Discrimination of Esterase and Peptidase Activities of Acylaminoacyl Peptidase from Hyperthermophilic Aeropyrum pernix K1 by a Single Mutation*

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It has been shown that highly conserved residues that form crucial structural elements of the catalytic apparatus may be used to account for the evolutionary history of enzymes. Using saturation mutagenesis, we investigated the role of a conserved residue (Arg\textsuperscript{526}) at the active site of acylaminoacyl peptidase from hyper- thermophilic Aeropyrum pernix K1 in substrate discrimination and catalytic mechanism. This enzyme has both peptidase and esterase activities. The esterase activity of the wild-type enzyme with p-nitrophenyl caprylate as substrate is ~7 times higher than the peptidase activity with Ac-Leu-p- nitroanilide as substrate. However, with the same substrates, this difference was increased to ~150-fold for mutant R526V. A more dramatic effect occurred with mutant R526E, which essentially completely abolished the peptidase activity but decreased the esterase activity only by a factor of 2, leading to a 785-fold difference in the enzyme activities. These results provide rare examples that illustrate how enzymes can be evolved to discriminate their substrates by a single mutation. The possible structural and energetic effects of the mutations on $k_{cat}$ and $K_m$ of the enzyme were discussed based on molecular dynamics simulation studies.

Acylaminoacyl peptidase (APH)\textsuperscript{2} belongs to the prolyl oligopeptidase (POP; EC 3.4.21.26) family of serine protease, which also includes dipeptidyl peptidase IV (EC 3.4.14.5) and oligopeptidase B (OB; EC 3.4.21.83) (1–3). The POP family is a relatively new group of serine peptidases and different from the classic serine proteases, trypsin and subtilisin, in several structural features and catalytic behaviors (4–8). Compared with the classic serine protease, the members of the new family are more similar to lipase: (i) the members of the POP family contain a canonical \(\alpha/\beta\) hydrolase fold, and the catalytic triad is covered by an unusual seven-bladed \(\beta\)-propeller (1); (ii) the enzymes share the same sequence order of catalytic residues (Ser . . . Asp . . . His) with lipase, which is different from that of the well known trypsin (His . . . Asp . . . Ser) and subtilisin (Asp . . . His . . . Ser); (iii) the POP family shares the same Gly-X-Ser-X-Gly motif with lipase (9). Polgár’s group has studied the structural and evolutionary relationship between the POP family and the microbial lipase family by comparing the segment near the catalytic residues (9). There is no significant sequence homology between lipases and peptidases, except for a 10-residue segment near the catalytic Ser. However, these two families have similar catalytic triads and a partially opened active site. In fact, APH shows comparable peptidase and esterase activities; it can catalyze both the removal of an \(N\)-acylated amino acid from blocked peptides and an acyl chain from esters. An understanding of the determinant for the peptidase and esterase activities of APH is an interesting issue, which may provide us with more information about the molecular evolution of the POP family.

The putative APH gene (APE1547) from the thermophilic archaeon Aeropyrum pernix K1 has been overexpressed in Escherichia coli, and the recombinant protein (apAPH) has been purified and characterized (10). The enzyme shows an optimal temperature at 90 °C for enzyme activity and is extremely stable. The recombinant protein showed hydrolytic activity for a wide range of substrates, including \(p\)-nitrophenyl alkanoate esters of varying alkyl chain lengths, \(p\)-nitroanilide (pNA)-labeled amino acids, and peptides. Of a series of \(Ac\)-amino acid-\(p\)NAs tested, apAPH shows the highest activity for \(Ac\)-Leu-\(p\)-NA. In an earlier study, the maximal esterase activity has been observed for the substrate \(p\)-nitrophenyl caprylate (\(p\)NPC8) (10). Both optimum substrates have bulky hydrophobic side chains. Recently, we have determined the crystal structure of apAPH complexed with an organophosphorus substrate (11), which is the first available APH structure. The structure of the complex unambiguously maps out the substrate binding pocket and the conserved Ser\textsuperscript{445}, Asp\textsuperscript{524}, and His\textsuperscript{556} catalytic triad. The substrate binding pocket has a hydrophobic environment, which provides an explanation for the substrate preference of apAPH for hydrophobic side chains. The putative S2 pocket is also a hydrophobic environment and is particularly rich in phenylalanines (Phe\textsuperscript{153}, Phe\textsuperscript{155}, Phe\textsuperscript{163}, and Phe\textsuperscript{171}). Arg\textsuperscript{526} is located in the S2 site, which is structurally equivalent to Arg\textsuperscript{125} in dipeptidyl peptidase IV and Arg\textsuperscript{643} in POP. The Arg\textsuperscript{526}, which is adjacent to the catalytic residue Asp\textsuperscript{524}, is completely conserved among all APHs. There is an obvious bias toward Arg at this position in the whole POP family (12), whereas Leu is favored at the same position in the hormone-sensitive lipase family (Table 1). The special location and the high conservation suggest that residue 526 may play a crucial role in substrate recognition and/or transition state stabilization.

The role of Arg\textsuperscript{526} counterparts in the prolyl oligopeptidase subfamily has been addressed in earlier studies. Crystal structures of prolyl oligopeptidase-substrate complexes from porcine muscle reveal that Arg involves itself in substrate binding by forming a hydrogen bond between the guanidine group and the main chain carbonyl group of the substrate (13). Arg\textsuperscript{526} pulls the negatively charged substrate away from the catalytically competent position (4). Moreover, structure-based mutagenesis of the Myxococcus xanthus prolyl oligopeptidase has revealed that Arg\textsuperscript{526} is involved in a salt bridge that acts as a latch for opening or closing the enzyme, and Arg\textsuperscript{526} also helps to secure the incoming peptide.

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2. The abbreviations used are: APH, acylaminoacyl peptidase; POP, prolyl oligopeptidase; OB, oligopeptidase B; apAPH, A. pernix K1 APH protein; pNA, p-nitroanilide; pNPC8, p-nitrophenyl caprylate.
TABLE 1

Sequence alignment of amino acids around Arg^526 for major representatives of APH, POP, and hormone-sensitive lipase (HSL)

| Enzyme                      | Residue numbering | APH524 | APH525 | APH526 | APH527 | APH528 |
|-----------------------------|-------------------|--------|--------|--------|--------|--------|
| **APHs**                    |                   |        |        |        |        |        |
| A. pernix K1                | Asp               | Ser    | Arg    | Thr    | Phe    |
| Caenorhabditis elegans      | Asp               | Leu    | Arg    | Val    | Val    |
| Rat liver                   | Asp               | Arg    | Arg    | Val    | Phe    |
| Human erythrocytes          | Asp               | Arg    | Arg    | Val    | Phe    |
| Bacillus subtilis           | Asp               | Asp    | Arg    | Cys    | Phe    |
| Fugu rubribies              | Asp               | Lys    | Arg    | Val    | Phe    |
| **POPs**                    |                   |        |        |        |        |        |
| Pyrococcus horikoshii       | Asp               | Asp    | Arg    | Val    | His    |
| Porcine brain               | Asp               | Asp    | Arg    | Val    | Val    |
| Bovine brain                | Asp               | Asp    | Arg    | Val    | Val    |
| Arabidopsis thaliana        | Asp               | Asp    | Arg    | Val    | Val    |
| **HSLs**                    |                   |        |        |        |        |        |
| AFEST                       | Asp               | Phe    | Leu    | Arg    | Asp    |
| BFAE                        | Asp               | Phe    | Leu    | Arg    | Asp    |
| EST2                        | Asp               | Phe    | Leu    | Arg    | Asp    |
| HSL                         | Asp               | Phe    | Met    | Leu    | Asp    |
| YBAC                        | Asp               | Phe    | Leu    | Leu    | Asp    |
| MOL                         | Asp               | Ile    | Leu    | Arg    | Asp    |

Substrate to the open form of the enzyme (14). The mutants R526A and R526Q showed a 4–5-fold increase in K_m. However, previous studies on this potential substrate-binding residue have not addressed how mutations affect the APH substrate specificity and the role of the Arg^526 in the evolution relationship between peptidase and esterase.

Here, we performed saturation mutagenesis, in which the Arg^526 was mutated to all other 19 amino acids, to investigate the importance of this residue in apAPH as well as its potential role in the POP family. High throughput screenings for both peptidase and esterase activities were undertaken with Ac-Leu-pNA and pNPC8 as substrates, respectively. Further kinetic studies of individual steps allow a deeper insight into the mechanism of mutation effect on substrate recognition and enzyme catalysis. Finally, molecular modeling studies of several mutants were carried out to elucidate the possible structural basis for the conversion of peptidase to esterase.

**EXPERIMENTAL PROCEDURES**

Reagents, Bacterial Strain, and Plasmid—Restriction enzyme DpnI was purchased from Promega (Madison, WI). Pfu DNA polymerase was purchased from Stratagene (Madison, WI). Ampicillin and isopropyl 1-thio-β-D-galactopyranoside were obtained from TaKaRa Shuzo (Otsu, Shiga, Japan). The substrate Ac-Leu-pNA was purchased from Sigma, and pNPC8 were purchased from Fluka (Buchs, Switzerland). Other chemicals were reagent grade. Vector pET-15b and E. coli strain BL21-CodonPlus (DE3)-RIL, which were used for cloning and expression, were purchased from Novagen (Madison, WI).

Oligonucleotides and Saturation Mutagenesis—Oligonucleotide primers used for saturation mutagenesis were synthesized by BioBasic (Shanghai, China). The R526X mutants of apAPH were constructed using the QuikChange site-directed mutagenesis protocol (15). The codon for residue 526 is randomized by the whole plasmid PCR using Pfu polymerase. The oligonucleotide primers used as forward and reverse primers were as follows: R526X_F (5’-GAACGACAGCNN-KACACCGCTGA-3’); R526X_r (5’-TCAGCGGTGTTKNNCGTGTGGTTC-3’). Here N represents any of the following A, T, G, or C. K represents G or T. The reaction used 16 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 18 min. After the amplification, the reaction was further incubated at 72 °C for 30 min and stored at 4 °C.

2 units of DpnI was then added into the final PCR solution, which was incubated at 37 °C for 1 h to eliminate the methylated template. The PCR product was transformed into freshly prepared competent E. coli BL21-CodonPlus (DE3)-RIL by electroporation with a gene pulsor (Bio-Rad).

Clones growing on 2YT plates were picked with a sterile toothpick into 96-well plates, which was filled with 200 μL of 2YT containing 50 μg/ml ampicillin. Cells were cultured by shaking the plates overnight at 37 °C. The plates were duplicated by transferring 5 μL of culture from each well into a new plate containing fresh medium and antibiotic. The original plates were stored, and the duplicated plates were shaken for an additional 3 h for cell growth. Then the cells were induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After 6 h of induction at 30 °C, cells were harvested by centrifugation at 3000 rpm for 30 min and readied for screening.

Screening of Mutant Libraries for Peptidase and Esterase Activities—The harvested cells were frozen and then thawed three times to release the enzyme and then resuspended in 200 μL of 50 mM phosphate buffer (pH 8.0). The turbidity of each well was measured at 600 nm by a Multiskan Ascent 96-well plate reader (Thermolabsystems, Franklin, MA). Crude bacterial extracts were subjected to heat incubation at 80 °C for 10 min and centrifuged in order to remove the heat-induced aggregated proteins. A 20-μL aliquot from each well was pipetted into a new 96-well plate, to which 200 μL of substrate solution containing 0.2 mM Ac-Leu-pNA or pNPC8 in 50 mM phosphate buffer at pH 8.0 was added.

The peptidase activity of apAPH was measured spectrophotometrically by the release of the p-nitroaniline from Ac-Leu-pNA. Hydrolysis of the substrate was performed for 10–20 min at 80 °C. The amount of released p-nitroaniline was determined by measuring the absorbance at 405 nm. The value of A_405 of each well was normalized by the corresponding A_400 and the ratio r = A_405/A_400 was used to estimate the activity of each clone.

The esterase activity of apAPH was measured by the release of p-nitrophenyl from pNPC8, which was released in a way similar to that described for the hydrolysis of Ac-Leu-pNA.

Protein Purification and Enzyme Assays—Wild-type apAPH protein and variants were expressed in E. coli BL21-CodonPlus (DE3)-RIL. The cells were harvested and suspended in 50 mM phosphate buffer, pH 8.0. After ultrasonication cell disintegration, the cell suspension was centrifuged at 8000 rpm for 30 min. The cytosolic fraction was heated at 80 °C for 30 min and centrifuged to remove heat-induced aggregated proteins. The supernatant was subjected to nickel-chelating chromatography. Fractions containing apAPH activity were pooled, concentrated, and dialyzed against 20 mM phosphate buffer (pH 8.0) overnight. The N-terminal His tag was cleaved using thrombin, as described in the pET system manual (Novagen, Madison, WI). The protein concentration was determined according to the Bradford method, and bovine serum albumin was used as the standard.

The peptidase and esterase activities were determined with Ac-Leu-pNA and pNPC8 as substrates, respectively. Enzyme assays were according to the method previously described (10), except that the enzyme was assayed in 50 mM phosphate buffer (pH 8.0) instead of the Tris-HCl buffer.

Measurement of Steady-state Kinetics—The kinetic parameters K_m and k_cat of wild type and the mutants were determined at 80 °C for Ac-Leu-pNA and pNPC8. The initial steady-state velocities of substrate hydrolysis were monitored for a minimum of six substrate concentrations. The following substrate concentration ranges were used: Ac-Leu-pNA (200–2000 μM) and pNPC8 (20–200 μM). The kinetic parameters were determined from the rates of hydrolysis by fitting the rates.
to a Lineweaver-Burk double reciprocal plot. All kinetic data were analyzed by linear regression using Microcal Origin 6.0. The S.E. value of each parameter was estimated from the curve fitting.

**Determination of Individual Rate Constants and Activation Energies for Hydrolysis of pNPC8 and Ac-Leu-pNA**—Individual rate constants defining the mechanism of substrate hydrolysis were extracted from the values of $k_{cat}/K_m$ obtained as a function of temperature from 40 to 90 °C in 50 mM phosphate buffer at pH 8.0. The $k_{cat}/K_m$ was determined under first-order conditions (i.e. at substrate concentrations lower than $K_m$) by using $k_{cat}/K_m = V_{max}/[E][S]$. All measurements were done in duplicates or triplicates, and the results for kinetic data were the mean of at least two independent experiments.

The substrate hydrolysis of Ser protease starts with the formation of the enzyme-substrate complex ES with a second-order rate constant $k_1$. After that, the substrate can either dissociate back into the solution with a rate constant $k_{-1}$, or react with the enzyme to form the acyl-enzyme complex EP with a rate constant $k_2$. The product $P$ is released at this step. At last, the acyl-enzyme complex EP is hydrolyzed, and the other portion of substrate $P''$ is released with a rate constant $k_3$ (16). The series of events of the mechanism can be defined as follows.

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EP + P' \xrightarrow{k_3} E + P''$$

**SCHEME 1**

The individual rate constants in Scheme 1 are related to the experimental values of $K_m$ and $k_{cat}$ by the following equations.

$$s = \frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \quad (Eq. 1)$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad (Eq. 2)$$

Because the Michaelis-Menten parameters $s$ and $k_{cat}$ are composite functions of the individual kinetic rates in Scheme 1, measurements of $s$ and $k_{cat}$ as a function of temperature can resolve all of the parameters from Equations 3 and 4 (16–19),

$$s = \frac{k_1 k_2}{k_{-1}} \exp \left(-\frac{E_1 + E_2}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right) + k_2 \exp \left(\frac{E_2}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right)$$

(Eq. 3)

$$k_{cat} = \frac{k_2 k_3}{k_2} \exp \left(-\frac{E_2 + E_3}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right) + k_3 \exp \left(-\frac{E_3}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)\right)$$

(Eq. 4)

where $E_r$ is the activation energy associated with the rate constant $k_r$, $R$ is the gas constant, $T$ is the absolute temperature, and $k_{cat}$ is the value of $k_r$ at the reference temperature $T_0 = 298.16$ K. From the temperature dependence of $s$ and $k_{cat}$ all of the parameters involved in Equations 3 and 4 can be obtained. Because $T$ can be set to any temperature, the parameters can be calculated for various temperatures.

The specificity constant $s$ is the same as $k_1$ when $k_2 \gg k_{-1}$ and is equal to $k_1/k_2/k_{-1}$ when $k_{-1} \gg k_2$. In the former limit, the slope in the In $s$ versus $1/T$ plot is $E_{-1}/R$, whereas in the latter, the slope is $(E_{-1} - E_1)/E_1/E_1/R$. Processes with low activation energy, like substrate diffusion into the active site or acylation, predominate at low temperatures, where $k_2$ becomes much larger than $k_{-1}$, and $s$ becomes equal to $k_1$. On the other hand, substrate dissociation has a higher activation energy and becomes predominant at high temperatures, where $s = k_1 k_2 k_{-1}$. The plot of In $s$ versus $1/T$ at low temperature allows the determination of $k_1$ and $E_{-1}$, whereas $k_1 k_2 k_{-1}$ and $E_{-1} E_1 E_2$ can be determined at high temperature. The curvature in the plot allows for determination of the four terms ($k_1, k_2, k_{-1}, E_{-1}, E_1, E_2$) in Equation 3 with the best fit parameters derived from the nonlinear least squares method using Microcal Origin 6.0. $k_{cat}$ is $k_2$ for both ester and peptide substrates (see “Results”). Together with the $k_{cat}$ obtained from the kinetic assay, the determination of all five parameters ($k_1, k_2, k_{-1}, E_{-1}, E_1, E_2$) can be made.

**Molecular Modeling**—All computations were performed with the InsightII package, version 2000 (Accelrys, San Diego, CA). The BIOPOLYMER module was used to create substrate molecular structures. All energy minimizations and molecular dynamics were performed with the DISCOVER module using the consistent valence force field. The 1.8 Å crystallographic structure of apAPH was used as the starting coordinates for calculations (Protein Data Bank code 1VE6). Hydrogen atoms were added according to the normal ionization state of the amino acids at pH 8.0. The atomic potentials were fixed according to the consistent valence force field atom types recommended by the manufacturer.

The advanced docking program, Affinity, was used to perform the automated molecular docking for ester substrate pNPC8. This procedure combines Monte Carlo and simulated annealing to dock a guest molecule with a host. The docked complexes of apAPH and ligands were selected according to the criteria of interacting energy combined with geometrical matching quality. Peptide substrate Ac-Leu-pNA was manually docked into the binding site of the enzymes and was oriented with the P1 Leu in the S1 pocket and Ac-O toward the Arg256. The selected pNPC8apAPH and Ac-Leu-pNAapAPH from the docking studies were subjected to energetic minimization before being used as the starting points for subsequent calculations.

Mutations were introduced with all mutant residues kept in the same orientation as their wild-type counterparts. Residues contained in a simulation area within 15 Å from atom Ne of His256 were allowed to move with the rest of the protein being fixed. A layer of 5 Å of explicit water was added to the surface of the assembly, and a nonbonded cut-off of 20 Å was fixed to reduce the time of calculation. Each structure was energy-minimized by applying 100 steps using the steepest descent method and followed by a conjugate gradient minimization procedure until a convergence of 0.001 kcal mol$^{-1}$Å$^{-1}$ was reached. A short molecular dynamics simulation was performed starting from the energy-minimized structures for 100 fs at 310 K to allow equilibration at 310 K. The actual simulation to explore conformational space was performed for 100 ps at the same temperature (time step = 1 fs). Snapshots were taken each picosecond to generate 101 different conformers. The trajectories obtained for each mutant were analyzed.
RESULTS

Saturation Mutagenesis and Screening Activities of both Esterase and Peptidase—Saturation mutagenesis was performed at position Arg\(^{526}\) using the modified QuickChange site-directed mutagenesis protocol as described under “Experimental Procedures.” With this approach, a random codon was introduced in a single PCR with one pair of complementary primer containing all possible mutations. We took advantage of the availability of the 96-microtiter well plate assay for the rapid screening for both esterase and peptidase activities of mutants. More than 200 clones from the saturation mutagenesis library were screened to ensure the probability that all 32 possible codons were covered with no less than 99% (20). To obtain the high screening sensibility, the optimal substrates for peptidase and esterase activities, which are Ac-Leu-pNA and \(pNPC8\), respectively, were chosen based on the compounds that were tested in our earlier studies.

The mutation library showed strikingly different effects on these two kinds of substrates. Nearly all mutants display a decreased peptidase activity with Ac-Leu-pNA as substrate. More than 80% of the clones showed an activity less than 20% of the wild-type enzyme. In contrast, they exhibited higher activity for \(pNPC8\). More than 90% of the clones showed esterase activity higher than the wild-type enzymes. Meanwhile, about 12% of the clones have activity more than 5-fold of the wild-type enzymes. These results suggest that residue 526 plays an important role in determining the peptidase and esterase activity of apAPH.

Steady-state Kinetic Analysis at 80 °C—The clones with obvious activity changes were sequenced. The screening data led us to identify specific mutants of interest for further enzymatic characterization. The hydrophobic Leu, Ile, and Val were chosen because they induced a significant increase in esterase activities (5–7-fold). Meanwhile, the least conserved R526E was chosen, because it is the most inactive variant for both Ac-Leu-pNA and \(pNPC8\). Ala has the smallest side chain, which could be used as a minimum reference to study the function of the other side chains. Lys, positively charged as Arg, could provide information on the electrostatic effects on the enzyme activity.

The kinetic parameters for wild-type apAPH and the selected mutants are shown in Table 2. All of the R526X mutants have reduced peptidase activity as measured by Ac-Leu-pNA hydrolysis. Generally speaking, the reduced activities are due to significant increases in \(K_m\) rather than \(k_{cat}\). Although the \(k_{cat}\) of R526E is dramatically decreased. The \(K_m\) of R526A is higher than the mutants with bulky hydrophobic side chains, such as R526V, R526I, and R526L suggesting that larger bulky hydrophobic side chains play a significant role in substrate binding. The charged mutants at this position changed the \(K_m\) most significantly; R526K and R526E had 25- and 14-fold increases in \(K_m\), respectively. In addition, these two residues also have effects on \(k_{cat}\). The positively charged R526K increased the \(k_{cat}\) by 3-fold, whereas the negatively charged R526E reduced the \(k_{cat}\) by 2 orders of magnitude.

The fact that all of these mutants increased the \(K_m\) suggests that it is the guanidine group of Arg\(^{526}\) that plays an unfavorable role for the binding of the substrate to the wild-type enzyme. This could be caused by its unique guanidine group rather than by the positive charge, since the Lys side chain is also positively charged.

All mutants showed an increased esterase activity with substrate \(pNPC8\), except that the R526E mutant decreases the activity slightly. The increase in esterase activity of the mutants is attributed to the increase in \(k_{cat}\) since the \(K_m\) is in the same range as the native enzyme (Table 2). These results indicate that the substitutions at this position affect the catalysis but not the substrate binding. The large hydrophobic mutants (Ile, Leu, and Val) increased the catalytic efficiency by 3.4–5.7-fold. R526K and R526A showed a small increase of 50–60%. It is worth noting that the R526E shows a \(K_m\) that is 2.6 times larger than the wild-type enzyme, suggesting that its negatively charged side chain might interfere with the substrate binding and decrease the affinity between the enzyme and \(pNPC8\).

The R526X mutants showed increased esterase activity and decreased peptidase activity, respectively. Therefore, their specificity toward two types of substrates has dramatically changed (Fig. 1). The ratio of the catalytic efficiency (\(k_{cat}/K_m\)) between esterase substrate and peptidase substrate for each mutant becomes much larger than the wild type. In the case of R526V, the esterase activity becomes ~150 times higher than the peptidase activity. A more dramatic effect occurred with mutant R526E, which essentially completely abolished the peptidase activity but decreased the esterase activity only by a factor of 2, leading to a 785-fold difference in the two enzyme activities. The above results unambiguously confirmed the importance of position 526 in substrate discrimination and illustrate that enzymes can be evolved to discriminate their substrates by a single mutation.

Reaction Mechanism Revealed by Individual Kinetic Constants—To gain a deeper insight into the catalytic role of the mutants, the wild-type apAPH and the Val, Glu, Lys, and Ala mutants were subjected to further kinetic analysis. The individual kinetic rate constants (\(k_1, k_{-1}, \text{ and } k_{2}\)) and the corresponding activation energies (\(E_1, E_{-1}, \text{ and } E_2\)) were calculated from the nonlinear Arrhenius plot for both substrates. This approach is based on the consideration that the specificity constant \(s\) and \(k_{cat}\) are temperature-dependent and obey the Arrhenius law. By measuring these constants at different temperatures, it is possible to obtain all of the individual rate constants together with the corresponding activation energies (see “Experimental Procedures”).

We first defined the rate-limiting step of the catalytic reaction by measuring the \(pNP\) (\(pNA\)) "burst." This burst is supposed to occur when the enzyme is added to the reaction mixture before the onset of the catalytic reaction, provided that the release of the \(P(k_j)\) is slower than the release of the \(P(k_e)\) (21) and the amplitude of burst is a linear function of the concentration of the enzyme. In our experiment, the immediate release of \(pNP\) (\(pNA\)) did not change for the wild-type and the

| Enzyme | Peptidase (Ac-Leu-pNA) | Esterase (pNPC8) | \(\text{E}_{\text{peptidase}}/\text{E}_{\text{esterase}}\) |
|--------|------------------------|-----------------|-----------------|
| \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) | \(k_{cat}/K_m\) |
| Wild type | 9.3 ± 0.5 | 0.4 ± 0.0 | 22.3 ± 3.8 | 6.6 ± 0.4 | 43.3 ± 2.8 | 152.4 ± 17.1 | 6.8 |
| R526A | 12.6 ± 0.4 | 4.3 ± 0.3 | 2.9 ± 0.1 | 9.6 ± 0.2 | 41.6 ± 2.6 | 230.7 ± 7.1 | 79.6 |
| R526E | 0.5 ± 0.0 | 5.8 ± 0.6 | 0.09 ± 0.01 | 8.1 ± 0.4 | 114.6 ± 7.7 | 70.7 ± 9.0 | 78.5 |
| R526K | 26.9 ± 1.3 | 10.5 ± 1.2 | 2.6 ± 0.3 | 9.4 ± 0.6 | 38.2 ± 2.8 | 246.1 ± 31.5 | 94.7 |
| R526I | 9.2 ± 0.1 | 2.1 ± 0.1 | 4.4 ± 0.6 | 20.8 ± 1.5 | 40.1 ± 3.6 | 518.7 ± 5.25 | 117.9 |
| R526L | 9.6 ± 0.4 | 1.3 ± 0.0 | 7.4 ± 0.8 | 28.5 ± 1.1 | 38.2 ± 2.9 | 746.1 ± 98.5 | 100.8 |
| R526V | 9.7 ± 0.3 | 1.7 ± 0.0 | 5.7 ± 0.6 | 30.9 ± 0.9 | 35.7 ± 1.6 | 865.5 ± 14.6 | 151.8 |

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mutants with either pNPC8 or Ac-Leu-pNA as substrates (data not shown here), suggesting that the rate-limiting step for both substrates is the acylation ($k_2$) with $k_{cat}$ close to $k_2$.

The individual kinetic constants and the corresponding activation energies for Ac-Leu-pNA hydrolysis are shown in Fig. 2A and Table 3. The parameters for R526E could not be obtained because of the extremely low activity. Compared with the wild-type apAPH, all of the mutants have an obvious lower $k_1$ for Ac-Leu-pNA hydrolysis. For example, the R526A mutant is low in $k_1$ by 8-fold. Since $k_{-1}$ is 1.4 times higher than that of the wild-type enzyme, the guanidine group of Arg$^{526}$ is not only involved in substrate binding but also stabilizes the bound substrate. R526V shows a 3.4-fold decrease in $k_{-1}$, and its $k_{-1}$ approaches that of the wild-type apAPH, suggesting that the hydrophobic side chain is not as efficient as the side chain of Arg to help Ac-Leu-pNA to diffuse into the active site. Surprisingly, the $k_{-1}$ of R526K is much larger than those of other mutants and native enzyme. It is about 5.3-fold that of the wild type, whereas other mutants have values similar to that of the wild type. From Fig. 2A, it can be seen that R526K has a higher specificity constant $s$ than R526A when $s$ is predominated by $k_1$ in the low temperature region. However, the $s$ of R526K becomes lower than R526A when $s$ is predominated by $k_{-1}$ at the high temperature region. These results suggest that the side chain of R526K may undergo some conformation rearrangements upon substrate binding, and the new conformation will have a favorable effect on the binding of the substrate. The effects of the mutants on $k_2$ are rather complex. R526V has a $k_2$ similar to that of the wild-type enzyme, whereas R526A and R526K showed increased acylation rate constants. Compared with the wild-type enzyme, the Arrhenius plots of all mutants have similar slopes in the low temperature region (Fig. 2), suggesting that they have similar activation energies for the formation of the enzyme-substrate complex ($E_s$). In contrast, the slopes of R526K and R526A showed a sharp decline at the high temperature region, suggesting the existence of a large active energy ($E_T$).

The individual kinetic constants and the corresponding activation energies for pNPC8 hydrolysis are shown in Fig. 2B and Table 3. All mutants showed increased $k_2$. Since $k_2$ is the rate-limiting step in the catalytic mechanism, revealed by burst experiments, its increase promotes the catalytic rate significantly. The R526V shows the highest $k_2$ (Table 3), suggesting that the hydrophobic residue at this position is more favorable for the formation of the acyl-enzyme complex. Thus, the increased catalytic efficiency of the R526V for pNPC8 is the combined effect of a 2-fold increase in $k_1$, a 5-fold decrease in $k_{-1}$, and a 4.7-fold increase in $k_2$. Together, they lead to a $k_{cat}/K_m$ that is 5.7-fold higher than the wild-type enzyme. The R526A also showed a 2-fold increase in $k_1$, which is the same as that of the R526V. However, its $k_2$ and $k_{-1}$ are similar to those of the wild type, suggesting that only large hydrophobic side chains are able to stabilize the bound substrate and accelerate the catalysis. This conclusion is consistent with the fact that the hormone-sensitive lipase family shows a strong bias toward large hydrophobic side chains at this position (Table 1). The $k_1$ of charged R526E and R526K, as well as wild-type enzyme, are 18–60% of that of R526A, suggesting that the charged residues in the hydrophobic core region of apAPH are unfavorable for the binding of esterase substrate. The effects of the mutants on the dissociation of the enzyme-substrate complex ($k_{-1}$) are rather complex. The R526A and the R526E showed a $k_{-1}$ that is similar to that of the wild-type enzyme. However, the $k_{-1}$ of the R526K and the R526V are decreased, suggesting that the enzyme-substrate complexes in these mutants are more stable.

Computational Models—To further understand the experimental results, molecular dynamics simulation studies were undertaken with selected apAPH mutants. In our previous work, the structure of apAPH and p-nitrophenyl phosphate complex maps out the S1 substrate binding pocket located in close proximity to the active site (Fig. 3A). The putative S2 pocket has a hydrophobic environment and is lined by Arg$^{526}$. It has been known that Arg$^{125}$ in dipeptidyl peptidase IV and Arg$^{434}$ in POP, which are structurally equivalent to Arg$^{526}$, stabilize the P2 residue carbonyl oxygen of peptide substrate in both dipeptidyl peptidase IV and POP. In apAPH, it is next to the catalytic triad residue Asp$^{525}$ in sequence. Its long positive side chain is pointed toward the S1 pocket (Fig. 3). Ac-Leu-pNA was manually docked into the binding site and oriented with the Leu side chain of the substrate in the S1 pocket and P2 carbonyl oxygen toward the guanidine group of Arg$^{526}$ (Fig. 3A).

For the ester substrate complex, since there is no ester substrate or substrate analogy complex structure for apAPH, the substrate binding is modeled by a detailed comparison with AFEST, which is a novel hyperthermophilic carboxylesterase from *Archeoglobus fulgidus* and classified as a member of the hormone-sensitive lipase group of the esterase/lipase superfamily (22–25). By structure-based alignment, the ester binding tunnel is proposed to overlap spatially with the peptide S1 pocket. The side chains of Phe$^{665}$ and Phe$^{688}$ and the main chain of

FIGURE 1. Mutation effect on the discrimination of Ac-Leu-pNA (white bars) and pNPC8 (black bars). WT, wild type.
Discrimination of Esterase and Peptidase by a Mutation

Gly368-Pro370 act as the two sides of borders of the ester tunnel for binding straight-chain fatty acids of ester (Fig. 3B). An automated docking module in InsightII was used to search for the possible binding site for ester substrate pNPC8 and to obtain the binding complex. The most stable complex maps the residues (Fig. 3B) that are consistent with the acyl binding site identified by a sulfonyl derivative in the AFEST complex structure. These two complex models (pNPC8-apAPH and Ac-Leu-pNA-apAPH) were used as the starting conformations for molecular dynamics simulation studies.

We performed the molecular dynamics simulations on the Glu and Lys mutants of apAPH in the presence of Ac-Leu-pNA. Lys was chosen because it has an increased value of $K_m$, although it is structurally similar to Arg in parental enzyme; Glu was chosen because it is the least active mutant. On the basis of the Ac-Leu-pNA-apAPH complex model, R526K and R526E are introduced by a side-chain substitute and energy minimization. After energy minimizations, the Ac-Leu-pNA-apAPH, Ac-Leu-pNA-R526K, and Ac-Leu-pNA-R526E complexes were subjected to 100-ps short dynamics simulations. Similarly, we performed molecular dynamics simulation studies on the Val and Glu mutants of apAPH in the presence of pNPC8. Val was chosen because it is the most active mutant for ester substrate; Glu was chosen because it is the only one that causes an increased $K_m$ and results in a strikingly large decrease of catalytic efficiency for the ester substrate.

During the simulation period for Ac-Leu-pNA-apAPH, one of the guanidine nitrogens (NH1) of Arg626 formed a hydrogen bond with the P2 carbonyl oxygen of Ac-Leu-pNA (Fig. 4A). Meanwhile, the other guanidine nitrogen (NH2) participated in forming a salt bridge with Glu88 OE (Fig. 5). This bidentate mode appears to play an important role in stabilizing the peptide substrate. As a result, the acetyl moiety of Ac-Leu-pNA is held tightly during the simulation. In the simulation of the Ac-Leu-pNA-R526E complex, we observed that the acetyl of the substrate is very unstable. The carboxyl terminus of Glu rotated significantly around the $C_\alpha-C_\beta$ bond. It is probably caused by the electrical repulsion of the negative charges between Glu526 and P2 carboxyl oxygen of the substrate Ac-Leu-pNA. The severe structure shift within the active site loop may account for its decreased turnover number to Ac-Leu-pNA.

In the Ac-Leu-pNA-R526K complex model, the original position of the positively charged NZ atom of Lys was within the hydrogen bonding distance (2.6 Å) to the P2 carbonyl oxygen of the ligand. After the short period of equilibration in simulation, the Lys526 side chain was subjected to the strong attraction of the negatively charged carboxyl terminus of Glu368, and then its NZ formed a salt bridge with OE of Glu28 (Fig. 5). No hydrogen bonds between Lys and the acyl oxygen of the substrate were observed during the entire simulations, which may account for the large $K_m$ of the R526K.

For the complex between R526V and substrate pNPC8, in the course of the 100-ps simulation, we observed that the large hydrolytic side chain seems to have a strong attraction on the C-1 to C-2 portion of the acyl moiety of the ester substrate (Fig. 4B). This significant structural shift may position the catalytic groups in a more favorable orientation for reaction. In the case of pNPC8/R526E, the simulation revealed that the side chain of this Glu rotated around its $C_\alpha$-$C_\beta$ bond and positioned toward the side of the ester binding tunnel. Its negatively charged side chain was in proximity to the middle acyl chain of pNPC8. The severely reduced hydrolytic interaction between the binding tunnel and the ester substrate may account for the decrease in $k_1$ and the increase in $k_{-1}$.

### Table 3

| Enzymes       | $k_1$ | $k_{-1}$ | $k_2$ | $E_1$ | $E_2-E_1$ |
|--------------|------|---------|------|------|----------|
| Ac-Leu-pNA   |      |         |      |      |          |
| Wild type    | 53.0±3.0 | 22.9±1.4 | 9.3±0.5 | 15.0±0.5 | 10.9±0.8 |
| R526K        | 9.8±1.3 | 117.0±5.3 | 26.9±1.3 | 14.3±0.6 | 19.1±1.0 |
| R526V        | 15.7±0.3 | 22.1±1.5 | 9.7±0.3 | 13.5±0.4 | 11.9±0.6 |
| R526A        | 6.7±0.3 | 30.6±2.2 | 12.6±0.4 | 15.4±0.2 | 20.1±0.7 |

**FIGURE 3.** Substrate-binding sites in apAPH. A, peptide binding site in the Ac-Leu-pNA-apAPH model shown in a ball-and-stick model. Ac-Leu-pNA is in gray. Hydrogen bonds are depicted as dashed lines in yellow. B, ester binding site in pNPC8-apAPH model shown in a ball-and-stick model. pNPC8 is in gray. The images were produced with PyMol.
DISCUSSION

Recently, the POP family has received much attention as a new group of Ser proteases. Although it has been suggested that the POP family has an evolutionary relationship with the microbial lipase family, little work has been done to determine the relationship between peptidase and esterase activity of the POP family. In this study, we performed saturation mutagenesis at the potential substrate binding residue Arg526 in apAPH and found that it is an important residue for the substrate specificity between peptidase and esterase. To our knowledge, this is the first study focusing on the substrate discrimination in acylaminoacyl peptidase of the POP family.

Arg526 is highly conserved among APHs (Table 1). The crystal structure of apAPH shows that Arg526 is at the active center and forms an ion pair network with Glu185 and Arg113 (Fig. 5). The molecular dynamics simulations showed that Arg526 is involved in peptide substrate binding by forming a hydrogen bond between the guanidine group and the main chain carbonyl group of the substrate. To our knowledge, this is the first study focusing on the substrate discrimination in acylaminoacyl peptidase of the POP family.

Arg526 is highly conserved among APHs (Table 1). The crystal structure of apAPH shows that Arg526 is at the active center and forms an ion pair network with Glu185 and Arg113 (Fig. 5). The molecular dynamics simulations showed that Arg526 is involved in peptide substrate binding by forming a hydrogen bond between the guanidine group and the main chain carbonyl group of the substrate (Fig. 3). As a primary part of the S2-P2 interactions, any substitution at position 526 results in reduced activity for Ac-Leu-pNA hydrolysis, as observed in the activity screening of the mutant library. The kinetic analysis of selected Arg526 mutants clearly showed a significant increase in $K_m$ (Table 2). These results suggest that the conservation of Arg526 in this position might be required for binding the substrate and for the stabilization of substrate. Surprisingly, R526K exhibited an unexpected larger increase in $K_m$. The molecular dynamics simulations showed that Lys526 formed a salt bridge with Glu185 but lost the S2-P2 hydrogen bond between the substrate and the enzyme (Fig. 5). The unique bidentate coordination of Arg526 cannot be replaced by any other residues, since it is involved not only in binding to the substrate but also in stabilizing the enzyme and substrate complex by forming the salt bridge network.

The screening results in our experiment show that the bulky hydrophobic side chains (Val, Ile, and Leu) at position 526 resulted in an increase of esterase activity. They have favorable effects on each step of the substrate hydrolysis, as illustrated by the individual rate constants of R526V. Compared with the wild-type enzyme, R526V showed a faster diffusion rate of the substrate into the active site, a more stable enzyme-substrate complex, and an enhanced acylation rate. The increased catalytic efficiency may be caused by the more twisted ester substrate, as suggested by the molecular dynamics simulation of the R526V mutant (Fig. 4).

Although the possible relationship between the POP family and the microbial lipase family was suggested by Polgár in 1992 (9), the direct evidence of their evolutionary relationship is still not known. Since three-dimensional structures are more conserved than sequences under evolutionary pressure, a comparison of protein structures is more powerful than a sequence comparison. A three-dimensional structure alignment by the combinatorial extension method (available on the World
Wide Web at cl.sdsc.edu/) (26) shows that the catalytic domain of apAPH can have very different effects on the peptidase and esterase activities for the long acyl chain ester (10, 27, 28). The structural and functional similarity between the two families clearly indicates they are evolutionarily related and might diverge from a common ancestor.

In conclusion, we found that a single mutation at position 526 of apAPH can have very different effects on the peptidase and esterase activities with Ac-Leu-pNA and pNPC8 as substrates. The substrate discrimination between esterase and peptidase appears to be associated with the polarity, volume, and conformation of substitutes, an important phenomenon that has not been recognized for the extensively studied POP family. This study provides the first direct evidence that the POP and lipase families are evolutionarily related, and Arg$_{526}^*$ may be an evolutionary marker.

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