Structural Basis for the Insensitivity of a Serine Enzyme (Palmitoyl-Protein Thioesterase) to Phenylmethylsulfonyl Fluoride*

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Palmitoyl-protein thioesterase-1 (PPT1) is a newly described lysosomal enzyme that hydrolyzes long chain fatty acids from lipid-modified cysteine residues in proteins. Deficiency in this enzyme results in a severe neurodegenerative storage disorder, infantile neuronal ceroid lipofuscinosis. Although the primary structure of PPT1 contains a serine lipase consensus sequence, the enzyme is insensitive to commonly used serine-modifying reagents phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate. In the current paper, we show that the active site serine in PPT1 is modified by a substrate analog of PMSF, hexadecylsulfonylfluoride (HDSF) in a specific and site-directed manner. The apparent $K_i$ of the inhibition was 125 μM (in the presence of 1.5 mM Triton X-100), and the catalytic rate constant for sulfonylation ($k_2$) was 3.3/min, a value similar to previously described sulfonylation reactions. PPT1 was crystallized after inactivation with HDSF, and the structure of the inactive form was determined to 2.4 Å resolution. The hexadecylsulfonyl was found to modify serine 115 and to snake through a narrow hydrophobic channel that would not accommodate an aromatic sulfonyl fluoride. Therefore, the geometry of the active site accounts for the reactivity of PPT1 with HDSF but not PMSF. These observations suggest a structural explanation as to why certain serine lipases are resistant to modification by commonly used serine-modifying reagents.

Palmitoyl-protein thioesterase-1 (PPT1) is a newly described lysosomal hydrolase that removes long chain fatty acids from lipid-modified cysteine residues in fatty acylated proteins (reviewed in Ref. 1). Deficiency of the enzyme leads to a lysosomal storage disease, infantile neuronal ceroid lipofuscinosis, which causes blindness, seizures, and cortical atrophy of the brain (2). Lysosomal inclusion bodies, termed granular osmiophilic deposits, accumulate in all tissues, and resemble lipofuscin deposits that occur during normal aging. In keeping with this important lipid-metabolizing role, PPT1 is one of the most abundant lysosomal enzymes in the brain (3). In contrast to most hydrolytic enzymes (such as proteases, lipases, esterases, and thioesterases), PPT1 is insensitive to the serine-modifying reagents phenylmethylsulfonyl fluoride (PMSF) and diisopropylfluorophosphate (DFP) (4). The insensitivity of PPT1 to these reagents was an important feature of PPT1 because it allowed for the identification of PPT activity in the presence of contaminating lipase and protease activities. We wondered whether there might be structural and/or mechanistic differences between PPT1 and these other lipolytic enzymes that would account for this observation. We have recently determined the three-dimensional crystal structure of PPT1 in the presence and absence of palmitoyl-CoA and shown that palmitate modifies serine 115 of the enzyme (5), confirming that a serine residue is the catalytic nucleophile.

In the current study, we show that although PPT1 is insensitive to inactivation by the serine-reactive reagent PMSF, the enzyme is covalently modified and inactivated by hexadecylsulfonyl fluoride (HDSF) by an active site-directed mechanism. The modified enzyme was crystallized, and the bound inhibitor was identified in the structure. The bound inhibitor defines a narrow, hydrophobic groove leading away from the active site that would not comfortably accommodate an aromatic sulfonyl fluoride. These findings suggest that the active site of PPT1 is readily susceptible to sulfonylation and that the insensitivity of PPT1 to PMSF is due to steric constraints related to the unique structure of the substrate-binding site.

EXPERIMENTAL PROCEDURES

Materials—Recombinant bovine PPT1 was overexpressed in the Sf9 cell baculovirus system and purified from serum-free SF-900II medium as described previously (6). The purity of the preparations were >90% as assessed by SDS-polyacrylamide gel electrophoresis, and the specific activity of the purified enzyme was 0.7–1.2 μmol of palmitoyl-CoA hydrolyzed per min per mg of purified protein. 3H-labeled palmitoyl-CoA was synthesized from 3H-palmitic acid as described previously (7). Hexadecylsulfonil fluoride was prepared as described (8).

Palmitoyl-CoA Hydrolyase Assays—The palmitoyl-CoA hydrolase ac-
Inactivation of PPT1 by Hexadecylsulfonylfluoride—Inactivation of purified recombinant PPT1 was carried out in buffer containing 50 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EDTA, and 0.1% Triton X-100 (buffer A) unless otherwise indicated. Triton X-100 (required to maintain solubility of the HDSF) was added to reaction mixtures from a concentrated stock solution (100 mM or lower) in Me₂SO. Incubations were carried out at 37 °C for varying amounts of time, and the inactivation reactions were stopped by 200-fold or greater dilution into ice-cold enzyme assay buffer. The inactivation was shown not to proceed during the assay under these conditions in control experiments (data not shown).

Kinetics of Inhibition—The inactivation of PPT1 was assumed to proceed via formation of an intermediate Michaelis complex as described previously (11). The reaction is assumed to follow the course shown in Equation 1.

\[
E + I \rightarrow (E-I) \rightarrow P
\]  
(Eq. 1)

where \( E \) is enzyme, \( I \) is inhibitor, \( E-I \) is the intermediate Michaelis complex, and \( P \) is the final inactivated product. When experiments are performed under pseudo-first-order conditions (i.e. HDSF well in excess over the enzyme) Equation 1 can be rewritten in the linear form as shown in Equation 2.

\[
1/k_t = 1/k_2 + K_0/[I]
\]  
(Eq. 2)

This equation describes the relation between the observed first-order rate constants for inactivation (\( k_2 \)), the rate of sulfonylation in the Michaelis complex (\( k_1 \)), the Michaelis constant \( K_m \), and the concentration of HDSF. Therefore, a double reciprocal plot of the observed first-order rate constants against HDSF concentration yields a straight line with a slope of \( k_1/k_2 \) and an intercept of \( 1/k_2 \) on the y axis. To determine the first-order rate constants, reactions were initiated by addition of 20–100 \( \mu \)M HDSF into reaction mixtures containing 1 \( \mu \)M of PPT1. Inactivation was followed by determining the activity remaining at closely spaced time intervals. Activities were expressed as the percentage of initial activity and plotted on a semi-log scale as a function of time. From the semi-log plots, the half-times of inactivation were determined. Observed pseudo-first-order rate constants were calculated using the following equation.

\[
k_2 = 0.693/t_{1/2}
\]  
(Eq. 3)

where \( t_{1/2} \) is the half-time of inactivation determined from semi-log plot. Once a first-order rate constant for each HDSF concentration was calculated, a double reciprocal plot of \( 1/k_t \) against \( 1/[I]\) was obtained. \( k_2 \) was calculated from the y-intercept, and \( K_m \) was determined from the slope as described above.

Crystallization of HDSF-inactivated PPT1—PPT1 (12 mg/ml in 20 mM HEPES, pH 7.0, 150 mM NaCl, and 0.01% Triton X-100) was inactivated by the addition of 1 mM HDSF. Crystals grew from sitting drop vapor diffusion experiments containing 3 \( \mu \)l of PPT1/HDSF and 2 \( \mu \)l of reservoir solution equilibrated against a 500-\( \mu \)l reservoir containing 55% polypropylene glycol 400 (Fluka) and 100 mM Bis-Tris, pH 6.5. Crystals were harvested from the drops in nylon loops and flash-frozen in liquid nitrogen.
RESULTS

In Fig. 1, the chemical structures of the alkylating agents used in this study, HDSF and PMSF, are shown for comparison with a natural substrate of PPT1, a palmitoyl cysteine thioester that normally occurs in the context of a lipid-modified protein or peptide (18). PPT1 prefers acyl chain lengths of 14–18 carbons (6), and no appreciable hydrolysis is observed for chain lengths under eight carbons. Previous work has also shown that there are no major constraints on the nature of the attached “leaving group,” because PPT1 readily hydrolyzes other long chain fatty acyl thioesters, such as palmitoyl-CoA (6) and S-palmitoyl thioglycoside (19). The current studies presented below utilized palmitoyl CoA as a substrate because of the availability of facile assays using [3H]palmitoyl-CoA. (However, the kinetic studies presented in Fig. 2A and B were obtained using either [3H]palmitoyl-CoA or [3H]palmitate-labeled Ha-Ras with virtually identical results [data not shown].)

Fig. 2A shows that incubation of PPT1 with PMSF (5 mM) had no appreciable effect on enzyme activity over that seen with solvent (Me2SO) alone, whereas incubation with HDSF (5 mM), an alkylating agent with a 16-carbon fatty acyl chain, resulted in a very rapid loss of enzyme activity, with 80% inactivation at 1 min and loss of measurable activity at 4 min. PPT1 has a broad pH optimum with a peak of pH 7.0 when palmitoylated protein or palmitoyl-CoA is used as the substrate. The rate of inactivation of PPT1 by HDSF as a function of pH paralleled this broad pH optimum with the exception that at pH above 7.5, the rate of inactivation became higher but was confounded by an HDSF-independent inactivation that was due to enzyme instability (data not shown). Therefore, the remainder of the studies were conducted at pH 7.0.

As illustrated in Fig. 2B, under pseudo-first-order reaction conditions (i.e., concentration of HDSF at 20–50 times over enzyme concentration), the inactivation of PPT1 by HDSF followed first-order kinetics as shown by the linear relationship between the natural logarithm of remaining activity and reaction time. No loss of activity was observed within the time period of inactivation when HDSF was omitted from the reaction mixture (data not shown). To determine the stoichiometry of inactivation, a precisely determined amount of enzyme was reacted with increasing amounts of HDSF under conditions where the reaction was expected to go to completion (2 h at 37 °C), and the remaining activity was determined (Fig. 2C). 1 mol of HDSF was needed to inactivate 1 mol of enzyme, suggesting that only one PPT1 functional group became modified in the course of inactivation.

In our kinetic study we have assumed that the inactivation of the enzyme by HDSF proceeds through the formation of an intermediate Michaelis complex, as is true for most of the serine hydrolases. To determine the kinetic constants for inactivation, we measured the rate of inactivation of PPT1 at increasing HDSF concentrations under pseudo-first-order conditions. We observed that a plot of the slopes of the natural logarithm of activity remaining became saturated at increasing inhibitor concentration, which is a characteristic of specific (site-directed) irreversible inhibition (20–22). A double reciprocal plot of the observed first-order rate constant of inactivation as a function of HDSF concentration is shown in Fig. 2D (open circles). The y intercept yields the reciprocal of the rate constant of sulfonlation in the Michaelis complex, and the slope (K_i/k_2) yields the inhibitor concentration at which the half-maximal rate is observed. From Fig. 2D, we derive a K_i of 125 μM and k_2 of 3.3/min for the inactivation of PPT1 by HDSF. The K_i does not represent a true Michaelis dissociation constant in this case, because HDSF is insoluble in aqueous solution and requires Triton X-100 (1.5 mM) for solubilization. In a micellar system, surface concentration in two dimensions is the relevant value, but because it is not possible to measure the surface concentration directly, the concentration is usually expressed as a mole fraction of inhibitor in the detergent micelle, irrespective of the total concentrations of inhibitor and detergent (23, 24). In this case, the K_i reflects a half-maximal rate of inactivation at a mole fraction of HDSF in the Triton X-100 micelle of 0.061, which is somewhat higher than the “affinity constant” of 0.022 measured in a similar experiment that examined the inactivation of Escherichia coli phospholipase A_2 by HDSF (8). The k_2 compares favorably with the inactivation of chymotrypsin by PMSF (3.1/min) (11) and with the k_2 measured for the inactivation of E. coli phospholipase A_2 with HDSF (6–17/min) (8). The experiments used to generate the curve in Fig. 2D in the absence of palmitoyl-CoA (open circles) were repeated in the presence of 15 μM palmitoyl-CoA (closed circles) and 25 μM palmitoyl-CoA (closed triangles), which are concentrations that are close to the K_i of PPT1 for palmitoyl CoA (40 μM; data not shown). As shown in Fig. 2D, substrate protection from the specific irreversible inhibition of PPT1 by HDSF was demonstrated, as reflected in the unchanged rate constant of sulfonlation (k_2) in the presence of substrate, whereas the inhibition constant K_i...
increased in the presence of substrate (to 235 and 273 μM at 15 and 25 μM palmitoyl-CoA, respectively). The protection of inactivation by substrate confirms an active site-directed mechanism.

HDSF-inactivated PPT1 was crystallized and its three-dimensional crystal structure determined at 2.4 Å resolution (Fig. 3). The binding of HDSF to PPT1 closely mimics the binding of substrate to PPT1 (5). The hexadecyl chain occupies the same hydrophobic groove that palmitate was shown to occupy in our previous structure (Fig. 4A), and serine 115 is covalently modified by the sulfonyl group. There are no significant differences observed between the structure of HDSF-inactivated PPT and the previously determined structures of PPT1 alone and PPT1 bound to palmitate.

Superposition of the crystal structure of PMSF-inactivated Pseudomonas carboxylesterase (Protein Data Bank code 1AUR) (Fig. 4B) with HDSF-inactivated PPT1 sheds some light on the molecular details of the specificity of HDSF over PMSF for inactivation of PPT1. The narrow hydrophobic groove leading away from the active site that provides substrate specificity in PPT1 begins with Met41 and Ile235 and is less than 5 Å wide at this point (Fig. 4A). The side chain of Met41 in PPT1 occupies the space where the aromatic ring of PMSF is found in the carboxylesterase structure (Fig. 4C). The side chain of Ile235 would block alternative conformations of PMSF from the active site, and the surrounding environment provides no space to accommodate alternate conformations of the Met41 side chain, which would make room for the PMSF. In contrast, HDSF is able to adopt a conformation that蛇 through the channel (Fig. 4A).

**FIG. 4.** A, HDSF occupies the narrow substrate-binding cleft in PPT1. B, in contrast, the *Pseudomonas* carboxylesterase active site, occupied here by PMSF, is more open and able to accommodate a wider range of substrates. C, *Pseudomonas* carboxylesterase residues and PMSF (red) superimposed on the PPT1 active site. The structures were aligned using LSQAB (36) from the CCP4 suite, using the α-carbons of the β sheet and the catalytic triad residues. Note that the side chain of Met41 occupies the space where PMSF is bound in the carboxylesterase structure, and the presence of Ile235 on the other side of the groove does not provide enough space for PMSF to adopt an alternative conformation.
Enzymes are often classified mechanistically on the basis of their susceptibility to inactivation by class-specific reagents. This classification scheme has served proteolytic enzymes well; for example, serine proteases are nearly universally sensitive to DFP, and classification on the basis of sensitivity to DFP applies to serine proteases such as the digestive proteases and the blood coagulation factors. Lipolytic enzymes have been more problematic, because sensitivity to class-specific reagents is not universal and because detailed reaction mechanisms for many classes of lipolytic enzymes are not well understood. For example, the enzymes lecinthin-cholesterol acyltransferase and lipoprotein lipase are readily inactivated by DFP, but the modification occurs not on the catalytic serine but on a second serine that is probably related instead to the substrate-binding site (25). Several additional secreted lipases, most notably bacterial lipases from *Staphylococcus hyicus* (26) and *E. coli* (phospholipase A2) (8) and pancreatic lipase (27, 28), are insensitive to DFP and PMSF and yet use serine as the catalytic nucleophile. In the case of the two bacterial lipases, the involvement of a serine residue in the catalytic mechanism was inferred by their sensitivity to the long chain alkylating agent HDSF (8, 29). Protein lipase has been well documented to utilize a classical serine-based triad mechanism based on analysis of its crystal structure indicates that PPT1 binds the long chain hydrocarbon in a narrow, hydrophobic groove that would not support this view, in that the narrow channel leading from the active site serine in PPT1 places severe constraints on the structures of molecules that could be accommodated there, either as substrates or inhibitors. In summary, we have shown that a lysosomal thioesterase, PPT1, is similar to several other “secreted” lipases in showing insensitivity to class-specific reagents DFP and PMSF. PPT1 is readily inactivated by a sulfonyl fluoride (HDSF) that is structurally more similar to its naturally occurring fatty acyl-containing substrates. The inactivation was shown to be clearly related to modification of a serine residue, which is in a position to act as the catalytic nucleophile in a classical serine-histidine-aspartic acid triad. The relatively closed conformation around the active site of PPT1 accounts for the insensitivity of PPT1 to the classical serine-specific reagent PMSF and provides a structural explanation for the resistance of serine lipases to inactivation by serine-modifying class-specific reagents.

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