Additive Effects of Acyl-Binding Site Mutations on the Fatty Acid Selectivity of *Rhizopus delemar* Lipase

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ABSTRACT: The fatty acid specificity and pH dependence of triacylglycerol hydrolysis by the *Rhizopus delemar* lipase acyl-binding site mutant Val206Thr + Phe95Asp (Val, valine; Thr, threonine; Phe, phenylalanine; Asp, aspartic acid) were characterized. The activity of the double mutant prolipase was reduced by as much as 10-fold, compared to the wild-type prolipase. However, the fatty acid specificity profile of the enzyme was markedly sharpened and was dependent on the pH of the substrate emulsion. At neutral pH, strong preference (10-fold or greater) for hydrolysis of triacylglycerols of medium-chainlength fatty acids ($C_{8:0}$ to $C_{14:0}$) was displayed by the variant prolipase, with no hydrolysis of triacylglycerols of short-chain fatty acids ($C_{4:0}$ to $C_{6:0}$) and little activity manifested toward fatty acids with 16 or more carbons. At acidic pH values, the fatty acid selectivity profile of the double mutant prolipase expanded to include short-chain triacylglycerols ($C_{4:0}$, $C_{6:0}$). When assayed against a triacylglycerol mixture of tributyrin, tricaprylin and triolein, the Val206Thr + Phe95Asp prolipase displayed a high selectivity for caprylic acid and released this fatty acid at least 25-fold more efficiently than the others present in the substrate mixture. When presented a mixture of nine fatty acid methyl esters, the wild-type prolipase showed a broad substrate specificity profile, hydrolyzing the various methyl esters to a similar extent. Contrastingly, the double mutant prolipase displayed a narrowed substrate specificity profile, hydrolyzing caprylic methyl ester at nearly wild-type levels, while its activity against the other methyl esters examined was 2.5- to 5-fold lower then that observed for the wild-type enzyme.

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KEY WORDS: Fatty acid specificity, lipase, methyl ester hydrolysis, molecular modeling, mutagenesis, protein structure, triacylglycerol hydrolysis.

Lipases (acylglycerol acylhydrolase, E.C. 3.1.1.3) are enzymes that catalyze the reversible cleavage of the ester bonds of triacylglycerols to yield free fatty acids, diacylglycerols, monoacylglycerols, and glycerol. Various lipases show degrees of regiospecificity, acyl selectivity, or stereospecificity toward their substrates (1–3). Lipases also catalyze the hydrolysis of a wide range of other water-insoluble esters, in some cases enantioselectively. For industrial application, lipases with pronounced selectivities (regio-, stereo-, or fatty acid) are of special interest because these specificities may be exploited to produce products not obtainable by conventional chemical catalysis (4,5).

The fungus *Rhizopus delemar* (*Rd*) (presently designated *R. oryzae*) produces extracellular lipases, one of which has been extensively characterized. This enzyme (*Rd* lipase), like the closely related *Rhizomucor miehei* and *Humicola lanuginosa* lipases, is termed a 1,3-specific lipase, because it exhibits strong preference for the hydrolysis of ester bonds at the *sn*-1 and *sn*-3 positions of a triacylglycerol. The *Rd* lipase has been purified to homogeneity (6), a complementary DNA encoding the enzyme has been cloned and sequenced (7), and the mature (269 amino acids) and proenzyme (includes 97 amino acid propeptide) forms of the lipase have been expressed to high levels in *Escherichia coli* (8). The crystal structure of *Rd* lipase has been solved at 2.6 Å resolution (9). The molecular architecture of the enzyme consists of a single, roughly spherical domain that contains predominantly parallel β-sheets with conserved α-helices packed on either side. The active center consists of a triad of Ser145, His257 and Asp204 (Ser, serine; His, histidine; Asp, aspartic acid), while an oxyanion hole, presumably formed in the active species by the hydroxyl and main-chain amide groups of Thr83 (Thr, threonine), is postulated to stabilize the tetrahedral transition state intermediate (9). Recently, additional functional groups that are critical for the electrostatic stabilization of reaction intermediates in *Rd* lipase have been identified (10). In *Rd* lipase, the catalytic center is buried beneath a surface loop or lid, which consists of a short amphipathic helix (11,12). The acyl-binding region of *Rd* lipase consists of a short hydrophobic trough on the enzyme surface beneath the lid. Upon adsorption to a lipid–water interface, the lid helix is displaced. This exposes the active site, creates the oxyanion hole, and increases the surface hydrophobicity of the enzyme in this region.

A major objective of protein engineering of lipases is to sharpen the substrate specificity of these enzymes with regard to the chainlength of the fatty acids whose esters they will hydrolyze. Joerger and Haas (13) initiated studies to alter chain-

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length selectivity of *Rd* lipase through site-directed mutagenesis of conserved acyl-binding groove residues. Using single-site mutagenesis, they found that the replacement of Phe95 and of Val206 (Phe, phenylalanine; Val, valine) with the remaining 19 amino acids resulted in the production of two variants, Phe95Asp and Val206Thr, that displayed modest increases in their specificity toward tricaprylin as a substrate, relative to triolein or olive oil. These studies, and those of Klein *et al.* (14), indicate that modest gains in fatty acid selectivity can be achieved by single amino acid substitutions in the acyl-binding groove of *Rd* lipase.

However, engineering a lipase with pronounced fatty acid selectivity may require the accumulation of multiple mutations. The crystal coordinates of wild-type *Rd* lipase indicate that Val206 and Phe95 are located in spatially distant parts of the acyl-binding groove of the enzyme (9). Because structural perturbations that result from amino acid substitutions are often accompanied by only localized changes in protein structure (15), changes in amino acid sequence in such spatially distant parts of the acyl-binding groove of the lipase may have cumulative effects on substrate specificity. Therefore, the two single-site mutations, shown by previous work to increase selectivity, were introduced into the same lipase gene, and the substrate selectivity and other properties of the resulting double mutant, Phe95Asp + Val206Thr, were determined. Because alteration of the electrostatics of an active site can cause significant changes in the pKa values of ionizable residues residing there (16), it was of particular interest to examine the pH-activity profile of this double-mutant lipase.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutants of *Rd* prolipase were generated with the Sculptor™ *in vitro* mutagenesis system (Amersham Corp., Arlington Heights, IL) as previously detailed (14). To construct the double mutant Val206Thr + Phe95Asp, single-strand DNA with the single mutation Phe95Asp was used as template for an additional cycle of site-directed mutagenesis. The sequence of the doubly mutated prolipase DNA in the regions of its mutations was determined as previously described (17). The double-mutant DNA was subcloned into plasmid pET 15b (Novagen Corp., Madison, WI) for the production of recombinant prolipase in *E. coli*.

Synthesis and purification of recombinant lipases. Escherichia coli BL21(DE3) cells were transformed with recombinant pET-prolipase plasmids and grown in liquid culture medium (M9ZB + 100 μg/mL carbenicillin) at 37°C as previously described (18). Synthesis of recombinant prolipase was induced by the addition of 10 μM isopropyl-β-D-thiogalactoside at a culture $O.D._{600} = 1.0$, and the resulting proteinaceous inclusion bodies were isolated (8). These were solubilized with 8 M urea, and the enzyme was refolded essentially as described (8) except that the refolded lipases were not further purified by affinity chromatography. Such complete purification was not required because the purpose of these studies was to characterize the double-mutant lipase, and it is known that the *Rd* enzyme is the only source of lipolytic activity in extracts from this system (Haas, M.J., and R.D. Joerger, unpublished observation).

Determination of lipase activity. Quantitative determination of lipase hydrolytic activity with single homotriacylglycerol substrates was accomplished titrimetrically in a VIT 90 Video Titrator (Radiometer, Copenhagen, Denmark). Standard emulsions contained 200 mM triacylglycerol, 15 mM $CaCl_2$ and 4.2% (wt/vol) gum arabic. Reactions were initiated by the addition of approximately 5 μg enzyme. Reactions were conducted at 26°C with a set-point pH of 7.0 unless stated otherwise. Lipase activity was calculated by computer from the maximum rate of titrant addition by means of a lipase titrimetric program (19). Titrimetric assays were run in duplicate or triplicate. For examining the pH dependence of catalysis, the Video Titrator set-point pH was modified to the desired pH value, and lipase activity was quantitated.

Competitive hydrolysis of triacylglycerols was examined at 26°C in reactions that contained 15 mM $CaCl_2$ and equiweight mixtures of tributyrin, tricaprylin, and triolein (2.0% wt/vol; Sigma, St. Louis, MO), brought to 5 mL with H_2O. Triacylglycerol mixtures were sonicated, and reactions were initiated by the addition of lipase (5–10 μg/mL reaction volume). Reaction pH was monitored and maintained at 7.0 with a VIT 90 Video Titrator. At selected times, reactions were quenched on ice, and 5 mg stearyl alcohol (in 100% ethanol) was added as an internal standard. Reaction mixtures were extracted with 5 vol of hexane, and 5 to 10 μL of the hexane phase was injected into a Hewlett-Packard (Avondale, PA) Model 1050 high-performance liquid chromatograph, equipped with a LiChrosorb Diol column (3 × 100 mm, 5 μm bead size; Chrompack, Inc., Raritan, NJ) that was then eluted with a gradient of isopropanol in hexane (14). Peaks eluting from the column were detected with an Alltech-Varex Mark III evaporative light-scattering detector (Deerfield, IL), operating at a drift tube temperature of 41°C and a nitrogen flow rate of 1.36 L/min. Standard curves were generated for tributyrin, tricaprylin, and triolein as detailed (14), and lipolytic activities were quantitated by measuring the disappearance of triacylglycerol substrates, compared to control reactions with boiled lipase.

Competitive hydrolysis of fatty acid methyl esters was examined in 1- to 2-mL reactions that contained 50 mM phosphate buffer (pH 7.0) and equiweight mixtures of the methyl esters of caprylic, capric, lauric, myristic, and palmitic acids plus either stearic, oleic, linoleic, or linolenic acid methyl ester (2% wt/vol of each methyl ester; Sigma). All methyl ester mixtures were liquid at the selected reaction temperature (28°C). Methyl ester mixtures were sonicated, and reactions were initiated by the addition of lipase (40 μg/mL reaction volume) and shaken at 330 rpm. At selected times, the reactions were sonicated to thoroughly remix them, and the free fatty acids in a 10-μL sample were silylated with *N,O*-bis(trimethylethylsilyl)trifluoroacetamide (BSTFA, 400 μL) in the presence of 200 μL dry pyridine. Silylation reactions

were incubated for 15 min at 70°C, cooled to room temperature, and 1 mL of hexane was added to each reaction. The solutions were then filtered through Millex-FH_{13} filter units (Millipore, Bedford, MA). Five μL of each sample was injected on-column into a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph with a 15-m nonpolar high-temperature column DB1-HT with an inner diameter of 0.32 mm and a film thickness of 0.1 μm (J&W Scientific, Folsom, CA). The helium flow rate was 4 mL/min with an injector temperature of 310°C and a detector temperature of 350°C. The initial oven temperature of 40°C was held for 5 min and then increased to 170°C at a rate of 4°C/min. Peaks on the chromatograms were identified by comparing their retention times to those of methyl ester standards and to fatty acid standards after derivatization with BSTFA as described above. Data collection and processing were conducted with a Hewlett-Packard 3396 Series II integrator. Quantitation of methyl ester hydrolysis was based on quantifying the appearance of free fatty acids after selected periods of incubation with lipase. Standard curves for each fatty acid were generated based on eight injections of mixtures of silylated fatty acid standards.

Molecular modeling. Molecular modeling was conducted with the SYBYL (version 6.0) software package (Tripos Associates, St. Louis, MO) on an SGI Indigo2 workstation (Silicon Graphics, Mountain View, CA). Molecular mechanics, molecular dynamics, and all related modeling simulations were conducted as detailed previously (14).

RESULTS AND DISCUSSION

Protein engineering provides a means of elucidating the structure–function relationships of lipases and potentially of tailoring the substrate selectivity of a lipase. The focus of the present research was to engineer a lipase with pronounced selectivity for medium-chain fatty acids. Joerger and Haas (13) conducted a systematic study of the replacement of four conserved acyl-binding groove residues of *Rd* lipase. These efforts resulted in the identification of two single-mutant enzymes, Val206Thr and Phe95Asp, that exhibited modest improvements in selectivity for medium-chain fatty acids. Against single substrates, these mutant enzymes were two- to threefold more active against tricaprylin than was the wild-type enzyme. Against mixed substrates, they were 40 to 80% more active toward tricaprylin than the parent enzyme. While the substrate selectivities of these mutant lipases were an improvement over that of the wild-type lipase, it became apparent that engineering a variant lipase that is truly fatty acid-specific will most likely require the accumulation of multiple mutations in the acyl-binding groove. To this end, we have employed site-directed mutagenesis to identify small substitutions, even single amino-acid replacements, that enhance the fatty acid selectivity of *Rd* lipase, and then combined these substitutions into a single lipase gene. A similar strategy has been used for increasing the stability of subtilisin E in organic solvents (20).

The pH dependence of hydrolysis of each of three triacylglycerols by the double-mutant prolipase is illustrated in Figure 1. Activity and substrate selectivity were strongly affected by pH. At pH 7.0, the enzyme exhibited pronounced selectivity for tricaprylin hydrolysis, no hydrolytic activity toward tributyrin, and an activity toward olive oil that was approximately 11-fold less than that toward tricaprylin. Below pH 7.0, tributyrin hydrolysis increased, peaking near pH 6.0. In contrast, activity toward tricaprylin and triolein decreased below pH 7. At and above pH 7.5, the double-mutant prolipase was inactive. This is in contrast to the wild-type prolipase, which displayed maximum hydrolytic activity between pH 7.5 and 8.0 (8). This inhibition of hydrolysis at alkaline pH was not due to denaturation of the double-mutant prolipase: full activity was restored by lowering the pH to 7.0 (data not shown).

The pH dependence of triacylglycerol hydrolysis by the single amino-acid mutants Val206Thr and Phe95Asp was subsequently examined to determine whether the unique pH profiles displayed by the double mutant could be ascribed to either (or both) of the single-point mutants. The single amino-acid mutants Val206Thr and Phe95Asp exhibited pH profiles similar to that of the wild-type prolipase and displayed maximum hydrolytic activity between 7.5 and 8.0 (data not shown). Hence, the unique pH profile of the Val206Thr + Phe95Asp double mutant cannot be ascribed to either of the single amino-acid substitutions alone. It is a consequence of the cumulative effect of both substitutions. One implication of the pronounced pH sensitivity of the substrate selectivity of the double-mutant prolipase is that, by precise pH control, it may be possible to selectively release mid-chain fatty acids from a mixed glyceride substrate. Another is that, by altering the reaction pH from 7.0 to 6.0 during a reaction, the substrate specificity of the double mutant would expand to include short-chain as well as medium-chain fatty acids.

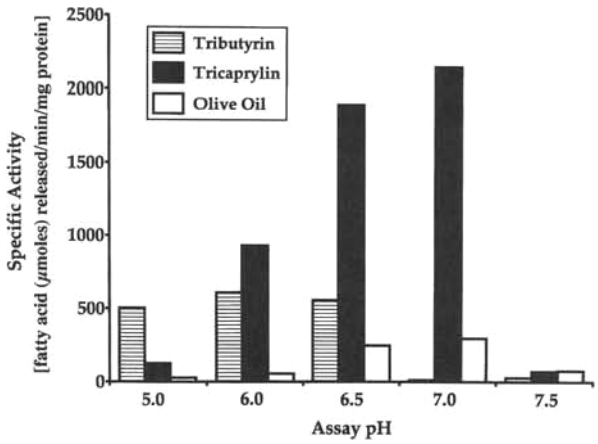

FIG. 1. Effect of emulsion pH on hydrolysis of triacylglycerols by the Val206Thr + Phe95Asp (Val, valine; Thr, threonine; Phe, phenylalanine; Asp, aspartic acid) prolipase. Specific activities were measured titrimetrically against single triacylglycerol emulsions of tributyrin, tricaprylin, or olive oil at the indicated emulsion pH. Each specific activity is the mean of two or three independent assays.

Comparison of the specific activities of the Val206Thr + Phe95Asp double mutant to the specific activities reported previously (13) for the respective single amino-acid mutants indicates that, in some cases, the effects of these substitutions on substrate selectivity against single homoacylglycerols are largely additive: The specific activity of the Phe95Asp mutant for tricaprylin was 5.7-fold greater than that toward olive oil, while the Val206Thr enzyme showed a 3.7-fold greater activity toward tricaprylin than toward olive oil (Ref. 13, Table 4). By comparison, at pH 7, the double-mutant Val206Thr + Phe95Asp displayed a 7-fold greater specific activity for tricaprylin than for olive oil (Fig. 1). In other instances, more profound changes occur. For example, the Phe95Asp and Val206Thr single mutations each reduce activity toward tributyrin by 1.5- to 2-fold, relative to the wild-type enzyme (13). But in the double-mutant enzyme, the activity toward this substrate was so greatly reduced as to be virtually undetectable. As a consequence, the double mutant hydrolyzed tricaprylin on the order of 100 times better than tributyrin (Fig. 1), while the parent single mutations hydrolyzed it only 6- to 7-fold more rapidly than tributyrin (13).

A more stringent test of the fatty acid selectivity of a lipase than the use of individual homotriglycerides involves the use of substrate mixtures of a variety of fatty acid acylglycerols. This can include blends of natural oils, mixtures of pure triacylglycerols, or mixtures of aliphatic esters (e.g., methyl esters). A substrate that consists of mixed triacylglycerols simultaneously presents a lipase with a variety of fatty acids at the lipid–enzyme interface and therefore should permit an accurate assessment of the substrate selectivity of the lipase. Thus, the hydrolytic activity of the Val206Thr + Phe95Asp mutant prolipase against a substrate mixture composed of tributyrin, tricaprylin and triolein was determined, with the amount of each triacylglycerol remaining after the incubation being determined by high-performance liquid chromatography (Fig. 2). The pH of these assays was held at 7.0, the pH where the double-mutant enzyme exhibited the highest chain-length specificity (Fig. 1). In agreement with the results of single substrate assays, the double-mutant prolipase displayed strong selectivity for tricaprylin. After 15 min of incubation, 25% of the tricaprylin initially present had been hydrolyzed, while only 1% of the triolein and none of the tributyrin were hydrolyzed. After 30 min of hydrolysis, more than 35% of the tricaprylin had been hydrolyzed, while less than 3% of the triolein and none of the tributyrin were hydrolyzed. After 60 min, the double-mutant prolipase still exhibited preference for tricaprylin hydrolysis, although the selectivity was not as absolute as during earlier reaction times: 60% of the tricaprylin had been hydrolyzed, while 15 and 17% of the tributyrin and triolein, respectively, were hydrolyzed. This is in contrast to the wild-type prolipase, which showed a modest approximately 2-fold preference for tricaprylin during the initial 15 min of competitive hydrolysis (see inset, Fig. 2) and no greater than a 2-fold difference in the hydrolyses of these three substrates at longer incubation times (13).

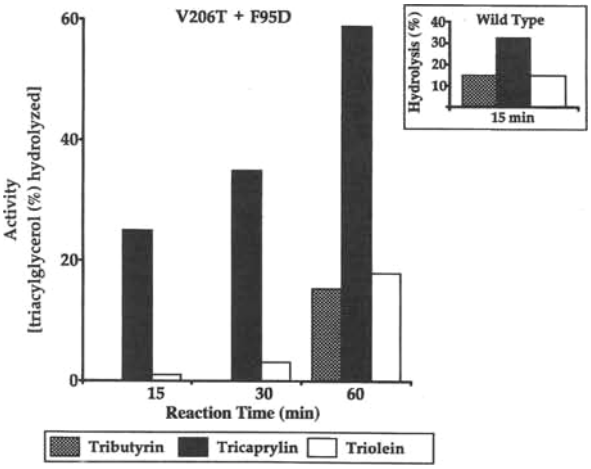

FIG. 2. Hydrolysis of triacylglycerol mixtures of tributyrin, tricaprylin, and triolein by Val206Thr + Phe95Asp mutant and wild-type *Rd* prolipase. Hydrolytic activities were determined by quantitating the amounts of unhydrolyzed triacylglycerols remaining after prolipase incubation with an equiweight mixture of tributyrin, tricaprylin, and triolein (pH 7.0; 26°C). The amount of each triacylglycerol remaining after various periods of incubation with enzyme was quantitated by high-performance liquid chromatography. Competitive hydrolysis assays were conducted a minimum of four times for each enzyme, and a representative data set is shown. See Figure 1 for abbreviations.

The striking, unprecedented, nearly exclusive hydrolysis of tricaprylin during the initial 30 min of these reactions paralleled the specific activities determined with single triacylglycerol emulsions. The decline in fatty acid selectivity with increased reaction times is a relatively common observation. Of greater significance, especially for future engineering efforts, was the apparent potentiation of specificity in the substrate competition experiments that was obtained by combining single-site lipase mutations into one enzyme. It has been previously demonstrated (Ref. 13, Table 5) under similar reaction conditions that the single-site mutations Val206Thr and Phe95Asp each resulted in a 2-fold greater hydrolysis of tricaprylin than of tributyrin, while the present study showed that the double mutant displayed at least a 20-fold difference (Fig. 2). This is consistent with, though not as striking as, the results with individual substrates (Fig. 1). Similarly, tricaprylin was hydrolyzed about six times faster than triolein by the double-mutant prolipase, whereas it was hydrolyzed only between 1.2- and 2-fold better by the singly mutated enzymes (13). It may be possible to obtain even greater fatty acid selectivity by combining additional mutations into a single lipase. The additivity and potentiation of impacts on substrate range also suggest that mutations at positions 95 and 206 exert their effects by independent modes of action.

To further characterize the substrate selectivity of the double-mutant prolipase, titrimetric assays of this and the wild-type enzyme were conducted at pH 7.0 with a more extensive range of triacylglycerol substrates (Fig. 3). The activity of the double-mutant prolipase was always less than that of the wild-type enzyme. (The parent single mutations reduce activity relative to that of the wild-type enzyme [13].) However, the degree of reduction of activity was different for different fatty acid chainlengths. The hydrol-

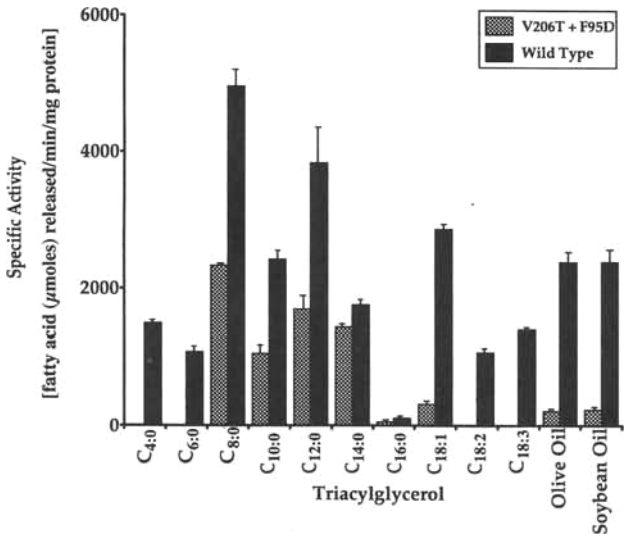

FIG. 3. Comparison between the specific activities of Val206Thr + Phe95Asp mutant and wild-type *Rd* prolipase in single-triacylglycerol emulsions. Specific activities were measured by titrimetric assay against single-triacylglycerol emulsions as described for Figure 1, except the reaction pH was maintained at 7.0 for all assays. See Figure 1 for abbreviations.

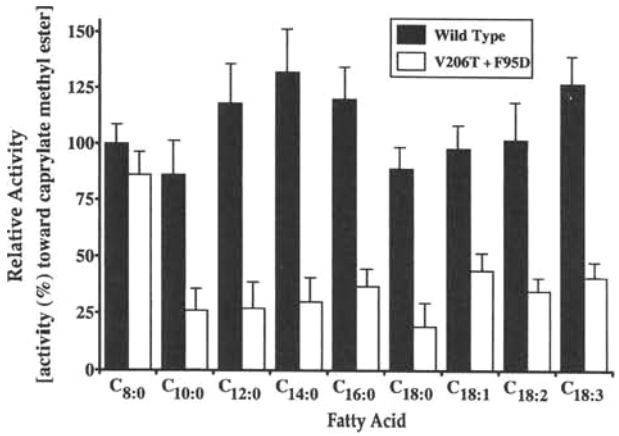

FIG. 4. Competitive hydrolysis of methyl ester mixtures by Val206Thr + Phe95Asp mutant and wild-type *Rd* prolipases. Quantitation of methyl ester hydrolysis was based on the appearance of free fatty acids after selected periods of incubation with lipase. Free fatty acids were derivatized with *N,O*-bis-trimethylethylsilyl-trifluoroacetamide and subsequently analyzed by gas chromatography. The quantity of caprylic acid released by the wild-type *Rd* prolipase was set at 100%. See Figure 1 for abbreviations.

ysis of tributyrin ($C_{4:0}$) and tricaprin ($C_{6:0}$) was below the detection limits, and its activity toward olive and soybean oils (both rich in 18-carbon fatty acids with 0 to 3 double bonds) was 10- to 12-fold lower than that of the wild-type enzyme. Similarly, the specific activity of the Val206Thr + Phe95Asp prolipase for triolein ($C_{18:1}$) was 9-fold lower than that observed for the wild-type enzyme [Activity was not determined toward tristearin ($C_{18:0}$) with the titrimetric assay because the physical nature of its emulsions at room temperature prevented an accurate assay]. The activity toward medium-chain substrates ($C_{8:0}$ to $C_{14:0}$) was reduced twofold, compared to the wild-type enzyme. The net result was that the double-mutant prolipase exhibited less activity but marked substrate selectivity for medium-chain substrates, compared to the wild-type enzyme.

The melting temperature of an acylglycerol depends on the position, chainlength, and degree of unsaturation of its fatty acids, and it affects its suitability as a lipase substrate. To avoid the possible artifactual effects of this phenomenon, fatty acid methyl esters, which are generally all liquids at room temperature, have been used in the characterization of lipase specificities (21). Accordingly, the fatty acid selectivity of the double-mutant prolipase was examined with a mixture of fatty acid methyl esters as substrate (Fig. 4). The wild-type *Rd* prolipase showed a broad substrate selectivity, with a slight preference for methyl esters of lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), and linolenic (18:3) acids. In contrast, the double-mutant prolipase displayed a much narrower substrate range. Its activity toward methyl caprylate was nearly equivalent to that of the wild-type enzyme, while its activity toward other fatty acid methyl esters was reduced 2.5- to 5-fold, relative to the wild-type enzyme. This selectivity of the double-mutant prolipase for methyl caprylate was evident at all time points examined (from 1 to 36 h of incubation) and at various methyl ester concentrations (data not shown).

The fatty acid specificity profile of the double-mutant prolipase was much narrower toward methyl esters than toward triglycerols (Figs. 2 and 3 vs. Fig. 4). This may reflect true differences between the esterolytic and lipolytic activities of this enzyme. On the other hand, it may reflect features of the substrates other than the chainlengths of their fatty acids. For example, Briand *et al.* (21) determined that the nature of the alcohol moiety of aliphatic esters markedly affected their hydrolysis by *Candida parapsilosis* lipase. It is possible that the identity of the alcohol moiety plays a similar role in determining the fatty acid specificity of the *Rd* lipase. The use of additional classes of esters (e.g., nitrophenyl, ethyl, etc.) in the characterization of this enzyme and its mutational derivatives may produce a clearer, more comprehensive view of substrate specificity.

The double-mutant lipase studied here exhibited a striking dependence of activity and substrate selectivity on pH. Previous reports have described changes in the pH dependence of catalysis by serine proteases after alteration of the surface charge of the enzyme (16,22). For example, Russell *et al.* (16) reported that site-directed mutation of residues at the rim of the active site of subtilisin altered the pH dependence of catalysis: Mutagenic removal of two negatively charged surface residues within 12 to 15 Å of the active site His lowered its pKa by as much as 0.4 units. The authors concluded that this change in pKa likely resulted from alterations in electrostatic interactions across the active site. Examination of the molecular model of the Val206Thr + Phe95Asp mutant of *Rd* lipase (Fig. 5) revealed that Thr206 and Asp95 lie within 6 and 17 Å, respectively, of the active site His257 (distance from α-carbon to α-carbon). Molecular dynamics simulations did not predict any gross changes in protein conformation as a result

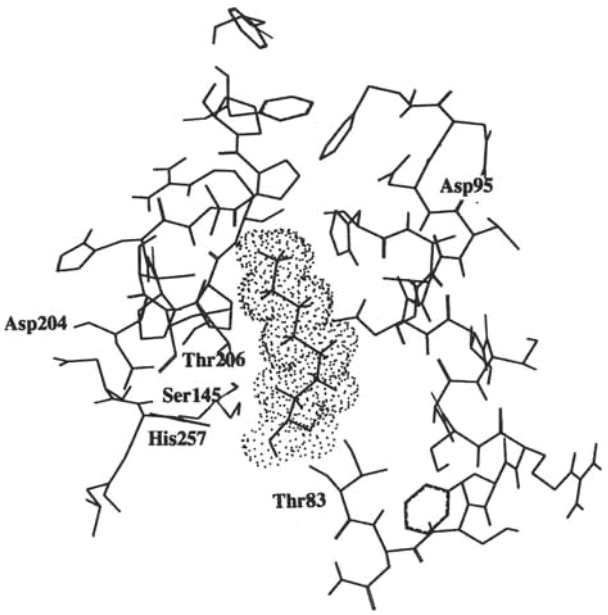

FIG. 5. Molecular model of the active site region of V206T + I95D mutant *Rd* lipase with the *sn*-3 acyl side-chain of a triacylglycerol substrate docked in the catalytic center. Only the *sn*-3 acyl side-chain of tricaprylin is shown (Van der Waals dots). The catalytic residues Ser145, Asp204, and His257 (Ser, serine; His, histidine) are labeled, as is Thr83, which comprises a portion of the oxyanion hole. Models of the docked substrate were calculated by energy minimization and molecular dynamics simulations with bond distance constraints as defined previously (Ref. 14). See Figures 1 and 2 for other abbreviations.

of the two introduced mutations. The observed changes in the pH dependence of enzymatic activity may reflect localized changes in electrostatic interactions in the active-site region. Alternatively, there may be fine-structure changes in the double-mutant enzyme that lead to the dramatic alteration in the pH dependence of its activity and substrate selectivity. What has been ascertained is that the effect of pH on activity is the result of the combined effect of both amino-acid substitutions because the single-site mutant lipases both displayed a pH sensitivity that was similar to that of the wild-type enzyme (data not shown). Future molecular modeling simulations, in conjunction with additional mutational analyses, will be necessary to determine the structural basis for the sharp pH dependence of catalysis that is exhibited by the double-mutant prolipase.

In summary, the present results confirm the primary role played by the amino acids at positions 95 and 206 in determining the fatty acid selectivity of *Rd* lipase and indicate that this selectivity can be further augmented by combining multiple single mutations into one enzyme. As further molecular determinants of fatty-acid selectivity are elucidated, it is anticipated that additional substitutions can be combined to produce lipases with unique substrate specificities. Engineering a lipase that exhibits sufficiently tight fatty-acid selectivity to allow its industrial use as a selective catalyst, however, may require more aggressive mutagenic strategies, such as random, cassette or combinatorial mutagenesis (23–25), to simultaneously introduce multiple mutations in the lipase. These approaches may permit a more rapid development of lipases with novel substrate specificities.

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