An Essential Histidine Residue Required for Fatty Acylation and Acyl Transfer by Myristoyltransferase from Luminescent Bacteria*

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The lux-specific acyltransferases are serine esterases responsible for preferential diversion of myristic acid from fatty acid biosynthesis to the luminescent system. In contrast to other acyltransferases, an acylated enzyme intermediate can readily be detected making it ideal for the study of the mechanism of acyl transfer. Although the transferase readily cleaves acyl carrier protein and acyl-CoA, an alternate more rapid and convenient assay involving the cleavage of p-nitrophenyl acyl esters was developed and applied in these studies. The cleavage of the oxysterols by the transferase was shown to have a similar dependence on fatty acid chain length and organic solvents as the cleavage of thioesters. Using this assay, it could be demonstrated that the Photobacterium phosphoreum transferase was inactivated at pH 6 with diethyl pyrocarbonate at a rate (73 \text{ s}^{-1} \text{ s}^{-1}, 10^\circ \text{C}) even faster than that reported for other enzymes with reactive histidyl residues at their active site. Spectral changes during chemical modification as well as restoration of activity by neutral hydroxyamine showed that the loss of activity was associated with modification of a single histidine residue. Replacement of the four histidine residues, conserved in all lux-specific acyltransferases, by asparagine demonstrated that cleavage of both thioesters and oxysterols by the P. phosphoreum acyltransferase as well as acylation of the enzyme was blocked on mutation of His-244 but not the other three conserved histidines (His-12, -52, and -75). These results suggest that the histidine residue near the carboxyl terminus (His-244) may be part of a catalytic triad essential for cleavage of acyl esters and transfer of the acyl group to the enzyme.

The light-emitting reaction in luminescent bacteria is catalyzed by luciferase, a heterodimer which oxidizes long chain aliphatic aldehydes in an oxygen and reduced flavin mononucleotide-dependent reaction (Meighen and Dunlap, 1993). The aldehyde substrate for this reaction is derived from the fatty acid biosynthesis pathway by a lux-specific fatty acid reductase multienzyme complex made up of acyl-CoA reductase, acyl-protein synthetase, and acyltransferase subunits. Fatty acids are activated in an ATP-dependent reaction and reduced in a NADPH-dependent reaction by the synthetase and reductase subunits, respectively. The transferase is responsible for specifically diverting myristic acid from fatty acid biosynthesis to the luminescent system by preferentially cleaving the myristoyl ester of acyl carrier protein (ACP)\(^1\) (Byers and Meighen, 1985a; Ferri and Meighen, 1991). Esterases with acyl chain-terminating functions similar to that of the lux-specific transferase are frequently found in eukaryotes. These esterases can exist as a domain of the multifunctional fatty acid synthase (Lin and Smith, 1978; de Renobales and Blomquist, 1984; Knudsen and Grunnet, 1982) or as separate enzymes such as the thioesterases in the mammary gland of non-ruminant mammals (Knudsen et al., 1976; Libertini et al., 1976), the uropygial gland of certain waterfowl (de Renobales et al., 1980), the pea aphid (Ryan et al., 1982) and Umbellulatara californica seeds (Pollard et al., 1991).

A common feature of most acyl chain-terminating enzymes is a serine at the active site, reminiscent of the active site of serine proteases, which catalyze triad consisting of a serine, histidine, and aspartate residue (Brenner, 1988). Recent x-ray crystallographic studies of triglyceride lipases have also identified a catalytic triad of serine, histidine, and aspartate/glutamate residues at the active site (Brady et al., 1990; Winkler et al., 1989; Schrag et al., 1991).

The lux-specific acyltransferases are also serine esterases (Ferri and Meighen, 1991). The dependence of the enzyme activity on a basic group with a pK\(_a\) of 6.3 also suggests the requirement for a histidine residue as part of a catalytic triad. Although an essential histidine residue in the rat mammary thioesterase II has recently been found (Witkowski et al., 1991), a catalytic triad similar to that of serine proteases and lipases has not yet been identified in acyl chain-terminating enzymes. Moreover, mutation of the critical histidine in rat mammary thioesterase indicated that this residue is not required for the nucleophilic attack by serine on acyl-CoA (Tai et al., 1993).

In contrast to other thioesterases, the lux-specific transferases can readily be labeled by acylation at the active site, perhaps as the turnover rate of these transferases are relatively low. Moreover, the Vibrio harveyi lux-specific transferase is currently the only thioesterase that has been crystallized (Swenson et al., 1992). In this study, a highly reactive histidine residue essential for acylation and function has been identified providing strong support for the proposal that a catalytic triad is part of the active site of serine thioesterases and in particular the lux-specific transferases.

**EXPERIMENTAL PROCEDURES**

**Materials**—[\(^{3}H\)]Myristic acid (30 Ci/mmol) was from Amerham Corp and purified by thin-layer chromatography. *Escherichia coli* ACP, diethyl pyrocarbonate, p-nitrophenyl acyl esters, and reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Sigma. [\(^{35}S\)]Methionine (600 Ci/mmol), ENHANCE, and Aqueous were from DuPont. Phosphate buffers were made by mixing the appropriate amounts of K\(_2\)HPO\(_4\) and Na\(_2\)HPO\(_4\).

**Strains and Enzymes**—The Photobacterium phosphoreum (NCMB844) acyltransferase was purified as previously described (Rod-

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\(^{1}\) The abbreviations used are: ACP, acyl carrier protein; DEPC, diethyl pyrocarbonate.
BamHI restriction enzymes and inserted into the SmaI and BamHI
site. Using the method described by Laemmli (1970) with 12% poly-
acrylamide resolving gels and 5% stacking gels, gels were stained in
gel electrophoresis sample buffer and incubating at 65°C for 20 min.

**Gel Filtration**—Five ml of [35S]methionine-labeled extract from E. coli
carrying the inactive H244N mutant was mixed with purified active
P. phosphoreum transferase, and the proteins were precipitated
from 50 ml of cells was resuspended in 1

**Gel Electrophoresis**—SDS-polyacrylamide gel electrophoresis was
performed by the method described by_Laemmli (1970) with 12% poly-
acrylamide resolving gels and 5% stacking gels. Gela were stained in
Coomasie Brilliant Blue R, destained, soaked in ENHANCE, dried
under vacuum, and exposed to Kodak X-Omat AR film overnight.

**Protein Assay**—The extinction coefficient of the transferase, calcu-
lated to be 32,000 M⁻¹ cm⁻¹ at 280 nm, gave a protein concentration 1.5
times greater than that determined by the Bio-Rad dye binding assay
(Bradford, 1976), with bovine serum albumin as the standard (Wall
et al., 1986). Consequently, protein concentrations determined by the Bio-
Rad dye binding assay were multiplied by 1.5.

**RESULTS**

**Cleavage of Oxyesters by Myristoyltransferase**—The thioesters,
[3H]myristoyl-CoA and [3H]myristoyl-CoA, have been used as the standard substrates to measure transferase activity. These assays are inconvenient and expensive, requiring a separate reaction mixture for each time point and extraction of the samples with organic solvents (e.g., hexane) to determine the amount of radioactive product by scintillation counting. A continuous spectrophotometric assay was therefore developed using p-nitrophenyl myristate as a substrate. In this assay, 0.05% Triton X-100 was added to maintain the higher concentrations of p-nitrophenyl myristate (up to 100 μM) in solution required for a spectrophotometric assay.

Fig. 1 shows the stimulation by glycerol of the cleavage of
p-nitrophenyl myristate and myristoyl-CoA. Due to the limitation
in solubility of p-nitrophenyl myristate, assays could only be
conducted near the Kᵦᵦ for this substrate (see below), whereas the concentration of myristoyl-CoA was well above its
Kᵦᵦ of 1 μM (Carey et al., 1984; Byers and Meighen, 1985a). In
spite of these differences and the assay conditions, the absolute
rates of cleavage of the two substrates at different glycerol
concentrations are very similar.

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**Lux-specific Myristoyltransferase**

The cleavage of myristoyl-CoA (O) and p-nitrophenyl myristate (•) by
the acyltransferase of P. phosphoreum was measured with increasing
glycerol as described under "Experimental Procedures." The cleavage of
p-nitrophenyl myristate by ethylene glycol (■) is also shown. The
organic solvent concentration is expressed in volume percent.

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**FIG. 1. Stimulation of transferase activity by organic solvents.**

The cleavage of myristoyl-CoA (O) and p-nitrophenyl myristate (•) by
the acyltransferase of P. phosphoreum was measured with increasing
glycerol as described under "Experimental Procedures." The cleavage of
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[Image 1x0 to 591x812]
Fig. 2. Lineweaver-Burk plot of p-nitrophenyl myristate cleavage by transferase. Assays were performed under the standard conditions with various substrate concentrations in the presence of 15 and 30% glycerol (G) and ethylene glycol (EG).

Fig. 3. Acyl chain length specificity of p-nitrophenyl acyl ester cleavage. The cleavage rates of p-nitrophenyl acyl esters by the P. phosphoreum (○) and V. harveyi (□) acyltransferases are expressed as a percentage of the rate of cleavage of p-nitrophenyl myristate.

20 mM neutral hydroxylamine completely restored transferase activity (data not shown), indicating that inactivation is due to modification of histidine and not primary amines.

Site-directed Mutagenesis of Histidyl Residues in Myristoyltransferase—To identify the histidine residue critical for the transferase activity, site-directed mutagenesis was performed on the luxD gene coding for the transferase from P. phosphoreum. Comparison of the amino acid sequences of the lux-specific transferases from different bacterial species (Fig. 6) shows that only 4 histidine residues are conserved in the transferases; 3 in close proximity to the amino terminus and 1 near the carboxyl terminus. Site-directed mutagenesis was used to convert each of the 4 conserved histidyl residues into asparagine residues as they are related in structure, polarity, and size and most often replace the nonconserved histidines in the luxtransferases (Fig. 6).

The wild type and mutated luxD genes were then inserted into a plasmid next to a promoter specific for T7 RNA polymerase and expressed in E. coli containing the T7 RNA polymerase (Tabor and Richardson, 1985). The activities of the mutant luxD gene products can be measured directly since E. coli has little myristoyl-ACP cleavage activity on extraction (Byers and Meighen, 1985b). Myristoyl-ACP cleavage activity can be detected in all extracts except cells containing luxD with a H244N mutation (Fig. 7). For H12N and H52N, activities are between 50 and 70% of wild type levels, while the activity has decreased to about 20% of the wild type level for the H75N mutant.

Elucidation of the rate of cleavage of the oxyester, p-nitrophenyl myristate, by the mutated luxD gene products could also be accomplished in E. coli extracts. After correction for activity by E. coli extracts alone, only the H244N mutant was found to be inactive with p-nitrophenyl myristate. Although the three histidine mutants near the amino-terminal region of the transferase have different relative activities with p-nitrophenyl myristate compared to myristoyl-ACP, these differences may reflect to some degree minor modulations in the interaction with the different substrates. Incubation of the H12N, H52N, and H75N cell extracts with 0.5 mM DEPC for 5 min blocked transferase activity consistent with modification of His-244 causing inactivation.

As addition of rifampicin to the cells blocks E. coli RNA polymerase but not T7 RNA polymerase, [35S]methionine labeling of the expressed proteins can be used as an indicator of the relative level of synthesis of the LuxD mutants. Each recombi-
nont plasmid containing a luxD gene produced transferase at comparable rates (Fig. 8A). Although some differences in transferase levels can be found in extracts from one experiment to another, the level of expression of the H244N mutant was higher than the active transferases in this experiment, clearly demonstrating that it is inactive.

Treatment of the cell extracts of each of the mutants with [3H]myristoyl-CoA shows that the serine active site can be acylated for all His to Asn mutants of luxD except for the H244N mutant (Fig. 8B). This result provides strong evidence that the histidine residue near the carboxyl terminus is essential for formation of the acylated intermediate and indicates that the histidine is involved in a charge-relay system increasing the nucleophilicity of the active site serine residue. In contrast, transferases in which any one of the three histidine residues near the amino terminus have been substituted can readily be acylated.

Although the P. phosphoreum H244N mutant was inactive, it was possible to determine its Stokes radius by gel filtration as the LuxD protein could be exclusively labeled with [35S]methionine. Gel filtration of the extract of E. coli containing the [35S]labeled LuxD mutant (H244N) and mixed with purified P. phosphoreum transferase showed that the radioactivity of the H244N mutant, and the activity for the wild type transferase eluted at the same position, demonstrating that the H244N mutant has the same Stokes radius ($R_s = 28 \text{ Å}$) as the native transferase (Wall and Meighen, 1986). Although the low expression of P. phosphoreum in E. coli prevented purification of the H244N mutant, recently we have obtained a much higher level of expression of the V. harveyi luxD in E. coli. Mutation of the corresponding histidine (H241, see Fig. 6) to asparagine also resulted in an inactive protein. However, due to its higher expression, the V. harveyi H241N mutant could be purified to homogeneity and its properties investigated. Both the fluorescence emission spectra for the tryptophan residues and their quenching by acrylamide were identical for the native V. har-
voyeri transferase and the H241N mutant, indicating the two proteins had very similar conformations with the tryptophans being in identical environments.²

DISCUSSION

The spectrophotometric assay reported in this study provides several advantages over the previous assay with myristoyl-ACP and myristoyl-CoA. The activity can be followed without having to stop the reaction at each time point, and the product does not have to be separated from the substrate.

This assay was therefore chosen to follow the transferase activity after modification by DEPC and site-directed mutagenesis. DEPC is specific for modification of histidine residues in the pH range 5.5-7.5 (Muhlrad et al., 1967; Pradel and Kassab, 1968; Miles, 1977). The increase in A₂₄₀, the absence of change in and restoration of activity with neutral hydroxylamine showed that inactivation is due to the modification of the equivalent of 1 histidyl residue. The rates of inactivation of the P. phosphoreum and V. harveyi transferases by DEPC are very large and currently exceed values reported for other enzymes including those with histidine residues at their active sites. Only a few rate constants of inactivation by DEPC approach that of the transferase such as the value of 49 M⁻¹ s⁻¹ for the rat mammary gland thioesterase II (Witkowski et al., 1991). Because the lux-specific transferases are structurally and functionally related, an essential histidine residue involved in a charge relay system would be expected to be conserved. Four histidine residues, conserved in the lux-specific transferases, were selected for site-directed mutagenesis. To minimize the possibility of causing inactivation resulting from a conformational change a histidine to asparagine substitution was chosen for the mutagenesis experiments. Asparagine appears to have been a suitable choice for the substitutions since three of the H244N mutants retained activity, whereas only H244N was inactive. Moreover, studies of the inactive P. phosphoreum H244N mutant and the equivalent V. harveyi H241N mutant showed that they had the same structural properties as the respective native transferases demonstrating that a major conformational change had not occurred on mutation.

By comparing the transferase amino acid sequence with that of eukaryotic acyl chain-terminating enzymes, similarities were observed around His-244 (Fig. 9), particularly with the duck thioesterase II. Moreover, the conserved His-244 is located near the carboxyl terminal while a critical serine residue is located in the amino-terminal region similar to the location of the proposed essential histidine and serine residues in esterases and their location in lipases with catalytic triads (Dwer-

² S. R. Ferri and E. A. Meighen, unpublished data.

FIG. 6. Comparison of the amino acid sequences of the lux-specific acyltransferases. Line 1, P. phosphoreum (Ferri and Meighen, 1991); line 2, P. leiognathi (Lee et al., 1991); line 3, V. fischeri (Baldwin et al., 1989); line 4, V. harveyi (Miyamoto et al., 1988); and line 5, X. luminescens (Meighen and Szittner, 1992). Dashes (-) refer to amino acids identical to those of P. phosphoreum, and the conserved histidine residues selected for site-directed mutagenesis are boxed.

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\begin{align*}
&\text{PSKSLA.} \\
&\text{ENEIfNRLADRVLASV*} \\
&\text{ENEL-ELA' } \\
&\text{E-RTPEM.} \\
&\end{align*}
\]
H75N, and H244N LwD mutants can be estimated to be 80, 130, 130, respectively, of that for the wild type transferase except for the H244N mutant. The results are presented as a percentage of myristate were performed at pH 8, as described under "Experimental Procedures." Samples (20 µg) were separated by SDS-polyacrylamide gel electrophoresis, fixed, and treated with ENHANCE. The relative amounts of the labeled proteins are given in the legend to Fig. 7. Shown are fluorograms resulting from overnight exposures. The position of the transferase (T) is indicated with the intensities of the bands varying between 60 and 120% of the wild type transferase except for the H244N LuxD mutant.

This histidine residue (Tai et al., 1993), suggesting that it is not required for the nucleophilic attack by the serine residue. In contrast, His-244 in the lux-specific myristoyltransferase is required for both acylation of the serine residue as well as activity, indicative of a role in enhancing the serine nucleophilicity and suggesting that a catalytic triad similar to that found in serine proteases and lipases is present.

REFERENCES