Articles

Conversion of Serine-114 to Cysteine-114 and the Role of the Active Site Nucleophile in Acyl Transfer by Myristoyl-ACP Thioesterase from *Vibrio harveyi*†

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ABSTRACT: The lux-specific myristoyl-ACP thioesterase (LuxD) is responsible for diverting myristic acid into the luminescent system and can function as an esterase and transferase as well as cleave myristoyl-CoA and other thioesters. The recently elucidated crystal structure of the enzyme shows that it belongs to the α/β hydrolase family and that it contains a typical catalytic triad composed of Asp^{211}, His^{241}, and Ser^{114}. What is unusual is that the nucleophilic S^{114} is not contained within the esterase consensus motif GXSXG although the stereochemistry of the turn involving S^{114} is almost identical to the nucleophilic elbow found in α/β hydrolases. In contrast to mammalian thioesterases, deacylation of LuxD was the rate-limiting step, with the level of acylated enzyme formed on reaction with myristoyl-CoA and the pre-steady-state burst of *p*-nitrophenol on cleavage of *p*-nitrophenyl myristate both being 0.7 mol/mol. Cold chase experiments showed that the deacylation rate of LuxD corresponded closely to the turnover rate of the enzyme with ester or thioester substrates. Replacement of S^{114} by a cysteine residue generated a mutant (S114C) that was acylated with the same pH dependence as LuxD but had greatly diminished capacity to transfer acyl groups to water or glycerol. The acyl group could be removed from the S114C mutant by neutral hydroxylamine, showing that a cysteine residue had been acylated. Mutation of H^{241} creating the double mutant, S114C·H241N, decreased acylation of the cysteine residue. These results provide direct kinetic and chemical evidence that S^{114} is the site of acylation linked to H^{241} in the charge relay system and have led to the recognition of a more general consensus motif flanking the nucleophilic serine in thioesterases.

Thioesterases are hydrolytic enzymes involved in a multitude of biochemical processes including bioluminescence (Ferri & Meighen, 1991), regulation of the intracellular levels of fatty acids and acyl-CoA[1] derivatives (Sanjanwala et al., 1987), dehalogenation of chlorinated aromatics (Scholten et al., 1991), cleavage of acyl groups from palmitoylated Ras proteins (Camp & Hoffman, 1993), and biosynthesis of polyketides (Donadio et al., 1991; Shen & Hutchinson, 1993; McDaniel et al., 1993; Kratzschmar et al., 1989; Raibaud et al., 1991). Perhaps one of the most important roles for thioesterases involves chain termination and release of fatty acids from the phosphopantetheine group that carries the acyl chain during fatty acid biosynthesis (Libertini & Smith, 1978; Lin & Smith, 1978; De Renobales et al., 1980; Voelker et al., 1992).

Animal thioesterases involved in release of fatty acids have been studied in the greatest detail and may be structurally related with a conserved serine nucleophile located in a GXSXG motif about 100 amino acids from the N-terminus (Poulose et al., 1985; Randhawa & Smith, 1987; Safford et al., 1987; Yang et al., 1988). The nucleophilic serine is believed to be part of a charge relay system along with a conserved histidyl residue in the carboxyl-terminal region of the thioesterase (Pazirandeh et al., 1991; Witkowski et al., 1991, 1992); only low levels of the acylated serine intermediate can be detected indicating that acylation is the rate-limiting step in the enzyme mechanism (Pazirandeh et al., 1991; Witkowski et al., 1994). Replacement of the active site serine by cysteine in rat mammary gland thioesterase II followed by cross-linking of the cystyl and histidyl residues showed that these residues are in close proximity (Witkowski et al., 1992). The thiol mutant of rat mammary gland thioesterase II as well as that of rat thioesterase I have relatively high activities, indicating that the position and orientation of the active site histidine and the nucleophile involved in the enzyme mechanism are relatively undisturbed on conversion of the active site serine to cysteine (Witkowski et al., 1992, 1994; Pazirandeh et al., 1991; Tai et al., 1993).

In luminescent bacteria, a lux-specific thioesterase (LuxD) that cleaves myristoyl-CoA and myristoyl-ACP is present and is responsible for diversion of tetradecanoic acid into the luminescent system (Byers & Meighen, 1985a,b; Ferri & Meighen, 1991). This enzyme interacts with a multienzyme fatty acid reductase complex that reduces the fatty acid

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[1] Abbreviations: CoA, coenzyme A; ACP, acyl-carrier protein; *p*-NP, *p*-nitrophenol; β-ME, β-mercaptoethanol; Vh, *Vibrio harveyi*; Xl, *Xenorhabdus luminescens*; Vf, *Vibrio fischeri*; Pp, *Photobacterium phosphoreum*; Pl, *Photobacterium leoignathi*.

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into the tetradecanal substrate required for the light-emitting reaction. The thioesterase can also exhibit esterase and transferase activities under some circumstances. Both a histidine and a serine residue have been implicated in the catalytic mechanism (Ferri & Meighen, 1994), and an acylated serine intermediate can be readily detected.

At present, LuxD from *Vibrio harveyi* is the only thioesterase whose crystal structure has been elucidated (Lawson et al., 1994). The molecule of LuxD, as described at 2.2 Å resolution, is a typical α/β hydrolase containing a "nucleophilic elbow" which is a tertiary structure motif consisting of a β strand and an antiparallel α helix connected by a sharp γ turn. The nucleophile is in the center of this turn and exhibits a strained secondary conformation of the ϵ-type (Brady et al., 1990; Winkler et al., 1990; Schrag et al., 1991; Derewenda & Derewenda, 1991; Ollis et al., 1992; Noble et al., 1993). The "nucleophilic" serine, S^{114}, is in close proximity to a histidine residue (H^{241}) previously identified as essential for catalytic activity (Ferri & Meighen, 1994). Surprisingly, the nucleophilic serine did not correspond to a previously identified serine (S^{77}), which was originally implicated in the catalytic mechanism on the basis of local sequence similarities to the so-called esterase consensus sequence GXSXG and mutational studies.

In the present experiments, the formation and turnover of the acylated intermediate of the *V. harveyi* thioesterase (LuxD) and its S114C mutant were investigated. In contrast to mammalian thioesterases, deacylation was shown to be the rate-limiting step for the native enzyme, and replacement of serine by cysteine greatly diminished the turnover of the enzyme. These studies have also demonstrated that S^{114} is indeed the site of acylation of *V. harveyi* LuxD and have led to the proposal of a more general sequence for the consensus motif flanking the nucleophilic serine in thioesterases.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Myristic acid (14 Ci/mmol) was from Amersham Corp. and was purified by thin-layer chromatography. [^{3}H]Myristoyl-CoA (14 Ci/mmol) was prepared from the radioactive fatty acid as previously described (Rodriguez et al., 1983). Acrylamide, *N*,*N*-methylenebis-(acrylamide), β-mercaptoethanol, SDS−PAGE molecular mass standards, and *p*-nitrophenyl myristate were all obtained from Sigma Chemical Co. Stock solutions of *p*-nitrophenyl myristate were prepared in 2-propanol. En^{3}Hance was from Dupont. Hyamine hydroxide and CytoScint were from ICN. Phosphate buffer was made by mixing the appropriate amounts of K_2HPO_4 and NaH_2PO_4.

Site-Directed Mutagenesis. A 1.6 kbp *Sac*I−*Bam*HI restriction fragment containing the entire *V. harveyi* LuxD gene encoding the thioesterase was inserted into the *Sac*I and *Bam*HI restriction sites of the M13mp19 sequencing vector (Messing, 1983). Site-directed mutagenesis was performed according to the method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) using the M13 *In Vitro* Mutagenesis Kit (Bio-Rad). Oligonucleotide primers to convert S^{114} to C^{114} and H^{241} to N^{241} were obtained from the Sheldon Biotechnology Center, McGill University.

Gene Expression. The bacteriophage T7 promoter−RNA polymerase system (Tabor & Richardson, 1985) was used to express wild-type and mutant *V. harveyi* LuxD in *Escherichia coli* K38. The LuxD gene was excised from the M13 sequencing vector by restriction with *Sac*I and *Bam*HI and inserted into the same sites in the pT7 plasmid. *E. coli* K38 cotransformed with the recombinant pT7 plasmid and the pGP1-2 plasmid were grown in enriched media (2% tryptone/1% yeast extract/0.5% NaCl/0.2% glycerol/50 mM KPO_4, pH 7.2) containing 100 μg/mL ampicillin and 40 μg/mL kanamycin at 30 °C until $OD_{660} = 1.0-1.2$. The temperature was increased to 42 °C for 15 min to induce the transcription of the T7 RNA polymerase on pGP1-2. Rifampicin (200 μg/mL) was then added to inactivate *E. coli* RNA polymerase and allow specific transcription of LuxD by the T7 polymerase followed by incubation of the cells for 2 h at 30 °C. Cells were harvested by centrifugation and pellets were stored at −20 °C. In some cases, the T7-expressed proteins were specifically labeled with [^{35}S]-methionine (Miyamoto et al., 1988).

Enzyme Activity. The esterase and thioesterase activities of the wild-type and mutant LuxD enzymes were determined from the rate of cleavage of *p*-nitrophenyl myristate and myristoyl-CoA, respectively. Thioesterase activity was measured by the conversion of 10 μM [^{3}H]myristoyl-CoA into hexane-soluble [^{3}H]myristic acid as described previously (Rodriguez et al., 1983) in 50 mM phosphate, pH 7.5, 0.1 mM β-mercaptoethanol, and 0.2% glycerol. Esterase activity was measured at pH 8.0 in 50 mM phosphate containing 0.05% Triton X-100, 0.7 mM β-mercaptoethanol, 1.4% glycerol, and 100 μM *p*-nitrophenyl myristate by following the change in absorbance at 405 nm using an extinction coefficient of 16 800 M^{-1} cm^{-1} for *p*-nitrophenol at pH 8.0. All assays were conducted at 23 °C.

Protein Acylation and Deacylation. To detect the acylated enzyme intermediates, the purified wild-type LuxD as well as its mutants was incubated in 50 mM phosphate, pH 7.5, 0.1 mM β-mercaptoethanol, and 0.2% glycerol with [^{3}H]-myristoyl-CoA (14 Ci/mmol) at room temperature. The acylation reaction was stopped by mixing with an equal volume of the SDS sample buffer (24 mM Tris-HCl/10% glycerol/0.8% SDS/10 mM β-mercaptoethanol/0.04% bromophenol blue). Deacylation was conducted under the same conditions as acylation except the acylated protein was diluted into cold 20 μM myristoyl-CoA before the reaction was stopped at different times.

Protein Assay. The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 32 000 M^{-1} cm^{-1} or the Bio-Rad dye binding assay (Bradford, 1976) with bovine serum albumin as the standard. The Bio-Rad protein concentration was multiplied by 1.5 to give the correct value (Wall et al., 1986).

Gel Electrophoresis and Autoradiography. SDS−PAGE was performed by the method described by Laemmli (1970) with 12% polyacrylamide resolving gels and 5% stacking gels. Gels were stained in Coomassie brilliant blue R-250, destained, soaked in En^{3}Hance, dried under vacuum, and exposed to Kodak X-OMAT film overnight to 3 days at −80 °C. The dried protein bands were cut from the gels and dissolved in 90% hyamine hydroxide at 37 °C overnight, CytoScint was added, and the radioactivity was counted. The recovery of protein from the gel was determined to be 87% by electrophoresis of known amounts of labeled acylated transferase prepared by SDS electrophoresis and electroelution from the gel.

Enzyme Purification. The recombinant wild-type and mutant thioesterases were purified as previously described (Byers & Meighen, 1985; Swenson et al., 1992) except inactive mutants had to be monitored either by labeling specifically with [^{35}S]methionine or by SDS−PAGE before and/or after acylation with [^{3}H]myristoyl-CoA. The LuxD proteins gave a single band of 34 kDa with a purity of greater than 95% on SDS−PAGE after purification. The protein was stored in 20% glycerol, 10 mM β-mercaptoethanol, and 0.2 M NaCl.

RESULTS

Identity of the Active Site Serine Nucleophile. The ability to readily measure the levels of an acylated enzyme intermediate for lux-specific thioesterase provides an excellent opportunity to investigate the acylation and deacylation of the serine nucleophile during the cleavage of fatty acyl esters. Acylation of *V. harveyi* LuxD with [^{3}H]myristoyl-CoA showed that maximum acylation is reached at the first time point (20 s); the level of acylated enzyme then decreased after 1−2 min due to the consumption of the acyl-CoA substrate, which was present initially in only a 4-fold molar excess over enzyme (data not shown).

Previous studies focused on S^{77}, initially implicated in the catalytic mechanism because of its location within a "consensus" sequence, GXSXG, found to contain the nucleophile in various lipases and esterases (Brenner, 1988). It was found that site-specific mutations of S^{77} indeed reduce the activity of LuxD to minimal levels (Ferri & Meighen,1991). However, the crystal structure of the enzyme clearly showed that the active site, located as in all α/β hydrolases on the carboxyl side of the central β-sheet and containing a typical chymotrypsin-like triad, contains S^{114}—not S^{77}. Furthermore, in spite of the presence of a typical nucleophilic elbow, the γ turn carrying the nucleophile does not conform to the GXSXG paradigm but shows a sequence of AXSXS. This anomaly was rationalized in terms of a slight "opening" of the elbow allowing the accommodation of small side chains between the β strand and the α helix. While the crystallographic evidence pointing at S^{114} was very strong—as there never has been a case of a fortuitous triad found in any protein—chemical and kinetic evidence supporting the role of this serine nucleophile in the charge relay system and the acylation/deacylation process is still required.

The effects of mutation of S^{114} to cysteine (S114C) provide direct chemical evidence that S^{114} is the site of acylation. The S114C mutant can be acylated as shown in Figure 1. However, on treatment of the denatured S114C mutant with 1 M neutral NH_2OH, the acyl group is readily removed as shown for cleavage of the acyl thioester linkage from the control enzyme, acyl-CoA reductase (r, Figure 1). In contrast, the acyl group covalently linked to serine in the wild-type enzyme is stable in the presence of NH_2OH. The cleavage of the acyl group by neutral NH_2OH provides direct evidence for an acyl thioester bond in the S114C mutant and provides strong evidence that residue 114 is the site of acylation.

Acylation. To determine the optimum conditions for acylation, the thioesterase was reacted with different concentrations and molar excesses of [^{3}H]myristoyl-CoA. Maximum acylation occurred between 0.9 and 10 μM myristoyl-CoA, provided that acyl-CoA was in a molar excess of at least two times that of the thioesterase (data not shown). A significant decrease in the level of acylation occurs at higher myristoyl-CoA concentrations presumably due to the formation of micelles on exceeding the solubility limit of the substrate (Jaeger et al., 1994).

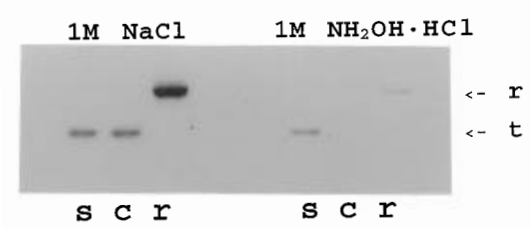

FIGURE 1: Effect of hydroxylamine on deacylation of the wild type and S114C mutant of *V. harveyi* LuxD. The purified enzymes (0.46 μM), *V. harveyi* LuxD, the S114C mutant of LuxD, and *P. phosphoreum* LuxC were reacted with 1.7 μM [^{3}H]myristoyl-CoA in 50 mM phosphate, pH 7.5, for 30 s, 5 min, and 1 min, respectively, before SDS−PAGE. After electrophoresis, the gels were soaked overnight in 50 mM phosphate, pH 8.0, containing either 1 M NaCl or 1 M NH_2OH·HCl before autoradiography. The positions of migration of the thioesterase (LuxD) and acyl-CoA reductase (LuxC) on the SDS gel are indicated by t and r, respectively. Samples: S, wild-type LuxD; C, S114C mutant; r, *P. phosphoreum* LuxC.

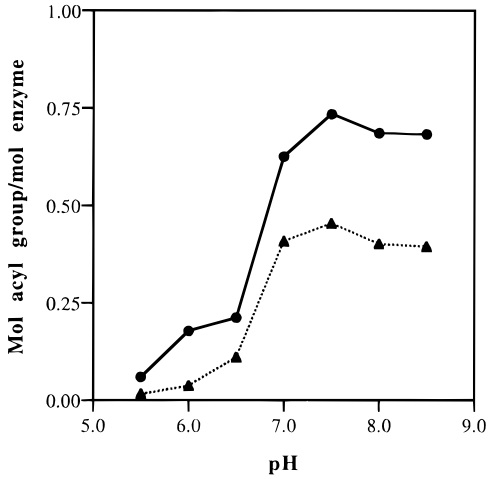

FIGURE 2: Dependence of the acylation of LuxD and its S114C mutant on pH. LuxD and the S114C mutant of LuxD, each at a concentration of 0.46 μM, were reacted with 1.7 μM [^{3}H]myristoyl-CoA for 30 s and 5 min, respectively, in 50 mM phosphate at different pHs. After SDS gel electrophoresis, the protein bands were excised and dissolved in hyamine hydroxide, the radioactivity was counted as described in Experimental Procedures, and the moles of acyl groups per mole of LuxD was calculated. Symbols: ●, wild type; ▲, S114C.

The dependence of acylation of the purified wild-type and S114C mutant thioesterases on pH are very similar, with maximum acylation of both proteins being observed at pH 7 and higher (Figure 2). A stoichiometry of 0.7 mol of myristic acid/mol of protein was found for the native enzyme upon acylation while the level of acylation of the S114C mutant reached only 0.45 mol/mol. However, the time dependence of acylation of these two enzymes is strikingly different (Figure 3). In contrast to the wild-type enzyme from which the acyl group is removed within a few minutes, the acylation of the S114C mutant increases slowly after an initial rapid phase and then remains at a relatively constant level for at least 1 h. This result indicates that the rate of deacylation of the mutant must be very low, as the level of acylation of the wild-type thioesterase decreases after only

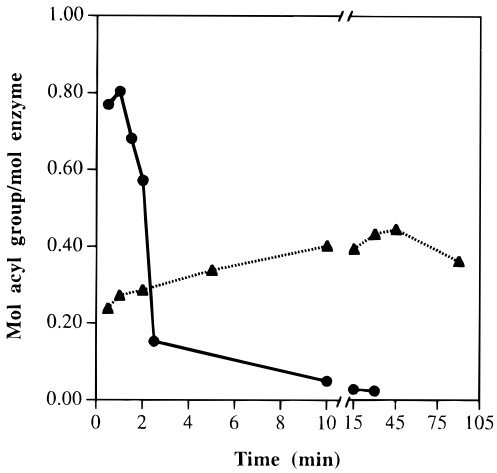

FIGURE 3: Dependence of acylation of LuxD and its S114C mutant on time. The enzymes were incubated with 1.7 μM [^{3}H]myristoyl-CoA in 50 mM phosphate, pH 7.5, for different times before SDS gel electrophoresis and analysis of the level of acylation as described in Figure 2. Symbols: ●, wild type; ▲, S114C.

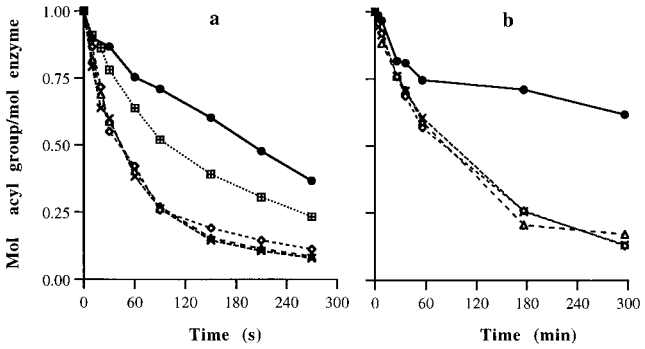

FIGURE 4: Deacylation of LuxD and its S114C mutant at different pHs in cold chase experiments. The (a) wild-type LuxD (1.6 μM) and the (b) S114C mutant (1.6 μM) were incubated with 6.1 μM [^{3}H]myristoyl-CoA at pH 7.5 for 30 s and 5 min, respectively. The acylation mixtures were then diluted with 3.5 parts of 20 μM cold myristoyl-CoA in the appropriate phosphate buffer (50 mM) so that the final solution was at the correct pH. The final buffer contained 0.1 mM β-mercaptoethanol and 0.2% glycerol for the wild-type LuxD and 0.1% glycerol and 0.05 mM β-mercaptoethanol for the S114C mutant. Samples were taken at different times for SDS gel electrophoresis and analysis of the level of acylation as described in Figure 2. Symbols: ●, pH 6.0; cross-hatched box, pH 6.5; ◇, pH 7.0; ×, pH 7.5; △, pH 8.0.

a few minutes of incubation due to enzymic cleavage of myristoyl-CoA.

Deacylation. The high incorporation of acyl groups into LuxD indicates that deacylation is the rate-limiting step in the enzyme mechanism. Analysis of the deacylation rate of the wild-type enzyme at different pHs shows that deacylation occurs with a rate constant of 0.9 min^{-1} at pHs above 7 and decreases by about 4-fold at pH 6 (Figure 4a). In these experiments, the enzyme was acylated at pH 7.5 and then diluted with cold myristoyl-CoA at the appropriate pH. The rate of deacylation of the S114C mutant was much slower (Figure 4b) with a rate constant of $<0.001-0.002$ min^{-1} ($t_{1/2}$ > 5 h) at pH 6.

As glycerol and other compounds with hydroxyl or thiol acceptors can stimulate the rate of cleavage by LuxD of myristoyl-CoA (Carey et al., 1984; Byers & Meighen, 1985a) and *p*-nitrophenyl myristate (Ferri & Meighen, 1994), a comparison of the rate of deacylation of the wild-type and mutant enzymes with different concentrations of glycerol was

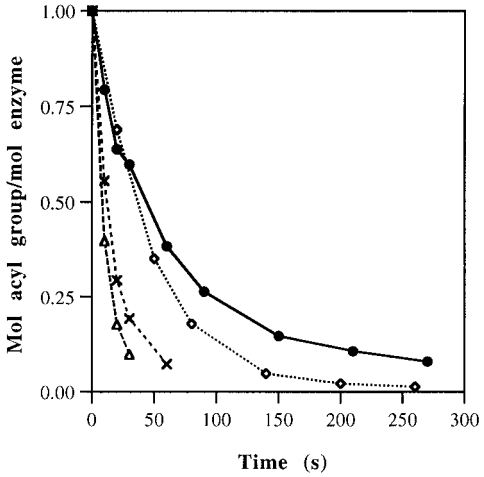

FIGURE 5: Effect of glycerol on deacylation of LuxD. Acylation was performed as described in Figure 4 for the wild-type LuxD. The acylation mixture was diluted into four parts of 20 μM cold myristoyl-CoA and 50 mM phosphate, pH 7.5, containing the indicated amount of glycerol, and the level of acylation was analyzed with time as described in Figure 2. Symbols: ●, without glycerol; ◇, 2.5% glycerol; ×, 5% glycerol; △, 10% glycerol.

Table 1: Kinetic Parameters of LuxD and Its S114C Mutant

	deacylation rate constant (min^{-1})	activity (k_{cat}) (min^{-1})	
		p-NP myristate	myristoyl-CoA
wild type	0.90	0.72[a]	0.7
+20 mM β-ME	1.9	1.4[a]	
+10% glycerol	5.0	5.6	
S114C	0.021	~0.02	0.02
+20 mM β-ME	0.12	0.08	
+10% glycerol	0.022	~0.02	

[a] The rate constants were corrected for the additional glycerol (~1% v/v) introduced by the high levels of enzyme in the assay with *p*-nitrophenyl (*p*-NP) myristate as substrate. Assay procedures are described in Experimental Procedures.

conducted. Figure 5 shows that the rate constant for deacylation of LuxD at pH 7.5 increases from 0.9 to 5 min^{-1} on increasing the glycerol concentration in the cold chase experiment to 10% (v/v). In sharp contrast, the rate of deacylation of the S114C mutant was not affected by glycerol even at the highest concentration tested.

Table 1 compares the first-order rate constants for deacylation of the wild-type and S114C mutant thioesterases to the rate constants for enzyme turnover (k_{cat}) measured with saturating concentrations of myristoyl-CoA and *p*-nitrophenyl myristate. The rate constants for turnover of the wild-type enzyme with *p*-nitrophenyl myristate or myristoyl-CoA as substrates (0.7 min^{-1}) are very close to the rate constant for deacylation (0.9 min^{-1}) even though slightly different conditions did prevail in the assays. A pre-steady-state burst of 0.7 mol of *p*-nitrophenol/mol of enzyme, in agreement with the level of acylation of LuxD by myristoyl-CoA, was observed in the assays with *p*-nitrophenyl myristate followed by a linear increase (correlation coefficient: $r^2 = 1.000$) in the amount of *p*-nitrophenol with time. Both glycerol and β-mercaptoethanol stimulated the deacylation rate of the wild-type LuxD and its turnover with *p*-nitrophenyl myristate as the substrate to the same degree (Table 1). These results provide strong proof that deacylation of LuxD is the rate-limiting step for the turnover of the enzyme with thioester and ester substrates.

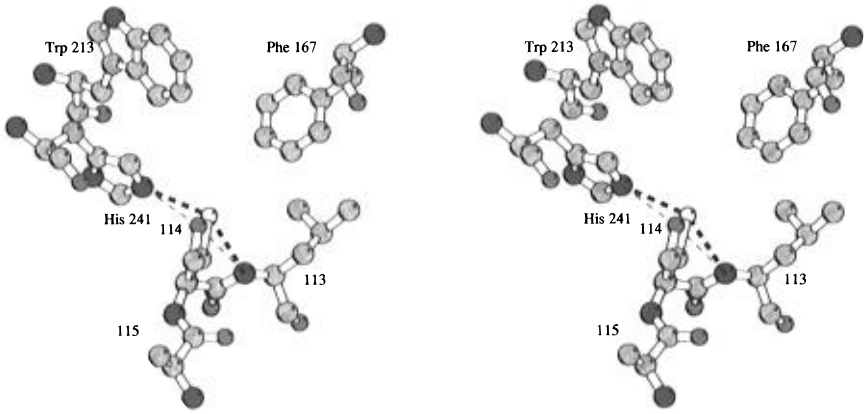

FIGURE 6: Environment of the active site of the wild type and the S114C mutant of *V. harveyi* thioesterase. Atomic coordinates for the wild type were taken from the X-ray study of Lawson et al. (1994). The putative location of the active site C^{114} of the S114C mutant has been calculated so as to minimize the nonbonded contacts. Atoms: Gray, carbon; red, oxygen; blue, nitrogen; yellow, sulfur. The Oγ of S^{114} has two close contacts shown in thin broken lines, a hydrogen bond to H^{241} Nϵ of 2.72 Å, and a predominantly van der Waals interaction with its own backbone amide nitrogen of 3.11 Å; respective distances in the S114C mutant model (thick lines) are 2.88 and 2.96 Å. Given the significantly larger radius of sulfur, the latter distances indicate a significant potential strain in the structure that may be relieved by conformational changes, the nature of which remains to be determined.

In contrast to the wild-type LuxD, the rates of cleavage of *p*-nitrophenyl myristate and myristoyl-CoA are very low for the S114C mutant and close to background levels. Glycerol had no effect on the deacylation of the S114C mutant. However, β-mercaptoethanol could still stimulate the deacylation of the S114C mutant, and a low level ofactivity could be measured with *p*-nitrophenyl myristate as substrate in the presence of this thiol acceptor.

S114C·H241N Double Mutant. The differences in the acylation and deacylation of the S114C mutant compared to that for the wild-type enzyme presumably reflect the larger radius of the sulfur atom, which in turn could distort its geometry with respect to other catalytic groups and in particular H^{241}. The model in Figure 6 shows that the distances between C^{114} and H^{241} and its own amide nitrogen are relatively close, taking into account the larger radius of the sulfur atom. Consequently, a double mutant, S114C·H241N, was constructed and expressed, and the mutant was purified to homogeneity, to determine if the active site histidine could still assist C^{114} in the catalytic mechanism. Comparison of levels of acylation of wild type, S114C, H241N, and S114C·H241N gave 0.80, 0.47, 0, and 0.06 mol of acyl group/mol of enzyme, respectively. As mutation of H^{241} significantly decreases the level of acylation of C^{114} in the double mutant S114C·H241N (0.06 mol/mol), H^{241} must be required for efficient acylation of the cysteine nucleophile. The ability to acylate C^{114} to a low level in the S114C·H241N double mutant and not S^{114} in the H241N mutant would reflect the higher nucleophilicity of the cysteine residue compared to the serine residue.

DISCUSSION

The mechanism of the lux-specific myristoyl-ACP thioesterase is similar to that proposed for mammalian thioesterases utilizing a Ser-His-Asp (or Glu) catalytic triad as part of the acylation and deacylation steps (Pazirandeh et al., 1991; Tai et al., 1993; Witkowski et al., 1991, 1994). However, certain properties of LuxD are quite different from those for the mammalian thioesterases. First, the enzyme is specific for 14-carbon acyl esters and thioesters with sulfhydryl and hydroxyl groups as well as water being capable of acting as acceptors of the acyl group (Ferri & Meighen, 1994). This specificity is consistent with tetradecanal being the natural substrate for the luminescence reaction (Meighen et al., 1982; Shimomura et al., 1974; Ulitzur & Hastings, 1978). Second, the rate of turnover is much lower than that of the mammalian thioesterases, with deacylation rather than acylation being the rate-limiting step. The slow turnover could well reflect the evolution of a system to supply a limited but sufficient level of free fatty acids for conversion into fatty aldehyde. Consequently, the primary function of the LuxD may be to collect myristoyl groups and act as an acyl carrier and storage form for release of the fatty acid under the appropriate physiological conditions.

The conversion of the active site serine of LuxD into cysteine (S114C) decreased the turnover of the enzyme with *p*-nitrophenyl myristate and myristoyl-CoA to close to background levels. The low activity of the S114C thioesterase mutant sharply contrasts with the high activity of similar mutants for mammalian thioesterase I or II (Pazirandeh et al., 1991; Tai et al., 1993; Witkowski et al., 1994) and corresponds more closely to the inactivation observed on replacement of the serine nucleophile by cysteine in esterases, lipases, and proteases (Neet & Koshland, 1966; Holm et al., 1994; Leuveling et al., 1994). For the S114C LuxD mutant, the loss of activity corresponded directly to a decrease in the deacylation rate. The high stability of the acylated cysteine intermediate particularly at lower pH and thiol concentrations should prove advantageous for elucidation of its crystal structure.

The low rate of deacylation of the S114C mutant appears to be due to a change in the geometry at the active site (Neet & Koshland, 1966) since the SH group would be a better leaving group than an OH group for reactions not limited by proton transfer to the nucleophile. Small differences in the orientation of the thiol group in the active site have been observed in the crystal structure of thiol trypsin (McGrath et al., 1989; Wilke et al., 1991) compared to the location of the serine nucleophile in trypsin.

The presence of the larger thiol group in the S114C mutant would result in relatively close contacts with H^{241} and its own backbone amide nitrogen (Figure 6), considering that the van der Waals and covalent radii of sulfur are approximately 0.4 Å greater than oxygen (Neet & Koshland,

	--β-> s ---α-->
Consensus	*o**o-s*oo-*o*-*
Vh	IGLIAASLSARVAYEV
Xl	FGMLASSLSARIAYAS
Vf	IGLIAASLSARIAYEV
Pl	IGLIASSLSARIAYEV
Pp	VGLIASSLSARIAYDI
Duck II	FALFGHSFGSFVSYAL
Rat II	FAFFGHSFGSYIALIT
Grs T	FAFLGHSMGALISFEL
Chicken I	YNAIGYSFGACVAFEM
Rat I	YRVAGYSFGACVAFEM
Rabbit I	VAGYSYG
Goose I	SFGACVAF
ORF2	LALFGHSMGAVIAHEV

FIGURE 7: Consensus sequence for thioesterases extending across the nucleophilic serine at the active site. The β strand and the α helix flanking S^{114} of *V. harveyi* LuxD along with a consensus sequence across this region are indicated at the top for the lux-specific thioesterases from *V. harveyi* (Vh), *X. luminescens* (Xl), *V. fischeri* (Vf), *P. phosphoreum* (Pp), and *P. leoignathi* (Pl) and the mammalian thioesterases II from rat and duck and the thioesterase I domains of chicken, rat (Naggert et al., 1988), rabbit (Hardie et al., 1985), and goose (Poulose, et al., 1981) fatty acid synthetase. The related sequence of a protein in the gramicidin biosynthetic operon in *Bacillus brevis* (Grs T) (Krätzschmar, 1989) and another protein encoded by the gene (ORF2) of the Bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus* (Raibaud et al., 1991) are also listed. An * corresponds to a large hydrophobic residue (I, F, L, M, V, Y); an o corresponds to a residue with a small side chain (−H, $-CH_3$, $-CH_2OH$). Exceptions in the sequences are underlined.

1966). This potential strain would be expected to cause a perturbation in the conformation and lead to a change in alignment of the residues. The lower acylation of the double mutant (S114C·H241N) compared to the S114C mutant does show, however, that H^{241} still interacts with the C^{114} nucleophile and assists in the acylation of the cysteine nucleophile.

In contrast to other serine thioesterases, the nucleophilic S^{114} of LuxD is not part of the consensus sequence motif of GXSXG. The active site serine (S^{114}) is located in a nucleophilic elbow between a β strand and an α helix (Lawson et al., 1994). Although this residue is not part of the standard consensus sequence, a comparison of the sequences extending across the β strand and through the α helix of myristoyl-ACP thioesterases from different luminescent bacteria to mammalian thioesterases does result in recognition of a more extended and general sequence motif (Figure 7). Out of the 15 positions flanking the active site serine, 7 positions are restricted to large hydrophobic residues and 5 positions contain residues whose side chains are small. The major differences between the sequences of the lux-specific thioesterases and the mammalian thioesterases are an arginine residue four amino acids after the serine and the presence of a residue with a small side chain just prior to the serine in LuxD. Alignment of the sequence flanking the serine (S^{77}) initially proposed to be at the acylation site in the motif GXSXG (Ferri & Meighen, 1991) gave mismatches at 5 of the 12 positions with the proposed consensus sequence (Figure 7). Dewerenda and Dewerenda (1991) have made an extensive analysis of the sequence of the region extending across the nucleophilic elbow for esterases and lipases and related it to the packing of the β strand and the α helix. A similar consensus sequence to that of the thioesterases appears to exist for these two classes of enzymes across the β strand but diverges to some degree in the α helix. The use of the extended consensus sequence could prove useful in distinguishing nucleophilic serines in thioesterases that are part of a charge relay system from "essential serines" such as S^{77} located in the standard GXSXG consensus motif. The structural motif of the nucleophilic elbow apparently shows a higher degree of flexibility than previously thought and that the changes in the mutual disposition of the β strand and the α helix may modify the requirements for specific amino acids buried at the interface.

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