Tryptophan Fluorescence of the lux-Specific *Vibrio harveyi* Acyl-ACP Thioesterase and Its Tryptophan Mutants: Structural Properties and Ligand-Induced Conformational Change†

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ABSTRACT: The lux-specific myristoyl-ACP thioesterase from *Vibrio harveyi* contains four tryptophan residues, Trp23, Trp99, Trp186, and Trp213. Replacement of each of these residues with tyrosine by site-directed mutagenesis coupled with fluorescence and quenching studies of the purified mutant and wild type thioesterases during catalysis has been used to probe ligand-induced conformational changes. Mutant W99Y retained high enzyme activity (80%) with W213Y and W23Y retaining intermediate activity and W186Y having the lowest activity (20%). The sum of the differential fluorescence spectra of the individual tryptophans was identical to the fluorescence spectrum of the wild type thioesterase, showing that mutation had not caused a major conformational change and energy transfer did not occur between the tryptophans. Fluorescence emission maxima and quenching by acrylamide revealed that Trp213 and Trp23 are in a polar environment and/or exposed to solvent while Trp186 appeared to be buried inside the molecule, consistent with the crystal structure of the thioesterase. The fluorescence intensities of the wild type, W23Y, W99Y, and W186Y thioesterases increased in direct correlation to their degree of acylation with myristoyl-CoA, while the fluorescence of the acylated W213Y mutant remained constant, showing that the enhancement of fluorescence was entirely due to interaction of the acyl group with Trp213. Acrylamide quenching of the acylated mutants showed that the accessibility of the tryptophans to solvent was differentially altered and that the quenching of W23Y was enhanced in contrast to the quenching of the other mutants, supporting a ligand-induced conformational change during enzyme turnover.

In luminescent bacteria, a lux-specific acyl-ACP thioesterase (LuxD) is responsible for providing myristic acid to the luminescent system (*1*−*4*). The LuxD protein interacts with a multienzyme fatty acid reductase complex which reduces the fatty acid to aldehyde for the light-emitting reaction. This enzyme cleaves myristoyl-CoA[1] and myristoyl-ACP and can also exhibit esterase activity as well as transfer acyl groups to various low-molecular weight alcohol and thiol acceptors (*2*). The recombinant lux-specific thioesterase from *Vibrio harveyi* has been purified to homogeneity (*2*, *5*), and its crystal structure represents the only X-ray structure determined for a thioesterase (*6*). Studies on the mechanism of this enzyme have greatly benefited from elucidation of its structure (*6*), identification of a catalytic triad similar to that of serine proteases and lipases, and the capability to readily detect an acylated enzyme intermediate (*7*, *8*).

Although knowledge of the crystal structure of a protein provides important information about its tertiary structure, much less is known about local structural fluctuations during enzyme catalysis. Spectroscopic techniques involving fluorescence emission (*9*) and collisional quenching of emission by solutes (*10*, *11*) can provide sensitive probes of the conformational states of proteins. The fluorescence properties of endogenous tryptophan residues can serve as local intrinsic probes for investigating the dynamic nature of the enzyme. Such fluorescence studies have been applied recently to a number of proteins with known X-ray structures (*12*−*22*)

The thioesterase of *V. harveyi* contains four tryptophan residues at positions 23, 99, 186, and 213 which are conserved in the lux-specific thioesterases from all species of luminous bacteria, including *Photobacterium phosphoreum*, *Photobacterium leiognathi*, *Vibrio fisheri*, and *Xenorhabdus luminescens* (*4*, *23*−*26*). As shown in Figure 1, Trp23 on the second central β-strand and Trp99 at the C-terminal end of the second α-helix appear to be in partially buried environments. Trp186 is buried deeply inside the molecule and is on a loop between two α-helices. In contrast, located on a loop between the seventh central β-strand and the adjacent α-helix, Trp213 appears well-exposed with its indole ring lying just above the ring of the His241 residue involved in the catalytic triad (*6*, *7*). The four tryptophan residues are quite distant from each other (13−33 Å) and thus may serve as independent reporters of the local structure of the enzyme in these regions. In this work, mutant enzymes containing a single substitution of tryptophan by tyrosine were constructed by site-directed

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[1] Abbreviations: CoA, coenzyme A; ACP, acyl-carrier protein; p-NP, *p*-nitrophenol.

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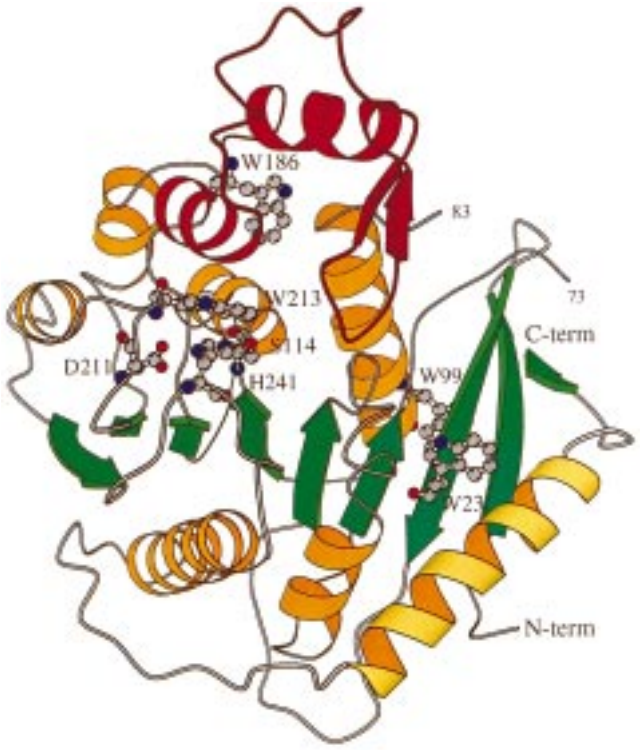

FIGURE 1: Schematic representation of the three-dimensional structure of the thioesterase from *V. harveyi*. The figure was generated using MOLSCRIPT (*27*). The atomic coordinates were taken from the X-ray study of Lawson et al. (*6*). The α-helices are yellow; the β-strands are green, and the putative "cap" subdomain is red. The catalytic triad and the four tryptophan residues are shown with gray carbon atoms, blue nitrogen atoms, and red oxygen atoms. The amino and carboxyl termini are denoted by the letters N and C, respectively. Residues 73 and 83 flank the surface loop that is invisible in the electron density map.

mutagenesis and purified to homogeneity. By comparison of the kinetic and fluorescence properties of the tryptophan mutants with those of the wild type thioesterase, the contributions of individual tryptophan residues to the fluorescence spectra, their solvent accessibility, and the identity of the residue(s) responsible for the spectroscopic changes induced by ligand binding have been determined.

EXPERIMENTAL PROCEDURES

Materials. Acrylamide, *N*,*N*-methylenebisacrylamide, β-mercaptoethanol, SDS−PAGE molecular mass standards, *p*-nitrophenyl myristate, and all coenzyme derivatives were obtained from Sigma Chemical Co. Stock solutions of *p*-nitrophenyl myristate were prepared in 2-propanol. [^{3}H]-Myristic acid (14 Ci/mmol) was from Amersham Corp. and purified by thin-layer chromatography. [^{3}H]Myristoyl-CoA (14 Ci/mmol) was prepared from the radioactive fatty acid as previously described (*28*). En^{3}Hance was from Dupont. Urea (ultrapure), hyamine hydroxide, and CytoScint were from ICN. Guanidine hydrochloride (sequential grade) was purchased from Pierce. Phosphate buffer was made by mixing the appropriate amounts of K_2HPO_4 and NaH_2PO_4. Media used to grow bacteria were purchased from DIFCO Laboratories. Restriction enzymes were from New England Biolabs. DNA sequencing kits were obtained from United States Biochemicals.

Site-Directed Mutagenesis. A 1.6 kb *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 M13 (mp19) sequencing vector (*29*). Site-directed mutagenesis was performed according to the method of Kunkel (*30*, *31*) using the M13 *In Vitro* Mutagenesis Kit (Bio-Rad). Oligonucleotide primers for converting W23 → Y23, W99 → Y99, W186 → Y186, and W213 → Y213 were 5′-GAACTTCACGTCTACGAAACGC-CCCC-3′, 5′-GTTTATCACTACCTGCAGACC-3′, 5′-C-GAGCATCACTACGATACCTTAG-3′, and 5′-CAACGAT-GATTACGTTAAGCAAG-3′, respectively, and were obtained from the Sheldon Biotechnology Center (McGill University).

Expression and Purification of Wild Type and Mutant Thioesterase. The bacteriophage T7 promoter−RNA polymerase system was used to express wild type and tryptophan mutant *V. harveyi* LuxD in *Escherichia coli* K38 (*32*). *E. coli* K38 cells 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 the OD_{660} equaled 0.9−1.1. The temperature was increased to 42 °C for 20 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. The purification procedures of the wild type and the mutant LuxD enzymes were similar to those previously described (*2*, *5*, *8*), except tryptophan mutants with low activities were monitored by SDS−PAGE after acylation with [^{3}H]myristoyl-CoA. Cells were lysed by sonication. The enzymes were purified by ammonium sulfate precipitation, DEAE-Sepharose chromatography, and gel filtration on G-100 Sephadex. The purified enzymes were stored in 20% glycerol, 10 mM β-mercaptoethanol, and 0.2 M NaCl at −20 °C.

Determination of Protein Concentrations. The wild type and the mutant enzyme concentrations were determined by using the method of Gill and von Hippel (*33*) on the basis of the UV absorbance at 280 nm. Extinction coefficients of the proteins were calculated from the number of tryptophan and tyrosine residues in the protein using the molar extinction coefficients tabulated by Edelhoch (*34*).

Kinetic Properties of the Enzymes. *(1) Cleavage Activities*. The cleavage activities of the wild type and tryptophan mutants were determined from the rate of cleavage of *p*-nitrophenyl myristate at pH 8.0 in 50 mM phosphate containing 20% glycerol, 0.05% Triton X-100, and 50 μM *p*-nitrophenyl myristate by following the rate of 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.

(2) Thioesterase Activity. The rates of cleavage of myristoyl-CoA by the wild type and tryptophan mutant enzymes were determined in the standard assay (*28*) with minor modifications. Seven microliters of 0.29 mM [^{3}H]-myristoyl-CoA (1.35 Ci/mmol) was incubated at 23 °C with an aliquot of the enzyme for 3 min in 50 mM phosphate (pH 7.5) and 10 mM β-mercaptoethanol, in a total volume

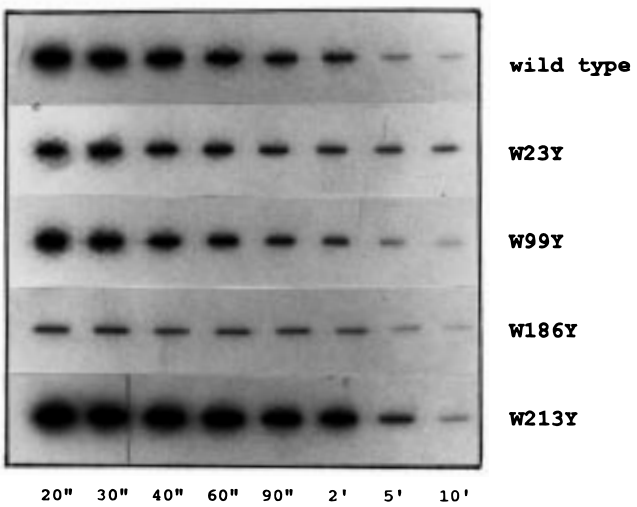

FIGURE 2: Comparison of the deacylation rates of the wild type thioesterase and its tryptophan mutants. The purified enzymes (0.46 μM) were reacted with 1.7 μM [^{3}H]myristoyl-CoA (14 Ci/mmol) in 50 mM phosphate (pH 7.5) at room temperature for 30 s (wild type), 2 min (W23Y, W99Y, and W213Y), and 10 min (W186Y). The acylated protein was then diluted into cold 20 μM myristoyl-CoA before stopping the reaction at the indicated times and the labeled protein resolved by SDS gel electrophoresis.

of 200 μL. The reaction was stopped with 10 μL of glacial acetic acid, and the solution was extracted with 1 mL of hexane. The hexane phase (0.5 mL) was counted in 10 mL of CytoScint with a 46% efficiency. The amount of enzyme in this assay was adjusted so that less than 50% of the substrate was converted into hexane extractable material.

Protein Acylation and Deacylation. To detect the acylated enzyme intermediate, the purified wild type LuxD as well as its tryptophan mutants was incubated in 50 mM phosphate (pH 7.5) with [^{3}H]myristoyl-CoA (14 Ci/mmol) at room temperature. The acylation reaction was stopped by mixing the solution 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 conditions similar to those of acylation except the acylated protein was diluted into cold 20 uM myristoyl-CoA before stopping the reaction at different times.

Gel Electrophoresis and Autoradiography. SDS−PAGE was performed by the method described by Laemmli (*35*) 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 for 3 days at −80 °C. The dried protein bands were cut from the gels, dissolved in 90% hyamine hydroxide at 37 °C, and incubated overnight in CytoScint at 37 °C, 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 thioesterase prepared by SDS electrophoresis and electroelution from the gel.

Fluorescence Measurements. Measurements of the fluorescence excitation and emission spectra of the wild type LuxD and its tryptophan mutants were taken on a Hitachi 3010 spectrofluorometer. The sample compartment was equipped with a magnetic stirrer and thermostated with a temperature regulator. The fluorescence cell was a 10 mm path length quartz cuvette. The intrinsic tryptophan fluorescence of wild type LuxD and the tryptophan mutants was measured at 20 °C in 50 mM phosphate at pH 7.5 in a 300 μL total volume unless stated otherwise. Excitation of samples was carried out at 296 nm with fluorescence emission scanned from 300 to 450 nm at 60 nm/min with excitation and emission band-pass set at 3 and 5 nm, respectively. Background emission was subtracted from the sample emission.

Dependence of Fluorescence on Enzyme Acylation. The fluorescence of the wild type enzymes and tryptophan mutants during acylation and deacylation was determined by adding aliquots of a 0.5 mM stock myristoyl-CoA to the enzyme solution. The tryptophan residues were excited at 296 nm, and emission was monitored continuously at 337 nm.

Quenching by Acrylamide. Fluorescence quenching studies were carried out by adding aliquots of freshly prepared 8 M acrylamide to the protein solution. Fluorescence quenching in the presence of substrate was carried out by incubating 5 μM myristoyl-CoA with 1 μM enzyme (30 s for the wild type, 2 min for W23Y, W99Y, and W213Y, and 10 min for W186Y) before adding acrylamide to the reaction mixture. Fluorescence of the enzyme was corrected for any dilution by the acrylamide.

RESULTS

Expression and Purification of Tryptophan Mutants of the lux-Specific Thioesterase. The tryptophan mutants W23Y, W99Y, W186Y, and W213Y of the thioesterase were overexpressed and purified as described previously for the native enzyme (*8*). All mutant proteins gave a single band of 32 kDa with a purity of >95% on SDS−PAGE. The yield of mutant W186Y was lower than those of the other mutants due to difficulty in extracting the protein from the cell.

Kinetic Parameters of Wild Type Thioesterase and the Tryptophan Mutants. Figure 2 shows that all four tryptophan mutants can be acylated with myristoyl-CoA and that the level of acylation decreases with time. The acylation levels of all mutants except W186Y are comparable to that of the wild type enzyme. The acylation rate for the W186Y mutant was much slower than that of the wild type thioesterase and required at least 10 min before maximum acylation was achieved.

In Table 1, the esterase and thioesterase activities, the first-order rate constants of deacylation, and the acylation levels of the wild type enzyme and the tryptophan mutants are compared. Mutation of tryptophan 186 resulted in the largest effects on kinetic properties. W186Y only displayed 17−19% of the esterase and thioesterase activities of the wild type enzyme with the deacylation rate showing a decrease to a comparable level (25%) which clearly indicated that the reduction in activity of W186Y is due to decreased turnover. On the other hand, mutation of tryptophan 23, 99, or 213 affected the (thio)esterase activities and deacylation rates to a much lower extent with mutants W23Y, W99Y, and W213Y, respectively, retaining 25, 77, and 46% of the esterase activity, 37, 64, and 59% of the thioesterase activity, and 41, 84, and 55% of the deacylation rate of the wild type thioesterase. The similarity in thioester cleavage and deacylation rates suggests that deacylation is the rate-limiting step

Table 1: Kinetic Parameters of the Wild Type Thioesterase and Its Tryptophan Mutants[a]

	activity (k_{cat}) (min^{-1})		deacylation rate constant (min^{-1})	mol of acyl groups/mol of enzyme
	p-NP myristate	myristoyl-CoA		
wild type	5.7 (100%)	0.93 (100%)	0.96 (100%)	1.0
W23Y	1.4 (25%)	0.35 (37%)	0.40 (41%)	0.61
W99Y	4.4 (77%)	0.60 (64%)	0.81 (84%)	0.73
W186Y	1.1 (19%)	0.16 (17%)	0.24 (25%)	0.20
W213Y	2.6 (46%)	0.55 (59%)	0.53 (55%)	1.0

[a] The rate constants for *p*-nitrophenyl myristate cleavage are higher than those for myristoyl-CoA cleavage due to the presence of 20% (v/v) glycerol in the assay. Assay procedures are described in Experimental Procedures. Note that the values for moles of acyl groups per mole of enzyme and deacylation rate were measured by extraction of the gel and determination of the radioactivity (all assays were conducted in triplicate). Acylation levels of the mutants and wild type thioesterase in Figure 2 are in good agreement with the results above with the changes arising due to small differences in exposure of each gel to the film.

for the tryptophan mutants as is the case for the wild type enzyme (*8*).

Steady State Fluorescence Spectra of the Wild Type Thioesterase and the Tryptophan Mutants. Construction of the tryptophan mutants allowed the contribution from each tryptophan to the overall protein fluorescence spectra to be assessed. For tryptophan residues, the intensity and spectra of their fluorescence emission will be dependent on the polarity of their microenvironment. In general, the less polar the microenvironment of specific tryptophans in the three-dimensional structure, the higher the intensity of the fluorescence and the greater the shift of the emission maximum to shorter wavelengths. Figure 3a shows the fluorescence spectra of the wild type and the difference spectra of the tryptophan mutants calculated by subtraction of their respective fluorescence emission from that of the wild type enzyme. Consequently, the results presented in Figure 3a give the net fluorescence spectra of the specific tryptophan residue missing in each of the mutants. Figure 3a shows that Trp186 provided the major contribution to the fluorescence intensity of the wild type enzyme followed in order by lower contributions by Trp99, Trp23, and Trp213.

The fluorescence spectrum of the wild type enzyme displayed spectral features typical of tryptophan emission with a λ_{max} of 337 nm. Removal of each tryptophan shifted the emission λ_{max} relative to that of the wild type enzyme and provided an indication of the local environments of these residues. Trp23 and Trp213 were slightly red shifted with λ_{max} values of 341 and 340 nm, respectively, indicating a relatively solvent-accessible environment for these two tryptophan residues. Trp99 had a λ_{max} of 338 nm, suggesting a mixed environment. Trp186 displayed a clear blue shift with a λ_{max} of 332 nm consistent with Trp186 being buried inside the molecule and not exposed to solvent as indicated from the crystal structure (Figure 1). The sum of the fluorescence spectra of the four tryptophan residues was almost identical (±2%) to that of the wild type enzyme (Figure 3b), showing that replacement of the tryptophans by tyrosines does not cause a major conformational change of the enzyme and that energy transfer between the tryptophan residues is insignificant, consistent with the four tryptophan residues being well separated in the thioesterase (Figure 1).

Effect of Substrate on Fluorescence. The steady state fluorescence emission spectra of the wild type thioesterase in the absence and presence of 5 μM myristoyl-CoA are shown in Figure 4. In the presence of myristoyl-CoA, there was a 30.3% net increase in fluorescence quantum yield. Since myristoyl-CoA itself did not fluoresce under these conditions, the increased emission intensity reflected a change in the exposure and/or environment of the tryptophan residues upon substrate binding, suggesting a possible conformational change associated with acylation of the enzyme. To investigate the fluorescence spectra of the thioesterase during the (de)acylation process, the fluorescence emission of wild type thioesterase was scanned with time (Figure 5). In the presence of different concentrations of myristoyl-CoA, the fluorescence intensity increased about 30% in all cases during the initial 7 s (inset of Figure 5) and then remained at a constant level for 4, 9, and 20 min at 2.5, 5, and 10 μM myristoyl-CoA, respectively (Figure 5). The intensities then decreased to the initial levels due to the consumption of the substrate.

Similar increases in spectra fluorescence intensity were also observed upon substrate addition to three of the four tryptophan mutants. As shown in Figure 6, in the presence of 5 μM myristoyl-CoA, W23Y, W99Y, and W186Y had 22, 24, and 6% increases in intensity, respectively, compared to the 30% increase for the wild type enzyme. The level of the increase in fluorescence intensity upon acylation of W23Y, W99Y, and W186Y relative to that of the wild type enzyme (73, 80, and 20%, respectively) corresponded very closely to the level of incorporation of the labeled acyl group (61, 73, and 20%, respectively) as listed in Table 1. In contrast, the fluorescence spectra of mutant W213Y remained unchanged upon substrate addition even though W213Y was fully acylated (Table 1). This result provides strong evidence that the increase in fluorescence intensity was entirely due to a change in the microenvironment of Trp213, probably implicating this residue in binding substrate and/or a conformational change associated with enzyme activation upon acylation.

On the basis of the deacylation rates and acylation levels of the wild type and the tryptophan mutants (Table 1), it would be predicted that the time before the decrease in fluorescence arising from the consumption of myristoyl-CoA would be in the following order: WT < W99Y < W23Y < W186Y. Figure 6 shows that this is indeed the case with W99Y and W23Y, taking approximately 17 and 30 min, respectively, and 5 times the amount of W186Y taking 40 min to consume 5 μM myristoyl-CoA, compared to approximately 9 min for the wild type enzyme (Figure 5). Due to the low level of acylation of W186Y and the slow turnover of the mutant, it was necessary to use 5 times more W186Y to observe the change in fluorescence intensity.

Quenching of Wild Type and Tryptophan Mutants by Acrylamide. To confirm if substrate binding caused a conformational change affecting the accessibility of individual tryptophan residues, the fluorescence quenching of the wild type and mutant thioesterases by acrylamide was investigated in the absence and presence of substrate. Figure 7 gives the Stern−Volmer plots based on the equation $F_o/F = 1 + K_dQ$, for acrylamide quenching of the wild type and tryptophan mutant thioesterases, where F_o and F are the fluorescence intensities in the absence and the presence of

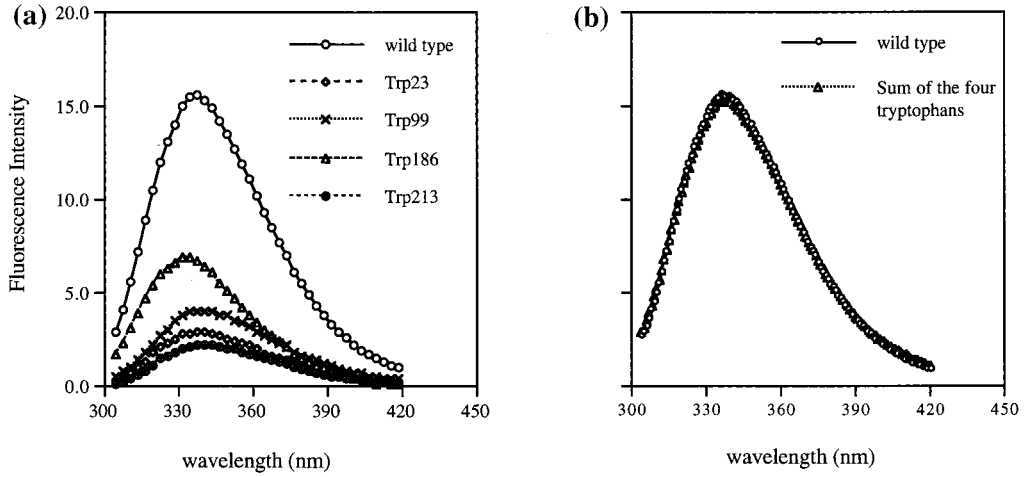

FIGURE 3: Contributions of each tryptophan to the fluorescence emission spectrum of the wild type enzyme. Fluorescence spectra of 1 μM enzyme in 50 mM phosphate (pH 7.5) were recorded at 20 °C on excitation of 296 nm. (a) Spectrum of the wild type enzyme and the difference spectra between the wild type and the tryptophan mutants. The difference spectra were calculated by subtracting the spectrum of a particular tryptophan mutant (e.g., W23Y) from that of the wild type enzyme to obtain the spectrum for a specific tryptophan (e.g., Trp23). (b) Comparison of the spectra of the wild type and the sum of the difference spectra of the individual tryptophan residues.

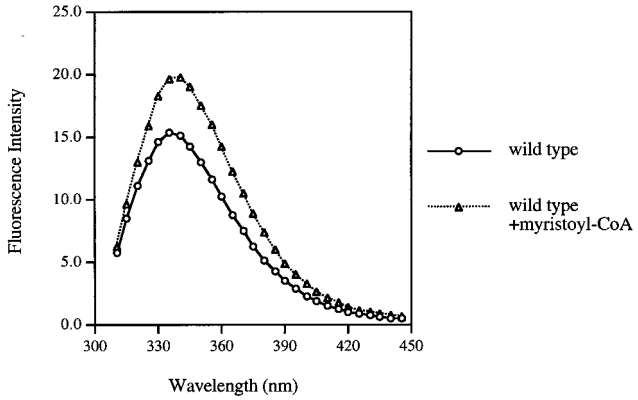

FIGURE 4: Effect of myristoyl-CoA on the fluorescence intensity of *V. harveyi* thioesterase. LuxD (1 μM) was incubated with 5 μM myristoyl-CoA in 50 mM phosphate (pH 7.5) at 20 °C for 30 s before the fluorescence emission spectra were recorded at an excitation wavelength of 296 nm.

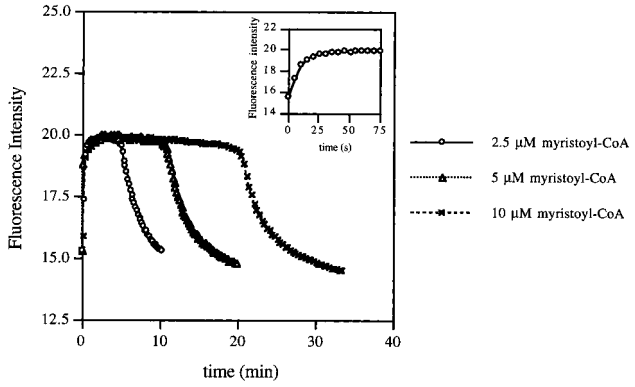

FIGURE 5: Fluorescence emission during acylation of LuxD with different concentrations of myristoyl-CoA. Fluorescence emission at 337 nm on excitation at 296 nm for LuxD (1 μM) during reaction with 2.5, 5, and 10 μM myristoyl-CoA in 50 mM phosphate (pH 7.5) at 20 °C. (Inset) Initial increase in fluorescence on acylation of LuxD with 5 μM myristoyl-CoA.

quencher, respectively, Q is the concentration of acrylamide, and K_d is the Stern−Volmer quenching constant. The curvature at >0.5 M acrylamide (data not shown) could be due to a static quenching component (*36*) or to a conformational change of the protein at high acrylamide concentrations.

The variation of the slopes (K_d) of the plots reflects the different overall accessibility of the intrinsic tryptophan residues to solvent. As shown in Figure 7a−d, mutants W213Y and W23Y had the lowest Stern−Volmer quenching constants. It should be noted that a large decrease in quenching of a mutant containing three tryptophans relative to that of the wild type enzyme containing four tryptophans reflects the loss of a highly quenched tryptophan. Consequently the tryptophans missing in W213Y and W23Y (Trp213 and Trp23, respectively) must be readily accessible to solvent and easily quenched. Removal of either of these highly quenched tryptophans thus causes a decrease in the relative quenching level of the respective mutant compared to the wild type enzyme. Similarly, the mutant W186Y has the highest quenching constant, and consequently, the missing Trp186 would be buried inside the protein and not readily quenched.

In the presence of myristoyl-CoA, the extent of quenching of the fluorescence intensity of the wild type enzyme decreased as might be expected if the tryptophans in general were less accessible to acrylamide (Figure 7a). However, the relative differences in the degree of quenching by acrylamide for the tryptophan mutants were quite distinct (Figure 7b−e). Removal of Trp23 (W23Y) actually resulted in an increase in the extent of quenching on reaction with myristoyl-CoA (Figure 7b), while substantial decreases in the extent of quenching were observed for mutants missing Trp99 (W99Y), Trp213 (W213Y), or Trp186 (W186Y).

The relative fluorescence of each mutant in its acylated [F_o(+S)] and nonacylated form (F_o) and the degree quenched [$1 - F(+Q)/F_o$] at 0.5 M acrylamide are summarized in Table 2. The degree of quenching of each mutant with 0.5 M acrylamide after acylation was also calculated from the Stern−Volmer plot [$1 - F(+S/Q)/F_o(+S)$] by taking into account the level of acylation of the respective mutant (Table 1). On acylation of the enzyme, the degree of quenching in W213Y, W186Y, and W99Y decreased 34−59%, although the effect of acylation on the extent of quenching of W186Y is particularly difficult to calculate due to the low degree of

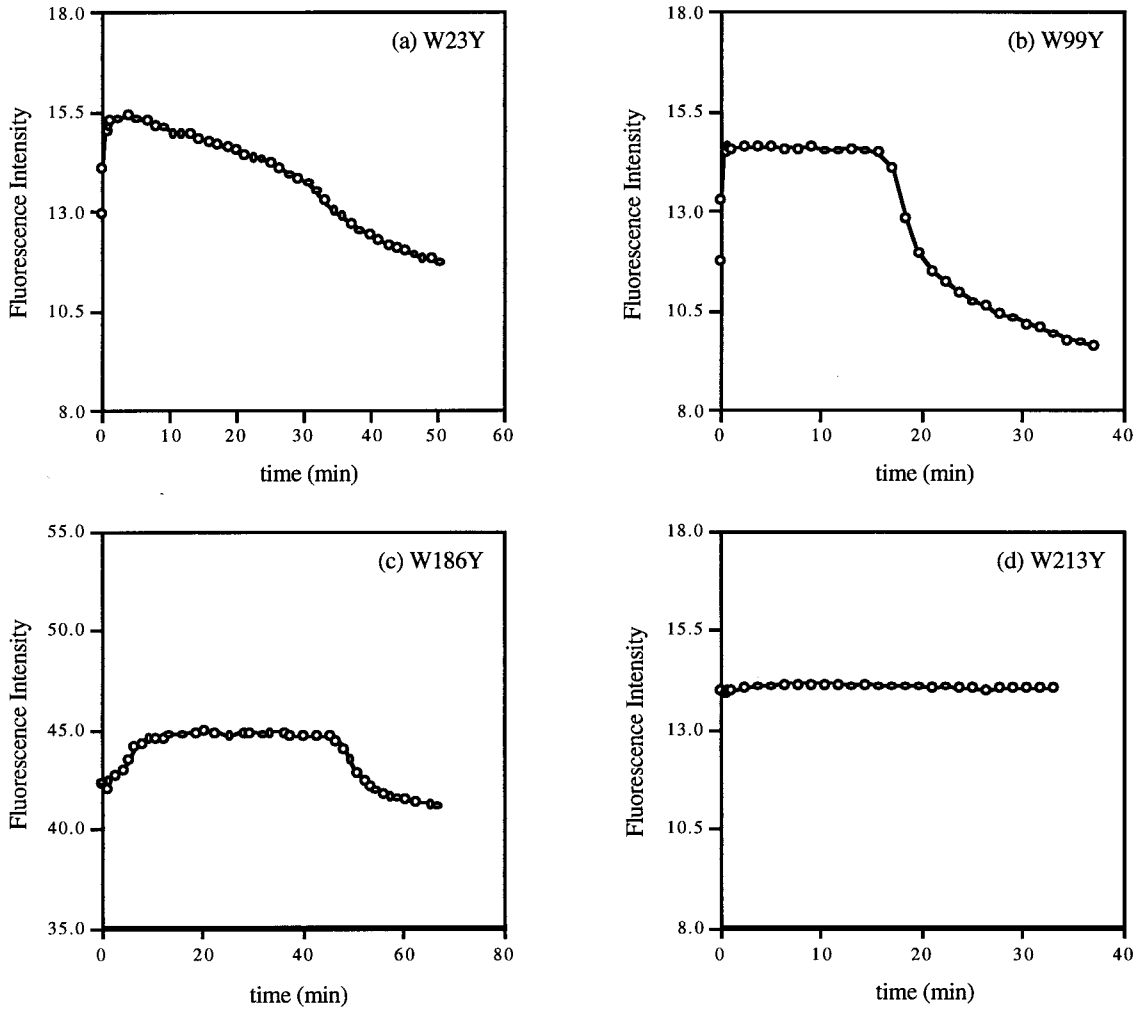

FIGURE 6: Dependence of fluorescence emission during acylation and deacylation of tryptophan mutants. Fluorescence was followed at 337 nm after excitation at 296 nm for the tryptophan mutants incubated with 5 μM myristoyl-CoA in 50 mM phosphate (pH 7.5) at 20 °C: (a) W23Y, (b) W99Y, (c) W186Y, and (d) W213Y. All samples were at a concentration of 1 μM except W186Y which was at a protein concentration that was 5 times higher.

acylation of this mutant (Table 2). In contrast, W23Y becomes more exposed on acylation with its ability to be quenched increasing by about 19%. These large differences in the direction and degree of quenching of each tryptophan on acylation of the enzyme suggest a major conformational change on interaction with substrate. Since the fluorescence of Trp213 is enhanced and the mutant missing Trp23 (W23Y) becomes more accessible to solvent on acylation of the thioesterase, it suggests that Trp186 and/or Trp99 become more exposed to solvent on acylation.

The quenched fluorescence of individual tryptophans at 0.5 M acrylamide was also calculated by subtracting the fluorescence of the mutant missing the respective tryptophan from the fluorescence of the wild type enzyme at the same acrylamide concentration (Table 3). The results confirmed that Trp186 and Trp99, with their degree of quenching being only 0.18 and 0.23, respectively, are buried inside this enzyme. On the other hand, Trp213 and to a lesser extent Trp23, with their degree of quenching being 0.84 and 0.64, respectively, are quite accessible to solvent and can be really quenched by acrylamide. The sum of the quenched fluorescence $F(+Q)$ at 0.5 M acrylamide of the individual tryptophans (63.5%) was in reasonable agreement (+4.7%) with that for the wild type thioesterase (58.8%) at this acrylamide concentration, considering the potential error involved in calculating the differential fluorescence quenching for each tryptophan.

DISCUSSION

The intrinsic fluorescence emission of the lux-specific thioesterase and collisional quenching of the fluorescence by acrylamide have been used to characterize the microenvironment of the individual tryptophans and their interaction with substrate. The results of these experiments have provided strong evidence for a conformational change upon substrate binding and acylation of the active site serine residue.

V. harveyi thioesterase has four tryptophan residues (Trp23, Trp99, Trp186, and Trp213) which are separated by 13−33 Å from each other in the three-dimensional structure (see Figure 1). Consequently, interactions or energy transfer between the tryptophans would not be expected, and each tryptophan should function as an independent and intrinsic monitor of the local environment. To delineate the contributions of individual tryptophans, each tryptophan was substituted by a tyrosine, which is one of two residues (Y or F) commonly substituted for tryptophans in mutants and/or closely related homologues (*18*, *20*, *37*−*44*). Although

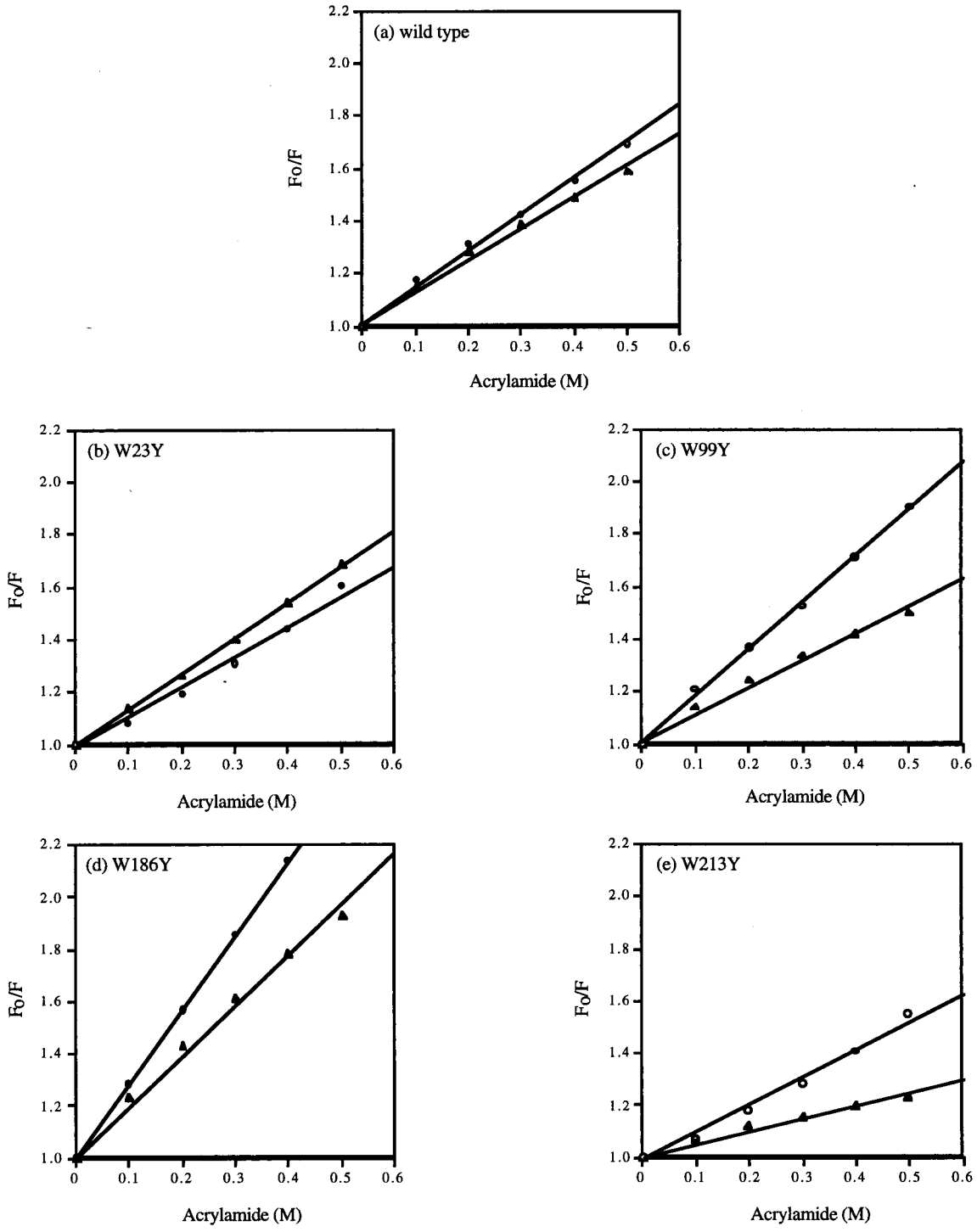

FIGURE 7: Stern−Volmer plots of F_o/F for LuxD and the tryptophan mutants vs acrylamide concentration in the absence and presence of myristoyl-CoA. Acrylamide was added to 1 μM enzyme in the absence (○) and presence (▲) of 5 μM myristoyl-CoA in 50 mM phosphate (pH 7.5) at 20 °C, and the fluorescence emission was measured at 337 nm with excitation at 296 nm.

phenylalanine has been used to replace tryptophan in many studies (*18*, *20*, *39*, *40*, *44*), tyrosine has been suggested to be the preferred substitution by some workers (*12*, *37*, *42*, *43*) due to the fact that its hydrophobicity and average buried volume are similar to that of tryptophan (*12*, *45*, *46*).

Although the ideal approach might be to substitute all but one tryptophan by tyrosine in the enzyme, and investigate the fluorescence properties of the specific tryptophan, this single-tryptophan-containing approach has only been accomplished in a few cases (*18*, *38*, *44*, *47*). In most cases, only one tryptophan is substituted (*14*, *17*, *43*, *48*, *49*). This is not unexpected as substitution of a tryptophan with tyrosine or phenylalanine would cause a local minor conformational perturbation, and the cumulative effect of multiple substitutions would in many cases substantially alter the functional activity and may cause a significant conformational change. For the *V. harveyi* thioesterase, substitution of each of the tryptophan residues decreased the activity to different extents with the largest loss in activity (~80%) occurring with mutant W186Y while mutants W99Y and W213Y retained more than 50% of their activity. The partial retention of activity by these mutants on one hand demonstrates that

Table 2: Fluorescence Intensities and Quenching of *V. harveyi* Thioesterase Mutants[a]

			degree quenched[c]	
mutant	F_o	$F_o(+S)$[b]	$1 - F(+Q)/F_o$	$1 - F(+S,Q)/F_o(+S)$
W23Y	81.9	112.2	0.36	0.43 (0.41)
W99Y	74.8	105.1	0.47	0.31 (0.35)
W186Y	56.8	87.1	0.59	0.24 (0.49)
W213Y	85.8	85.8	0.34	0.20
wild type	100	130.3	0.41	0.38

[a] Fluorescence intensities have been normalized to 100 for the wild type thioesterase in the absence of substrate and quencher. All enzymes are at a concentration of 1 μM. [b] Fluorescence of the acylated thioesterase calculated on the basis of an increase of 30.3% in that of the wild type thioesterase arising solely from an enhancement in fluorescence of Trp213 on acylation of the enzyme. [c] Calculated at 0.5 M acylamide from the Stern−Volmer plots in Figure 7 after correction of $F(+S,Q)/F_o(+S)$ for the presence of nonacylated thioesterase in the W23Y, W99Y, and W186Y mutants. The uncorrected values are given in parentheses.

Table 3: Fluorescence Intensities and Quenching of Individual Tryptophan Residues in *V. harveyi* Thioesterase[a]

Trp residue	F_o	$F(+Q)$	degree quenched $[1 - F(+Q)/F_o]$
Trp23	18.1	6.5	0.64
Trp99	25.2	19.4	0.23
Trp186	43.2	35.3	0.18
Trp213	14.2	2.3	0.84
wild type	100	58.8	0.41

[a] Fluorescence intensities of individual tryptophans, F_o, were obtained from Table 2 by subtraction of the fluorescence of the mutant missing the respective tryptophan from the wild type fluorescence. The quenched fluorescence, $F(+Q)$, of each tryptophan was calculated by subtracting the quenched fluorescence of the mutant missing the respective tryptophan from the quenched wild type fluorescence at 0.5 M acrylamide obtained from the Stern−Volmer plots.

substitution of one tryptophan with tyrosine only causes a minor conformational perturbation but clearly suggests that multiple substitutions would inactivate the enzyme and might create a substantial conformational change. Consequently, in these experiments, the fluorescence spectrum of individual tryptophans and the effect of acrylamide quenching were deduced by difference spectroscopy by subtraction of the fluorescence intensity of the mutant missing the respective tryptophan from the fluorescence intensity of the wild type thioesterase containing all the tryptophans. This approach was possible as there are a limited number of tryptophans in the thioesterase which were well separated from each other, precluding energy transfer and interactions. This conclusion was confirmed by demonstrating that the sum of the difference fluorescence spectra for the four tryptophans was identical to the fluorescence spectrum of the wild type thioesterase.

The fluorescence maximum of Trp186 exhibited a 5 nm blue shift and had the maximum contribution to the fluorescence spectrum of the wild type thioesterase, showing it is in a nonpolar or hydrophobic environment consistent with its location in the crystal structure that indicates it is buried inside the molecule. In contrast, the fluorescence of Trp23 and Trp213 experienced 3 and 4 nm red shifts, respectively, and had a low contribution to the fluorescence spectra, indicating that they are in a more polar environment and exposed to the solvent. As extraction of the W186Y mutant thioesterase from the cell was more difficult than for the other proteins and inclusion bodies were detected, substitution of this residue might affect the folding process. However, once extracted, the W186Y mutant could be purified in a normal manner, and its ability to be acylated and turnover could be readily demonstrated.

To more directly investigate the exposure of each tryptophan residue to solvent, quenching experiments with acrylamide were also conducted. Quenching with acrylamide is advantageous as it does not significantly interact with the proteins and can penetrate the protein matrix to a certain degree without being influenced by local charges and can quench buried tryptophan residues in dynamic structures with high flexibility (*36*, *50*). Quenching studies demonstrated that Trp213 and Trp23 were the most exposed to solvent, Trp99 is in a mixed environment, and Trp186 is buried inside the molecule.

These studies also demonstrated a large change in the fluorescence emission of the thioesterase on mixing with myristoyl-CoA which could be directly related to the interaction of the acyl group with Trp213. The increase in fluorescence followed by the subsequent return to the original level of fluorescence was directly correlated with the acylation of the active site serine residue and the subsequent deacylation which occurred on cleavage of all the substrate. The fluorescence intensities of the acylated enzymes were 30, 22, 24, and 6% higher for the wild type, W23Y, W99Y, and W186Y, respectively, than the corresponding values for unacylated enzymes, while the fluorescence intensities of the acylated and nonacylated W213Y mutant were identical. These increases correlated very well with the degree of acylation (Table 1; 1.0, 0.61, 0.73, and 0.20 for the wild type, W23Y, W99Y, and W186Y, respectively) but differed completely from that for W213Y (1.0 mol/mol), showing that changes in the fluorescence intensity upon acylation are due to interaction of the acyl group with tryptophan residue 213 and that significant interactions do not occur directly with the other tryptophans. This result agrees with the crystal structure which shows that Trp213 is in close proximity to His241 in the Ser-His-Asp catalytic triad (Figure 1).

Acrylamide quenching studies of the wild type and the tryptophan mutants showed that the quenching of each mutant was changed to a different degree upon acylation of the active site serine residue. As the four tryptophans are well separated in the crystal structure and only the fluorescence of Trp213 is affected by acylation, the results strongly support a conformational change in the protein upon acylation resulting in a differential change in the accessibility of each tryptophan residue to acrylamide. The quenching studies showed that upon acylation of the thioesterase, W99Y, W186Y, and W213Y were quenched to a lower degree than in the nonacylated form, suggesting a overall lower accessibility of these enzymes to solvent. In contrast, mutant W23Y was more readily quenched after acylation, consistent with at least one of the tryptophans being more exposed to solvent upon acylation and showing that this effect cannot be simply due to steric shielding by the acyl group. The conformational change is highly reminiscent of that observed for lipases and esterases in which the substrate binding pocket is believed to be "capped" by a conformation change after reaction with substrate (*51*, *52*). Indeed, a "cap" for the active site has been proposed for the lux-specific thioesterase (*6*) (red in Figure 1). A capping of the active

site upon acylation at serine could result in a major change in conformation and thus be responsible for the differential accessibility to acrylamide of the mutants upon acylation as well as explain why suitable crystals of the acylated thioesterase have not been generated for structural analysis. The application of fluorescence in measuring the intrinsic fluorescence of tryptophan residues provides an important and noninvasive approach for investigating substrate and conformational changes of the lux-specific thioesterase in an aqueous environment.

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