Trp-107 and Trp-253 Account for the Increased Steady State Fluorescence That Accompanies the Conformational Change in Human Pancreatic Triglyceride Lipase Induced by Tetrahydrolipstatin and Bile Salt*

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The conformation of a surface loop, the lid, controls activity of pancreatic triglyceride lipase (PTL) by moving from a position that sterically hinders substrate access to the active site into a new conformation that opens and configures the active site. Movement of the lid is accompanied by a large change in steady state tryptophan fluorescence. Although a change in the microenvironment of Trp-253, a lid residue, could account for the increased fluorescence, the mechanism and tryptophan residues have not been identified. To identify the tryptophan residues responsible for the increased fluorescence and to gain insight into the mechanism of lid opening and the structure of PTL in aqueous solution, we examined the effects of mutating individual tryptophan residues to tyrosine, alanine, or phenylalanine on lipase activity and steady state fluorescence. Substitution of tryptophans 86, 107, 253, and 403 reduced activity against tributyrin with the largest effects caused by substituting Trp-86 and Trp-107. Trp-107 and Trp-253 fluorescence accounts for the increased fluorescence emissions of PTL that is stimulated by tetrahydrolipstatin and sodium taurodeoxycholate. The largest contribution is from Trp-107. Contrary to the prediction from the crystal structure of PTL, Trp-107 is likely exposed to solvent. Both tetrahydrolipstatin and sodium taurodeoxycholate are required to produce the increased fluorescence in PTL. Alone, neither is sufficient. Colipase does not significantly influence the conformational changes leading to increased emission fluorescence. Thus, Trp-107 and Trp-253 contribute to the change in steady state fluorescence that is triggered by mixed micelles of inhibitor and bile salt. Furthermore, the results suggest that the conformation of PTL in solution differs significantly from the conformation in crystals.

Lipases belong to a large gene family of proteins characterized by a common protein structure (1, 2). Included in this family are pancreatic triglyceride lipase (PTL2 triacylglycerol acylhydrolase, EC 3.1.1.3) and its close homologues pancreatic triglyceride lipase related proteins 1 and 2 (3). Not only do these pancreatic lipases have highly conserved primary structures, their x-ray crystal structures are essentially identical (4–6). Each contains two domains, a globular N-terminal domain consisting of an α/β hydrolase fold and a C-terminal domain consisting of a β-sandwich structure. A striking feature of these lipases and many others is the presence of a surface loop termed the lid domain. Together with the β5 loop and β9 loops of the N-terminal domain, the lid domain sterically hinders access of substrate to the active site. In this conformation, PTL cannot hydrolyze substrate, and the existence of another conformation was proposed (6).

Subsequently, a second, open conformation of PTL was identified in studies of the crystal structure of the PTL-colipase complex (7, 8). In these studies, the investigators obtained crystals of the complex in the presence and absence of detergent and phospholipid mixed micelles. Without micelles, the lid domain remained in the same closed position as observed in the PTL structure even though colipase clearly bound to the C-terminal domain (8). With micelles, the lid domain and the β5 loop adopted new conformations (7). A large hinge movement of the lid moved the domain away from the active site to form new interactions with colipase. The lid movement opened and configured the active site to generate a conformation compatible with catalysis. Additionally, the movement exposed a large hydrophobic surface on the PTL-colipase complex, a surface that likely contributes to the anchoring of the complex on the substrate interface.

Although x-ray crystallography studies clearly demonstrated two conformations of PTL and other lipases, these only provide a static picture of what may be the beginning and end of the process. The mechanism that triggers lid opening and the presence of intermediate conformations remains speculative. Initially, many assumed that a lipid-water interface triggered the conformational change (9). However, a number of studies using inhibitors, small angle neutron scattering, neutron diffraction, and monoclonal antibodies suggest that the lid can open in solution (10–14). In these studies, it was variously suggested that bile salt micelles and colipase or bile salt micelles alone were sufficient to trigger lid opening. The presence of a lipid substrate was not required.

None of these studies addressed the relative contribution of bile salts and colipase to the lid opening. A recent paper described the use of electron paramagnetic resonance spectros-
copy combined with site-directed spin labeling to monitor conformational changes in the PTL lid and to determine the effect of bile salts and colipase on lid opening (15). A cysteine was substituted for Asp-250 in the lid domain, and a paramagnetic probe was linked at that site. Using this method, the authors observed a mixture of closed and open conformations of the lid in the presence of bile salt micelles alone. Colipase by itself did not induce lid opening, but in the presence of bile salt micelles, colipase increased the relative concentration of PTL in the open conformation. Although the spin labeling did not have dramatic effects on the activity of the labeled PTL, it may not be benign. The presence of the probe may alter the kinetics of lid opening and may explain why a portion of PTL always stayed in the closed position.

Another spectral method to follow conformation changes in proteins is fluorescence spectroscopy of native tryptophan. After systematically mutating the three tryptophans to alanine, investigators measured the binding of Thermomyces lanuginosus lipase and the mutants to mixed micelles of cis-parinaric acid and bile salt by fluorescence quenching and fluorescence resonance energy transfer (16). The measured values correlated with lid opening and depended on the presence of the single tryptophan in the lid. PTL shows a large increase in tryptophan fluorescence when incubated with a lipase inhibitor, tetrahydrolipstatin (THL), in the presence of bile salts (11). It was suggested, but not demonstrated, that the fluorescence change reflected movement of the lid domain. Because PTL contains seven tryptophan residues including one in the lid, Trp-253, the interpretation of this study is quite complicated. Another study monitoring time-resolved fluorescence of PTL and several tryptophan mutants demonstrated that Trp-30 makes a significant contribution to the tryptophan fluorescence of PTL (17). The lid tryptophan, Trp-253, had a low quantum yield and contributed considerably less to the overall tryptophan fluorescence. This report did not include investigations of PTL fluorescence in the presence of bile salts or colipase. Consequently, the assumption that the large increase in steady state fluorescence of PTL in the presence of THL and bile salt results from changes in the environment of the lid domain tryptophan remains unproven.

To determine whether the increased tryptophan fluorescence of PTL in THL and bile salt represents a conformational change in PTL, we measured the effect of tryptophan substitution mutations on the activity and intrinsic steady state fluorescence of PTL. Each of the seven tryptophans was mutated to tyrosine. Selected tryptophans were mutated to alanine or phenylalanine. Each mutant PTL was expressed and purified. We monitored the effect of bile salts, colipase, THL, and mixtures of these compounds on the steady state fluorescence of PTL.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Manipulations of DNA were done by standard methods (18). Mutations were introduced into the wild type PTL cDNA in the pHILS1 vector by polymerase chain reaction using the Stratagene QuikChange site-directed mutagenesis kit as per the manufacturer’s protocol (19). Oligonucleotide primers were designed to introduce the desired mutations and were generally 27 bp long. Transformations into Epicurian Coli XL-1 Supercompetent cells were performed as described in the QuikChange protocol. Minipreps were performed using either the Qiagen plasmid mini kit or the Qiagen QiAprep miniprep kit. The presence of the desired mutation was confirmed by dideoxynucleotide sequence analysis of the complete cDNA using the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit. The DNA was transformed into yeast by electroporation and positive clones selected as previously done (19, 20).

**Protein Methods**—The mutant lipases were expressed and purified as previously described (19). Protein concentrations were determined by amino acid analysis and by spectrophotometry at 280 nm using an extinction coefficient of $E_{1%}^\text{L} = 1.2$. The purified proteins were analyzed by SDS-PAGE and staining with GELCODE Blue stain (Pierce) to confirm the homogeneity of the isolated protein.

**Standard Lipase Activity Measurements**—The lipase activity of wild type and mutant PTL was determined in bulk by measuring the release of fatty acids from mechanically stirred emulsions of tributyrin, trioctanoin, or triolein as previously described (21, 22). Unless otherwise stated, pure colipase from porcine pancreas or pure recombinant, human colipase was added at a molar excess of 5. The lipolytic activities are expressed in international units/milligram of enzyme. One unit corresponds to 1 μmol of fatty acid released per min.

**Inhibition by THL**—For the incubations, 10 μg of PTL was added to 1 ml of 0.1 M Tris-HCl, pH 8.0, containing 4% dimethyl sulfoxide for a concentration of $2 \times 10^{-7}$ M. Five separate incubations were set up: 1) no other additions; 2) 10-fold molar excess of THL (Sigma); 3) 10-fold molar excess of THL and 10 μg of colipase; 4) 10-fold molar excess of THL and TDC to a final concentration of 4 mM; and 5) 10-fold molar excess of THL, TDC to a final concentration of 4 mM and 10 μg of colipase. Each mixture was incubated at room temperature. 100-μl aliquots were removed at time 0 and every 15 min for activity assays against tributyrin. For the samples that did not contain colipase, 1 μg of colipase was added to the assay. Inhibition was calculated as a percentage of the activity in the control sample (i.e., no other additions). The results were fitted to decay functions using SigmaPlot 10 (Systat Software, Inc).

**Steady State Fluorescence Spectroscopy**—Fluorescence measurements were performed at room temperature on a Fluoromax-2 (Jobin Yvon, Inc) spectrofluorimeter using a 1-cm quartz cell. The excitation and emission monochromators were set at 4-nm slit widths. Excitation was at 295 nm to selectively excite tryptophan residues. Emission was monitored between 305 and 400 nm. The average of five separate scans was analyzed. Quenching experiments with acrylamide were accomplished by adding 6-μl aliquots of an 8.4 M acrylamide stock to 3 ml of protein solution up to a final concentration of 0.41 M acrylamide. The fluorescence intensity changes were recorded at the maximum emission wavelength for the mutant PTL and corrected for dilution. The quenching curves were carried out after incubating PTL with THL for 6 h at room temperature. In preliminary experiments, longer incubation times did not alter the result. The Stern-Volmer plates were created with the simplified equation $23, 24$ $F_0/F = 1 + K_{SV}Q$, where $F_0$ and $F$ are the fluorescence intensities in the absence and presence of the
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**TABLE 1**
Specific activity and steady state fluorescence measurements of PTL and the tryptophan mutants
The specific activity was determined against tributyrin in 4 mM NaTDC as described under “Experimental Procedures.” The results are the means ± S.D. Fluorescence measurements were done in 0.1 M Tris-HCl, pH 8.0, with 4 mM NaTDC, 4% dimethyl sulfoxide. 20-fold molar excess of THL, and 5-fold molar excess of colipase and compared with the fluorescence of each lipase in 0.1 M Tris-HCl, pH 8.0, with 4% dimethyl sulfoxide. The excitation wavelength was 295 nm. The slits were set at 4 nm. The relative fluorescence was determined at the maximum intensity and normalized to PTL. The maximum fluorescence intensity and maximum emission intensity (λmax) are the means ± S.D.

| Lipase | Specific activity | λmax | Relative fluorescence | I330/I350 |
|--------|------------------|------|----------------------|-----------|
| PTL    | 7,200 ± 250      | 338 ± 1 | 1.0                  | 0.81      |
| W17Y   | 7,800 ± 130      | 338 ± 1 | 1.39 ± 0.20          | 0.71      |
| W30Y   | 7,400 ± 360      | 326 ± 1 | 0.40 ± 0.06          | 1.12      |
| W86Y   | 1,200 ± 80       | 338 ± 1 | 1.4 ± 0.15           | 0.80      |
| W107Y  | 1,880 ± 65       | 328 ± 1 | 0.82 ± 0.10          | 1.04      |
| W253Y  | 5,100 ± 300      | 339 ± 1 | 1.85 ± 0.30          | 0.75      |
| W339Y  | 7,200 ± 250      | 339 ± 1 | 0.60 ± 0.04          | 0.74      |
| W403Y  | 3,900 ± 195      | 339 ± 1 | 1.10 ± 0.20          | 0.75      |

**TABLE 2**
Peak shift and change in maximum fluorescence intensity for PTL and the tryptophan mutants induced by NaTDC, THL, and colipase
Fluorescence measurements were done in 0.1 M Tris-HCl, pH 8.0, with 4 mM NaTDC, 4% dimethyl sulfoxide, 20-fold molar excess of THL, and 5-fold molar excess of colipase and compared with the fluorescence of each lipase in 0.1 M Tris-HCl, pH 8.0, with 4% dimethyl sulfoxide. The excitation wavelength was 295 nm. The slits were set at 4 nm. The percentage of change in maximum fluorescence intensity (ΔI) is the mean ± S.D.

| Lipase | Peak shift | ΔI |
|--------|------------|----|
| PTL    | +4         | 93 ± 7 |
| W17Y   | +4         | 100 ± 9 |
| W30Y   | +4         | 128 ± 8 |
| W86Y   | +4         | 85 ± 9 |
| W107Y  | 0          | 9 ± 1  |
| W253Y  | 0          | 27 ± 8 |
| W339Y  | +4         | 88 ± 4 |
| W403Y  | +4         | 86 ± 7 |

**RESULTS**

**Functional Properties of the Tryptophan Mutants**—To determine the contribution of each tryptophan residue to the steady state fluorescence of PTL, we mutated each individual tryptophan to tyrosine. This substitution was selected based on substitution preferences showing that tyrosine is a well tolerated naturally occurring substitution for tryptophan (25–27). Additionally, tyrosine has low absorption at 295 nm, and its emission signal is insignificant compared with that of tryptophan (28). Wild type and tryptophan mutants of PTL were created and expressed in *Pichia pastoris* as described under “Experimental Procedures.” Each of the proteins was efficiently secreted into the medium, indicating that protein folding was not dramatically altered. All were purified and purity assessed by SDS-PAGE and Coomassie Blue staining (data not shown).

We next determined the activity of each mutant in the pH-stat assay (Table 1). Each assay included the test lipase, 0.11 M tributyrin in 4 mM sodium taurodeoxycholate with and without a 5-fold molar excess of recombinant human colipase. Neither PTL nor any of the mutants had activity in the absence of colipase. All were reactivated by colipase, indicating that they could bind colipase. Three of the mutants, W17Y, W30Y, and W339Y, had normal activities, indicating that none of these residues affects catalysis directly or indirectly. The remaining mutants had lower than normal activity ranging from 17 to 70% of PTL activity. The changes in activity are similar to those reported for W17F, W30F, W86F, W252F, and W402F PTL mutants (17). The colipase requirement and presence of activity indicate that the mutants are not grossly misfolded.

**Steady State Fluorescence**—The maximum emission wavelength for PTL (λmax = 338 ± 1) corresponds well to previously reported values (11, 17). Only the tyrosine substitutions of Trp-30 and Trp-107 shifted the maximum emission wavelength significantly (λmax = 328 ± 1). The blue shift suggests that Trp-30 and Trp-107 are exposed to solvent. This conclusion is supported by the change in the I330/I350 ratio. Tryptophan residues in proteins have a λmax = 330 when they are buried in nonpolar regions of the protein and a λmax = 350 when they are completely exposed to water (29). Higher I330/I350 ratios indicate a relatively hydrophobic environment for the tryptophan residues. PTL has an I330/I350 ratio of 0.81, whereas the ratio increases for both W30Y and W107Y, suggesting that they are in a relatively polar environment. The ratio decreases for all other mutants, except W86Y, consistent with a more hydrophobic environment for Trp-17, Trp-253, Trp-339, and Trp-403. The tyrosine substitutions also affected the relative fluorescence at maximum intensity for the mutants (Table 1). The largest effect was seen for the W30Y substitution, which showed a decrease in fluorescence consistent with the large contribution of Trp-30 to the quantum yield of PTL (17). The largest increase in fluorescence was observed for W17Y, W86Y, and W253Y in agreement with the suggestion that these residues have weak fluorescence and act as acceptors in Trp-Trp homotransfer (17).

We then examined the effect of NaTDC, THL, and colipase on the emission spectra of PTL and each of the tryptophan substitution mutants to identify the residues that contribute to the increased emission of PTL in the presence of those substances. As previously reported, the inclusion of bile salt and THL increased the emission intensity and red-shifted the λmax of PTL (11). A 93 ± 7% increase in emission intensity at the peak and a 4-nm red shift was observed (Table 2 and Fig. 1). The spectral changes agree with the earlier study (11). W17Y, W86Y, W339Y, and W403Y had increases in emission intensity and a red shift similar to those seen for PTL (p < 0.05), indicating that these residues did not contribute significantly to the observed spectral changes. In contrast, substitution of Trp-30 led to a significant increase in peak emission intensity (p = 0.002), and substitutions of Trp-107 and Trp-253 caused a decrease in peak emission intensity (p = <0.001) (Table 2 and Fig. 2). Interestingly, the substitution of Trp-107 and not Trp-253 had the greatest effect on the change in peak emission intensity.
Although tyrosine residues should have low emission signals compared with tryptophan when excited at 295 nm, their behavior may differ in individual proteins. Because tyrosine to tryptophan resonance energy transfer can occur, any significant tyrosine fluorescence may affect overall protein fluorescence (30, 31). Thus, we substituted alanine for the tryptophans of most interest, Trp-30, Trp-107, and Trp-253. Yeast transformed with W30A and W253A vectors expressed and secreted mutant lipase in good quantities. In contrast, yeast transformed with W107A did not secrete any detectable amounts of recombinant lipase (40 colonies were screened by immunoblot and lipase assay), suggesting that the substitution was not tolerated. We then created a phenylalanine substitution mutant for Trp-107. The W107F mutation was easily detectable in the media of transformed yeast. Each mutant was expressed and purified for further characterization.

The activities of W30A and W253A were similar to those of the corresponding tyrosine mutants (Tables 1 and 3). W30A and W30Y had near normal activity, whereas W253A and W253Y had ~65 to 70% of normal activity. W107F had higher activity than did W107Y, 60% versus 25% of the PTL activity. The peak fluorescence intensity of W30A and W107F relative to PTL were similar to the changes seen with W30Y and W107Y, whereas the peak fluorescence of W253A was nearly identical to that of PTL and lower than that of W253Y (p = 0.01) (Tables 1 and 3). As with the tyrosine substitution mutants, the W107F and W253A mutants did not have a red shift in peak intensity, nor did they show a large increase in peak intensity when incubated with NaTDC, THL (Tables 2 and 3). Interestingly, the W253A substitution had a decrease in peak fluorescence in the presence of NaTDC, THL, and colipase.

**Fluorescence Quenching by Acrylamide**—To determine whether there was a change in the accessibility of Trp-107 and Trp-253 in the presence and absence of NaTDC and THL, we performed quenching experiments with acrylamide, an uncharged polar molecule that can quench both solvent-exposed and buried tryptophan residues (Fig. 3). Each of the curves appeared linear when fit to the Stern-Volmer equation ($R^2 > 0.99$ for each linear regression). The slope of the curves was similar for PTL and W253Y (4.4 and $4.1 \, M^{-1}$). In contrast, the slope of curve for W107Y was less steep ($2.3 \, M^{-1}$). For each lipase, adding NaTDC decreased the slope, but the change was small (for PTL, 3.8 $M^{-1}$; for W107Y, 2.1 $M^{-1}$; and for W253Y, 3.8 $M^{-1}$). The inclusion of THL with NaTDC increased the slope. In the case of PTL and W253Y, the slope approached that of the lipase alone (4.1 and 4.0 $M^{-1}$, respectively). In the case of W107Y, a larger change was observed, and the slope increased above that of W107Y alone (2.6 $M^{-1}$).

**Conformational Change of PTL**—To determine the contributions of bile salt, colipase, and THL to the increase in emission intensity for PTL, we measured emission in the presence of each component singly and in combinations (Table 4). When added alone, each component increased peak emission fluorescence indicating the potential interactions. The addition of colipase had the smallest effect and only NaTDC caused a shift in the $\lambda_{max}$ of PTL, a red shift of 2 nm. Including NaTDC with colipase did not alter the spectra beyond the effects of NaTDC alone. Similarly, the PTL spectra in the presence of THL and colipase resembled that obtained in the presence of THL alone. Comparable changes were observed with W107Y and W253Y. Only when PTL was incubated with both NaTDC micelles and THL were the full spectral changes observed. The addition of colipase to this incubation caused no additional change in the peak shift or peak emission intensity.

**Influence of NaTDC and Colipase on THL Inhibition Rates of PTL**—Another potential measure of the conversion of PTL from the inactive to the active conformation is the rate inhibition by THL in the presence of potential activators. THL in the Me$_2$SO-containing buffer was able to slowly inactivate PTL reach-
TABLE 3
Activity and change in maximum fluorescence intensity for PTL and the W30A, W107F, and W253A mutants induced by NaTDC, THL, and colipase

| Lipase  | Specific activity | Peak shift | Relative fluorescence | ΔI |
|---------|------------------|------------|----------------------|----|
|         | units/mg | nm | %                  |
| PTL     | 7200±120 | +4 | 1.0                 | 90±8 |
| W30A    | 6348±150 | +4 | 0.49±0.09           | 108±10 |
| W107F   | 4400±80  | 0  | 0.89±0.13           | 14±1 |
| W253A   | 4700±10  | 0  | 1.07±0.06           | −17±3 |

FIGURE 3. Stern-Volmer plots of quenching of PTL, W107Y, and W253Y with acrylamide. The conditions for measuring fluorescence are given in the legend for Fig. 1. Excitation was at 295 nm, and emission was measured at 340 nm. The symbols are defined in the figure legend. The best fit linear regression lines are shown.

TABLE 4
Change in maximum fluorescence intensity induced by colipase, NaTDC and THL for PTL, W107Y, and W253Y

| Addition | PTL | W107Y | W253Y |
|----------|-----|-------|-------|
| Hcol     | 7±1 | 7±2   | 9±0.8 |
| NaTDC    | 14±9| 12±5  | 15±6  |
| THL      | 15±8| 8±6   | 10±3  |
| hcol, NaTDC | 16±7 | 10±4  | 12±8  |
| hcol, THL | 12±5 | 9±2   | 19±7  |
| NaTDC, THL | 88±7| 8±3   | 22±6  |
| hcol, NaTDC, THL | 93±7| 9±4   | 27±8  |

FIGURE 4. Time course for the inhibition of PTL by THL in the presence of TDC and colipase. PTL (0.1 mg/ml) was incubated in 0.1 M Tris-HCl, pH 8.0, and 4% dimethyl sulfoxide with the indicated additions. The aliquots were removed at the indicated times to determine remaining activity in an assay with tributyrin in 4 mM NaTDC with a 5-molar excess of colipase. The symbols are defined in the figure legend. The best fit regression lines are shown.

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To determine which residues in PTL account for the large increase in fluorescence emission when PTL is incubated with THL and bile salts, we replaced each tryptophan in PTL with a tyrosine. Each mutant was characterized for activity against tributyrin, and the fluorescence emission spectra were recorded with and without THL, NaTDC, and colipase. We then confirmed our results with alanine or phenylalanine substitutions for selected tryptophans. Our results allow several conclusions. First, Trp-86 and Trp-107 contribute to the catalytic mechanism of PTL. Second, contrary to the prediction from the crystal structure of PTL, Trp-107 is not buried and is likely exposed to solvent. Third, changes in Trp-107 and Trp-253 fluorescence account for the increased fluorescence emissions of PTL that is stimulated by THL and NaTDC. Most likely, the increase represents the conformational changes that occur as PTL moves from the inactive to active conformation. Fourth, both THL and NaTDC are required to produce the increased fluorescence in PTL. Alone, neither is sufficient. Fifth, colipase does not significantly contribute to the conformational changes leading to increased emission fluorescence. Finally, the conformation of PTL in solution differs significantly from the conformation observed in crystals.

The decreased activity of Trp-86 and Trp-107 mutants likely reflects the location of the residues near or in the catalytic site. Trp-86 sits near the active site and may contribute to one acyl chain-binding site (32). Substitutions at this position could alter substrate binding and hinder efficient catalysis. Although Trp-107 does not appear to contribute directly to the catalytic site, it forms contacts with the side chain of His-76 in the β5-loop. This loop contributes to the active site and undergoes
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a conformational change when the lid domain opens (7). A change in the Trp-107–His-76 interaction may alter the conformation of the β5-loop and inhibit activity of the Trp-107 mutants. The greater effect of the W107Y substitution compared with the W107F substitution suggests that the hydroxyl group on tyrosine hinders interactions between residues 107 and 76.

Similarly, the reduced activities of the lid domain residue mutants, W253Y and W253A, likely reflect the importance of this domain in the function of PTL, particularly in forming an acyl chain-binding site (33, 34). The decreased activity of W403Y is unlikely related to changes in the catalytic site because the residue is located in the C-terminal domain a long distance from the catalytic site. Trp-403 is located in close proximity to the β5’-loop that is important for PTL function and near Lys-400 that contributes to the collapse-binding site (22, 35–37). Disruption of the local structure in this region by a substitution mutation in position 403 could alter the function of the PTL–colipase complex.

The largest effects on the fluorescence emission spectra were observed with the W30Y and W107Y lipases. Both substitutions produced a large blue shift in the wavelength of the emission maximum as well as a large increase in the $I_{330}/I_{350}$ ratio. These observations indicate that both residues are likely solvent-exposed. These observations conform to the calculated fraction of solvent exposure for Trp-30 based on the crystal structure and confirm the previously reported results for a W30F mutant (17). The result for Trp-107 was unexpected. Trp-107 is completely buried in the crystal structure, and its calculated fraction of solvent exposure is 0.00 (17). Quenching experiments with acrylamide also suggest that Trp-107 is relatively solvent-exposed. W107Y showed much less quenching compared with PTL. This effect was present even at low acrylamide concentrations. Because the most accessible residues are quenched at low concentrations, the results are compatible with a solvent-accessible location for Trp-107 rather than diffusion of acrylamide into the core of the protein. These unexpected findings suggest that the structure of PTL in solution differs from that found in the crystals.

We confirmed that the presence of THL and NaTDC produced a red shift in the maximum intensity and a large increase in the fluorescence intensity of PTL (11). These changes occurred for each substitution mutant except for the Trp-107 and Trp-253 mutants. These mutants did not have a red shift of the maximum fluorescence intensity. Only a small increase in peak fluorescence was observed for W107Y or W107A. An increase in fluorescence intensity was detected in the W253Y mutant, although it was ~3-fold smaller than the increase for PTL. W253A had a decrease in peak fluorescence. The difference in emission change between W253Y and W253A suggests there may be energy transfer from Tyr-253 to a tryptophan. In the open lid position, Tyr-253 moves away from Trp-30 (21.7 Å) and Trp-107 (17.9 Å) but remains ~14 Å from Trp-80, well within the Förster distance for tyrosine to tryptophan energy transfer (9–18 Å). These results clearly demonstrate that the change in the environment of Trp-253 as the lid moves from the closed to open conformation contributes to the fluorescence changes in the presence of THL and bile salts. In addition, a change in the environment of Trp-107 appears to make a significant contribution to the spectral changes. This change may occur as part of the conformational rearrangement of PTL during the transition from the closed to open form. As the lid opens, the β5-loop also rearranges to configure the oxyanion hole. Because Trp-107 contacts the β5-loop in the closed conformation, its microenvironment likely changes when the open conformation is adopted.

The mechanism for the increased emission intensity is not readily apparent from our data and published structures. Luthi-Peng and Winkler (11) speculated that the increased emission intensity resulted from decreased quenching of fluorescence present in the closed conformation. They noted that energy transfer could occur among the five N-terminal tryptophan residues and suggested that efficient quenching of the tryptophan acting as an acceptor would decrease fluorescence in the closed conformation (7, 8). With movement of the lid domain, they conjectured that the quenching was removed and peak emission fluorescence increased. Trp-107 and Trp-30 seem to be ideal candidates for participation in the proposed mechanism. Trp-107 is within 10 Å of Trp-30, the residue that dominates PTL fluorescence, in the closed conformation. Moreover, a recent study provided evidence for energy transfer from Trp-30 to the neighboring tryptophans (17). If energy transfer occurs from Trp-30 to Trp-107 and Trp-107 is quenched in the closed but not the open form, then an increase in emission intensity could occur with a conformational change. In the closed conformation, Trp-107 contacts His-76, a residue in the β5-loop. Histidines are important quenchers of tryptophan fluorescence in proteins, and His-76 is in position to strongly quench Trp-107. In the closed conformation the two residues are within 2.9 Å. With movement of the β5-loop, His-76 moves away from Trp-107, although they are still within 5.5 Å. The movement could decrease quenching and increase fluorescence. If this mechanism is correct, it predicts that substitutions of Trp-30 would decrease the change in emission when THL and bile salts are included. In fact, we observed a significant increase in emission at $\lambda_{max}$ for W30Y and W30A. This finding makes it unlikely that Trp-30 is an important donor for energy transfer to Trp-107 and could suggest that Trp-30 is an energy acceptor that quenches Trp-107 or Trp-253 or both. Furthermore, the observation that the $\lambda_{max}$ of W107Y showed a large blue shift suggests that Trp-107 is not highly quenched in the closed conformation. If it were, no change or a small red shift would have occurred because of the large contribution of Trp-30 to the fluorescence of PTL (17).

A simple model of energy transfer from Trp-30 to a quenched Trp-107 also does not explain the role of Trp-253 in the observed increase in emission intensity at $\lambda_{max}$ when PTL is incubated with THL and bile salts. Trp-253 clearly contributes as demonstrated by our observation that the emission increase was dampened significantly in the W253Y lipase and eliminated with the W253A substitution.

A previous study found that Trp-253 is weakly fluorescent in the closed conformation of PTL (17). Trp-253 lies quite near several potential quenching groups in the closed conformation, His-76, His-264, and Cys-182 (17). Trp-253 moves further way from each of these residues in the open conformation of PTL, and its intrinsic fluorescence may increase. The increase in the
intrinsic fluorescence of Trp-253 is likely small because the THL and NaTDC only increased the fluorescence of W107Y by \( \sim 9\% \). Alternatively, the fluorescence of Trp-253 may increase in the open conformation through energy transfer from another tryptophan. Based on our data, the most likely residue to participate in Trp-Trp homotransfer of fluorescence resonance energy is Trp-107. A model including limited quenching of Trp-107 and strong quenching of Trp-253 in the closed conformation with increased fluorescence of Trp-107 and decreased quenching of Trp-253 with fluorescence resonance energy transfer from Trp-107 to Trp-253 is consistent with our data. Still, it is likely that other tryptophan residues contribute to a complex network of energy transfer and quenching.

Regardless of the mechanism, the observation that the two tryptophans whose microenvironment changes when PTL adopts the open conformation are also the ones contributing to the increased emission intensity supports the hypothesis that the change in fluorescence intensity reflects the conversion of PTL to the open conformation and that fluorescence can be used to monitor this conversion. Our finding that the combination of NaTDC and THL produces the largest increase in maximum fluorescence and the most efficient inhibition of PTL as well as the earlier report showing identical kinetics of enzyme inactivation and the spectral changes support this conclusion (11). Studies of T. lanuginosus lipase fluorescence provide additional support for the notion that the fluorescence spectral changes correlate with lid movement (16, 38). In these studies, the fluorescence properties of a T. lanuginosus lipase mutant where three of the four tryptophans were substituted leaving on the lid domain tryptophan Trp-89 intact were studied. Both concluded that changes in T. lanuginosus lipase fluorescence induced by detergents or by mixed micelles of cis-parinaric acid and NaTDC reflected a change in Trp-89 and the conformation of the lid domain.

Incubation of PTL with micellar concentrations of NaTDC increased emission intensity as previously reported (11). These authors suggested that the fluorescence increase indicated an interaction of NaTDC with PTL. The interaction could occur with exposed tryptophans in PTL. Alternatively, the result could occur if NaTDC shifted a fraction of PTL into the open conformation as suggested by a study monitoring lid opening with site-directed spin labeling (15). From EPR measurements, the authors estimated that 40–50% of PTL converted to the open conformation in the presence of micellar NaTDC concentrations. If that is the case, then there is not a comparable increase in fluorescence intensity. Furthermore, these authors interpreted their data as showing an increase in the open conformation when colipase was included with NaTDC. This was not the case with fluorescence or THL inhibition demonstrated in this study. Colipase had little to no effect on either process. Also, there was no difference in the spectral changes when either the W107Y or W253Y lipases were incubated with NaTDC. If NaTDC induced an open conformation, we would expect these mutants to have less fluorescence increase than did PTL. The explanation for the apparent difference between fluorescence and EPR is not clear because the structural correlates for changes in either EPR or tryptophan fluorescence are both inferred and not directly demonstrated. It may be that NaTDC alters the conformation of the lid domain without fully configuring the active site, thereby reducing the mobility of the spin label without producing the changes necessary to alter fluorescence. Another possibility is that bile salt micelles interact directly with the spin label and restrict its mobility. The labeled residue, Asp-250, is fully exposed to solvent in both the closed and open conformations so NaTDC micelles could interact with the spin label in the closed conformation (15). Colipase could augment binding of micelles in the region of Asp-250 without the lid domain opening.

The fact that diethyl p-nitrophenyl phosphate inhibited PTL and the spin-labeled PTL only in the presence of NaTDC does not demonstrate that NaTDC alone induces lid opening as suggested (15). This is analogous to what we observed in this work. The fluorescence change was maximal when both THL and NaTDC micelles were present. Lipase inactivation by diethyl p-nitrophenyl phosphate requires the formation of mixed micelles (14). Likewise inhibition of PTL by THL is more efficient in the presence of bile salts, most likely because THL and bile salts form mixed micelles (39). We postulate that mixed micelles trigger the efficient opening of the lid domain, although we cannot exclude the possibility that the effect is because mixed micelles increase the concentration of THL near the active site.

In conclusion, we have demonstrated that the large increase in PTL steady state fluorescence in the presence of NaTDC and THL corresponds to a change in the microenvironment of Trp-107 and Trp-253. Because the crystal structures of PTL in the closed and open conformation demonstrate changes in the environment of both residues, increased tryptophan fluorescence likely reflects the conversion of PTL from the closed to open conformations. The full fluorescence change requires the presence of mixed micelles, in this case THL and NaTDC. Colipase contributes little to the conformation change, although it may stabilize the lid in the open position (40). Conversion of PTL into the open form most likely occurs at an interface.

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