl label was observed (Fig. 3A).

The V. harveyi [3H]acyl-protein preparation exhibited remarkably similar properties to a commercial sample of pure E. coli [3H]palmitoyl-ACP. Both labeled proteins comigrated on NaDodSO4/PAGE at a significantly lower apparent molecular weight than the corresponding free E. coli or V. harveyi ACP (as visualized by labeling with β-[3H]halaline) (Fig. 3). This phenomenon has been reported earlier (21) and is attributable to the increased binding of NaDodSO4 by the acylated protein. The presence of at least two ACP bands in

Fig. 2. Pulse-chase labeling of E. coli extracts with [3H]tetradecanoic acid: effect of added V. harveyi or P. phosphoreum acyltransferase. Cell-free extracts (120 μg of protein) of E. coli alone (A) or with V. harveyi 32-kDa acyltransferase (6 μg) (B) or P. phosphoreum 34K (6 μg) (C) were pulse-labeled with [3H]tetradecanoic acid (lanes P) and chased with unlabeled tetradecanoic acid (lanes Ch). The experiment was performed as described in the legend to Fig. 1 except that the reaction contained 50 mM phosphate (pH 8) and 25 mM MgSO4. Proteins were separated by NaDodSO4/PAGE; a 6-day fluorogram is shown.

Fig. 3. Comparison of V. harveyi and E. coli ACP derivatives. Proteins were separated by NaDodSO4/PAGE; proteins and radioactive activity were visualized by Coomassie blue staining (A) and fluorography (2 days) (B), respectively. Lanes: 1, V. harveyi AFM mutant labeled in vivo with β-[3H]halaline (40 μg of protein); 2, E. coli ACP (10 μg); 3, V. harveyi [3H]acyl-18-kDa-protein (25,000 cpm); 4, E. coli [3H]palmitoyl-ACP (40,000 cpm); 5, protein markers (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.2 kDa).
phosphoreum acyltransferases

1. **mM**

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- hexane-soluble radioactivity (enzyme)
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- in this strain could a thioester bond at acyltransferases of conversion time and table all combinations
- ysis 7) (lanes E.
- 32-kDa [3H]acyl-18-kDa-protein to
- NaOH/MeOH saponification 6.5,
- [3H]palmitoyl-ACP in the presence of this strain could reflect proteolytic degradation or different
- A range of enzyme concentration (1–5 μg) and incubation time (4–10 min) was used to ensure linearity in acyl-protein cleavage. The hexane-soluble radioactivity was corrected for blank values, minus enzyme (≤2000 cpm). The results are presented relative to the rates in 50 mM phosphate (pH 7), for which the values [in cpm/(min.μg of enzyme)] are indicated in parentheses in each case.

**V. harveyi** labeled with β-[3H]alanine (Fig. 3B) suggests that the multiple [3H]fatty acyl-labeled (18-kDa) bands observed in this strain could reflect proteolytic degradation or different

- with both *V. harveyi* [3H]acyl-protein and *E. coli* [3H]acyl-ACP, the acyl group was completely liberated by NaOH/MeOH saponification or treatment with hydroxylamine at pH 6.5, the latter indicating the presence of a thioester bond (22).

Both the *P. phosphoreum* 34K and the *V. harveyi* 32-kDa acyltransferases were capable of catalyzing the complete conversion of either *E. coli* [3H]palmitoyl-ACP or *V. harveyi* [3H]acyl-18-kDa-protein to form a labeled, hexane-extractable product (Table 1). This conversion was dependent on time and enzyme concentration, and TLC analysis indicated that fatty acid is the major product of the reaction in phosphate buffer at pH 7 (Fig. 4). In general, similar responses to variations in the solvent composition were observed for all combinations of enzyme and substrate (Table 1). Hydrolysis of the [3H]acyl-protein bond was stimulated in the presence of high concentrations of phosphate or glycerol phosphate, whereas organic alcohols, such as glycerol and ethylene glycol, promoted the transfer of the fatty acyl moiety to the alcohol acceptor (Fig. 4). Little effect of 2-mercaptoethanol on the reaction was observed. These effects, which are somewhat different than those noted previously with [3H]tetradecanoyl-CoA as a substrate (6, 11), were not examined further, because the dilute substrates of unknown specific activity available for this study precluded the measurement of kinetic parameters.

On the basis of the above physical, chemical, and enzymatic data we conclude that the *V. harveyi* [3H]acyl-18-kDa-protein is acyl-ACP. It should be emphasized that appreciable cleavage of this [3H]acyl-ACP preparation to a hexane-soluble product was only observed in the presence of the 32-kDa (or 34K) acyltransferase; in *E. coli* extracts, or in those of *V. harveyi* mutants lacking this enzyme, the substrate was not hydrolyzed under the conditions used. This is in agreement with an earlier report indicating that acyl-ACP is relatively stable to thioesterase attack in *E. coli* (23).

The demonstration that the *V. harveyi* 32-kDa acyltransferase can cleave acyl-ACP in vitro, combined with earlier evidence that it is required to generate fatty acids for bioluminescence in vivo (8), suggests the following simple model for the function of this enzyme.

**Fatty acid synthesis**

- Acyl-ACP → Fatty acid
- Aldehyde + Fatty acid light

**Phospholipid synthesis**

According to this scheme the 32-kDa acyltransferase (and, by analogy, the *P. phosphoreum* 34K enzyme) works at the level of acyl-ACP to divert fatty acids from lipid synthesis to the bioluminescence pathway. It is tempting to speculate that these enzymes have a specificity for 14-carbon (as opposed to 16- or 18-carbon) acyl-derivatives, since tetradecanoic acid appears to be the preferred substrate for bioluminescence (10) while the longer fatty acids are more prevalent in membrane phospholipids (13). Alteration in the fatty acid composition as the result of the action of specific thioesterases has been reported in a number of other systems (24–27).

If this is the case in *V. harveyi*, the 32-kDa enzyme could have

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**Table 1.** [3H]Acyl-protein cleavage by *V. harveyi* and *P. phosphoreum* acyltransferases

<table>
<thead>
<tr>
<th>Solvent composition</th>
<th><em>V. harveyi</em></th>
<th><em>P. phosphoreum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>50 mM phosphate (pH 7)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 20 mM 2-mercaptoethanol</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>+ 5% isopropanol</td>
<td>2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>+ 30% ethylene glycol</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>+ 30% glycerol</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>1 M phosphate (pH 7)</td>
<td>13</td>
<td>23</td>
</tr>
<tr>
<td>1 M glycerol 3-phosphate (pH 7)</td>
<td>13</td>
<td>18</td>
</tr>
</tbody>
</table>

Acyl-protein cleavage assays were carried out as described in the text using either *V. harveyi* [3H]acyl-18-kDa-protein (54,000 cpm) (columns A) or *E. coli* [3H]palmitoyl-ACP (60,000 cpm) (columns B).

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![Fig. 4. Thin-layer chromatography and fluorography (4 days) of the hexane-soluble products of [3H]acyl-protein cleavage by *V. harveyi* 32-kDa acyltransferase. Reaction mixtures in 50 mM phosphate (pH 7) contained *V. harveyi* [3H]acyl-18-kDa-protein and 1.8 μg of enzyme (A) or *E. coli* [3H]palmitoyl-ACP and 4.5 μg of enzyme (B), with the following additions: 1 M phosphate (no enzyme) (lanes 1), 1 M phosphate (pH 7) (lanes 2), 50 mM phosphate alone (lanes 3), 30% (vol/vol) ethylene glycol (lanes 4), 30% (vol/vol) glycerol (lanes 5), 5% (vol/vol) isopropanol (lanes 6), or 50 mM 2-mercaptoethanol (lanes 7). Incubation time was 10 min except for lanes 2 (2 min).](image-url)
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a significant effect on growth and the development of bioluminescence. Studies with E. coli have indicated that the particular chain-length distribution of acyl-ACP species may play a major role in the determination of membrane fatty acid composition (12, 13).

The results described here do not rule out the possible involvement of other fatty acyl precursors in bioluminescence. Tetradecanoyl-CoA has been shown to be a substrate of both V. harveyi (11) and P. phosphoreum (6) acyltransferases in vitro, although both strains contain other bioluminescence-related enzymes that could directly utilize this substrate for aldehyde synthesis (4, 28). Membrane lipids may also be candidates for acyltransferase-mediated fatty acid supply, since they represent a large storage form of compounds that are more inert in the cell than free fatty acids or acyl-CoA. The stimulation of acyl-CoA (6, 11) and acyl-ACP cleavage by organic solvents and phosphate suggests that the mechanism of the 32-kDa and 34K enzymes may involve the channeling of acyl groups to and from specific acceptors, perhaps in a membrane environment. Whether compounds other than acyl-ACP could play a role in this process remains to be elucidated.

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