Enzyme Catalysis

Molecular Basis of Perhydrolase Activity in Serine Hydrolases**

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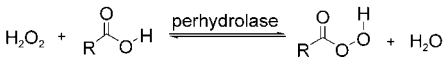
Catalytic promiscuity is the ability of one active site of an enzyme to catalyze several different chemical transformations.[1,2] Investigations and control of catalytic promiscuity are key to understanding the evolution of new enzymatic activities and the design of new enzyme-catalyzed reactions for synthesis. An example of a potentially useful catalytic process is the perhydrolase activity of esterases and lipases.[3,4] Herein we demonstrate a 2600-fold increase in the specificity of an esterase to catalyze perhydrolysis over hydrolysis. The procedures presented herein may be used for the design of other enzymes with altered nucleophile selectivities and that catalyze stereoselective oxidations.

Perhydrolases (previously known as metal-free haloperoxidases) contain a Ser–His–Asp catalytic triad and catalyze the reversible formation of peracids from carboxylic acids and hydrogen peroxide [Eq. (1)]. Perhydrolysis presumably takes



(1)

place with an esterase-like mechanism in which a carboxylic acid first reacts with the active site serine group to form an acyl-enzyme intermediate, which reacts with hydrogen peroxide to form a peracid.[5–9] The switch from water as the nucleophile to hydrogen peroxide could be considered a change in substrate selectivity, but the very different chemical reactivity of the respective products (carboxylic acid versus peroxycarboxylic acid) makes this an example of an alternate catalytic activity.

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Perhydrolysis might be an inherent side activity of serine hydrolases;[10] esterases and lipases both catalyze slow perhydrolysis of carboxylic acids.[3,11] However, other evidence suggests that perhydrolase activity is distinct from hydrolase activity. The perhydrolase activity of lipases and esterases is much lower than their esterase activity, and, conversely, the esterase activity of perhydrolases is much lower than their perhydrolase activity. Some serine hydrolases (subtilisin, for example) do not exhibit perhydrolase activity which suggests that the Ser–His–Asp catalytic triad is not the only determinant for perhydrolase activity. Bugg recently proposed an alternate mechanism for perhydrolysis in which the catalytic serine stabilizes the carboxylic acid substrate with a hydrogen bond instead of forming an acyl-enzyme intermediate, but there is currently no experimental evidence for this proposal.[12]

Recent X-ray crystallographic structures of two enzymes from *Pseudomonas fluorescens*, an aryl esterase (PFE) that shows low perhydrolase activity[13] and a homologous perhydrolase (CPO-F), show similar active sites and no clear structural basis for distinguishing their different activities.[14] Initial work to convert PFE into a perhydrolase focused on the individual replacement of three amino acids that differ in PFE and CPO-F, each of which have C^{α} atoms within 14 Å of O^{γ} of the conserved Ser94 group: Met95 (C^{α}–O^{γ} = 4.5 Å), Tyr69 (C^{α}–O^{γ} = 14 Å), and Thr122 (C^{α}–O^{γ} = 9.9 Å). However, none of the mutant PFE forms showed a significant increase in perhydrolase activity. The Met95Thr mutation decreased esterase activity from 13.7 to 3.4 U mg^{-1} and perhydrolase activity to below the detection limit (< 0.02 U mg^{-1}). This decrease may result from a shift in the backbone amide of position 95, which forms part of the oxyanion hole. The esterase and perhydrolase activities of the Tyr69Met and Thr122Pro mutant forms are similar to those of the wild-type protein (Table 1).

As a direct structural comparison of PFE with CPO-F did not reveal a clear means to increase its perhydrolase activity, we aligned the amino acid sequences of six hydrolases and six perhydrolases to determine the essential residues for each activity. The alignment identified 15 amino acids common to most esterases and 57 amino acids common to most perhydrolases. Figure 1 summarizes the results in a Venn diagram similar to that used by Rothman and Kirsch to map conserved residues in related proteins.[15] Not surprisingly, PFE contains most of the amino acid residues common to esterases. However, PFE has amino acid substitutions at 14 positions out of the 57 amino acids common to most perhydrolases. We hypothesized that substitution of one or more of these 14 residues would provide an increase in perhydrolase activity and a decrease in esterase activity, and that the residues closest to the active site are those most important for catalytic activity. Three amino acid residues are within a sphere of 12 Å about the O^{γ} atom of catalytic Ser94: Pro29, Glu99, and Ile227. The Leu29Pro, Asp99Glu, and Phe227Ile mutants of PFE were obtained by site-directed mutagenesis with complementary mutagenic PCR primers. DNA sequencing confirmed the mutations and the three proteins were expressed and purified as described previously.[13]

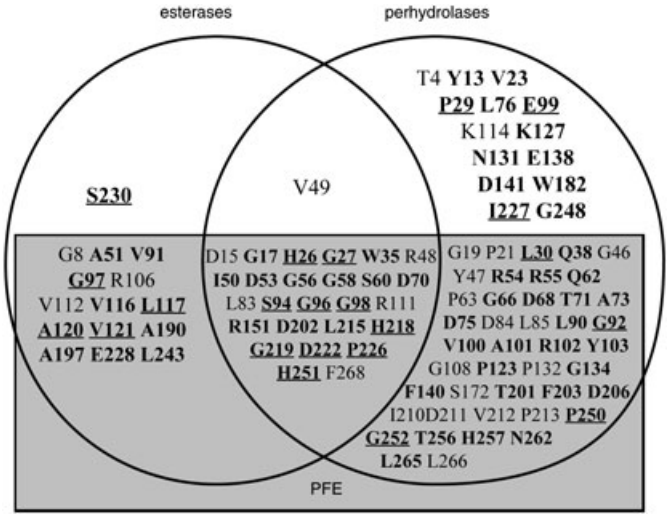

Figure 1. Conserved residues present either in esterases (left), or in perhydrolases (right), or shared between both enzyme types. Amino acids present in PFE are included in the gray box. All residues are numbered according to the primary sequence of PFE, and the kind of amino acid refers to the conserved group in the respective enzyme type. Residues are coded according to their distance from the O^{γ} of the conserved catalytic serine (underlined and boldface: < 12 Å; boldface: 12–20 Å; all others: > 20 Å).

Table 1: Rate of hydrolysis and perhydrolysis in PFE variants.

Enzyme	Hydrolysis [U mg^{-1}][a]	$\Delta\Delta G_{mut:wt}$ [kcal mol^{-1}][b]	Perhydrolysis [U mg^{-1}][c]	$\Delta\Delta G_{mut:wt}$ [kcal mol^{-1}][b]
wild-type	14	0	0.24	0
Leu29Pro	0.14	2.7	6.8	−2.0
Tyr69Met	18	−0.17	0.30	−0.13
Met95Thr	3.4	0.83	< 0.02	> 1.5
Asp99Glu	8.6	0.28	0.12	0.44
Thr122Pro	15	−0.062	0.18	0.17
Phe227Ile	38	−0.60	0.23	0.050

[a] Hydrolase activity assays were performed in *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer (5 mM, pH 7.2) at 25 °C; 1 U = hydrolysis of 1 mmol substrate min^{-1} (*p*NPAc) and 1 mmol oxidized product min^{-1} (H_2O_2). [b] Energy equivalent of the rate change relative to wild-type PFE: $\Delta\Delta G = -RT(\ln x)$ for an *x*-fold rate change at T = 300 K. [c] Perhydrolase activity determined in acetate buffer (0.7 M, pH 5.5) at 25 °C; 1 U = consumption of 1 μmol substrate (monochlorodimedone) min^{-1}. The error limit for kinetic assays is estimated at 20%.

The Leu29Pro mutation shifted the behavior of the enzyme from esterase to perhydrolase activity. Under the conditions assayed (Supporting Information), perhydrolase activity increased 28-fold from 0.24 to 6.8 U mg^{-1} for the bromination of monochlorodimedone.[16] Furthermore, the hydrolytic activity toward *p*-nitrophenyl acetate (*p*NPAc) decreased 100-fold from 14 to 0.14 U mg^{-1} (Table 1). The level of perhydrolase activity was higher than that of a wild-type perhydrolase from *P. fluorescens* (3.8 U mg^{-1}), but lower than that of the perhydrolases from *P. putida* (12 U mg^{-1}) and *P. pyrrocinia* (47 U mg^{-1}).[14,8]

The Asp99Glu and Phe227Ile mutants showed no increase in perhydrolase activity. For Asp99Glu, both the perhydrolase and hydrolase activities were lower than that of wild-type PFE: 0.12 and 8.6 U mg^{-1}, respectively (Table 1). The perhydrolase activity of Phe227Ile was statistically equivalent to that of the wild-type enzyme, whereas hydrolysis mediated by Phe227Ile (38 U mg^{-1}) was close to threefold higher than that catalyzed by the wild-type enzyme (14 U mg^{-1}; Table 1). Interestingly, residue 227 is at the periphery of the active site. The distance between O^{γ} of Ser94 and C^{α} of Phe227 is 11.5 Å; furthermore, the residue points toward the surface of the protein and is not part of the substrate-binding pockets.[13]

Kinetic characterizations of wild-type and Leu29Pro PFE showed that the major contributing factor to the observed perhydrolysis rate increase is V_{max}, which is 50-fold higher in the mutant (11 U mg^{-1}) than in the wild-type (0.22 U mg^{-1}). The perhydrolase reaction follows ping-pong kinetics with acetate and hydrogen peroxide. We measured the apparent kinetic constants for this reaction at a constant acetate concentration of 0.7 M. These values point to a 14-fold increase in catalytic efficiency (V_{max}/K_M) for perhydrolysis of acetic acid from 13 to 180 U mg^{-1}M^{-1}, which corresponds to a decreased activation-energy barrier of 1.6 kcal mol^{-1} at 300 K. The Leu29Pro mutation also caused a 50-fold decrease in V_{max} for the hydrolysis of *p*NPAc, whereas K_M increased threefold (Table 2). Altogether, this changed the catalytic efficiency 165-fold, which corresponds to an elevated activation-energy barrier for hydrolysis of 3.0 kcal mol^{-1} at 300 K.

Table 2: Kinetic parameters for *p*NPAc and apparent kinetic parameters for hydrogen peroxide.[a]

Enzyme, substrate	V_{max} [U mg^{-1}]	K_M [mM]	V_{max}/K_M [U mg^{-1} M^{-1}]
wild-type, *p*NPAc	143 ± 9	2.8 ± 0.4	51000
Leu29Pro, *p*NPAc	2.8 ± 1.1	8.9 ± 1.1	310
wild-type, H_2O_2	0.22 ± 0.02	17 ± 4	13
Leu29Pro, H_2O_2	11 ± 1	58 ± 12	180

[a] Reaction conditions as in Table 1.

The hydrolytic activity of Leu29Pro toward unactivated esters decreased much less dramatically, ranging from no change to an eightfold decrease (Table 3). The rate-determining step for hydrolysis may differ between activated esters (such as *p*NPAc) and unactivated esters. For activated esters, the acylation is most likely fast and deacylation would therefore be rate-determining.[17] For unactivated esters, the rates of acylation and deacylation are similar. The effect of the Leu29Pro mutation on the rate of hydrolysis of activated and unactivated esters suggests that the rate of *p*NPAc hydrolysis is lowered by a decrease in the rate of the deacylation step.

Molecular modeling was used to identify the molecular basis of the increase in perhydrolase activity caused by the Leu29Pro mutation. The model starting structure was prepared from the crystal structure of PFE (PDB ID: 1VA4). The Leu29Pro mutation was obtained by mutating position 29 of the wild-type structure scaffold. After energy minimization, an overlay of the proline residue in Leu29Pro PFE on the proline residue in CPO-F (PDB ID: 1A8S) confirmed identical conformations (results not shown). Assuming that perhydrolysis takes place with an esterase-like mechanism, we modeled the second tetrahedral intermediate to represent the transition state for hydrogen peroxide attack on the acyl enzyme. All modeled structures showed productive hydrogen bonds for catalytic triad residues and oxyanion-hole residues. In Leu29Pro PFE, a hydrogen bond was found between the carbonyl oxygen atom of Trp28 and the peroxide hydroxy group, with an O–O distance of 2.7 Å (O···H–O angle = 133°) (Figure 2a). Similarly, this hydrogen bond length in CPO-F was 2.7 Å (O···H–O angle = 127°). These distances are within the typical values for a hydrogen bond between the oxygen atom of a hydroxy group and a carbonyl oxygen atom: 2.8 ± 0.1 Å.[18] In contrast, the corresponding hydrogen bond in wild-type PFE is much weaker, with an O–O distance of 3.2 Å (O···H–O angle = 114°) (Figure 2b).

Table 3: Rate of hydrolysis and relative rates for several ester substrates.

Substrate	Wild-type [U mg^{-1}][a]	Leu29Pro [U mg^{-1}][a]	Fold rate change[b]
p-nitrophenyl acetate[c]	14	0.14	100
ethyl butyrate	1.3	0.16	8.1
ethyl valerate	0.65	0.083	7.8
propyl propionate	0.91	0.18	5.1
butyl propionate	0.57	0.14	4.1
ethyl propionate	0.75	0.19	3.9
methyl propionate	0.38	0.33	1.2

[a] Specific activity of ester hydrolysis determined in BES buffer (5 mM, pH 7.2) at 25 °C containing *p*-nitrophenol (0.51 mM) and substrate (5 mM); 1 U = 1 μmol protons generated from hydrolysis min^{-1} (Supporting Information). [b] The rate of hydrolysis by Leu29Pro over that of wild-type enzyme. [c] Experimental value, [*p*NPAc] = 0.3 mM.

Previous analysis of the CPO-F X-ray crystallographic structure found that Pro29 adopts a *cis*-peptide conformation, which may favor the reaction of hydrogen peroxide over water.[9] In that work, the perhydrolase activity of CPO-F was attributed to the difference in nucleophilic strength between hydrogen peroxide and water. Our results suggest that a hydrogen bond formed between the carbonyl oxygen atom of the enzyme and the peroxide nucleophile is the molecular basis for the increased perhydrolase activity. A peroxide hydroxy–carbonyl hydrogen bond could favor the perhydrolase reaction through orientation of the hydrogen peroxide substrate in a catalytically productive manner. The increased rate of perhydrolysis has an energy equivalent of 1.6 kcal mol^{-1}, which is consistent with a new hydrogen bond, as a hydrogen bond between a neutral donor/acceptor pair typically contributes 1–2 kcal mol^{-1} to substrate selectivity.[19,20]

To identify the molecular basis of the decreased hydrolytic activity of Leu29Pro toward *p*NPAc, we modeled the deacylation step of the hydrolysis. In wild-type PFE, modeling suggests that a water molecule can bridge the carbonyl oxygen

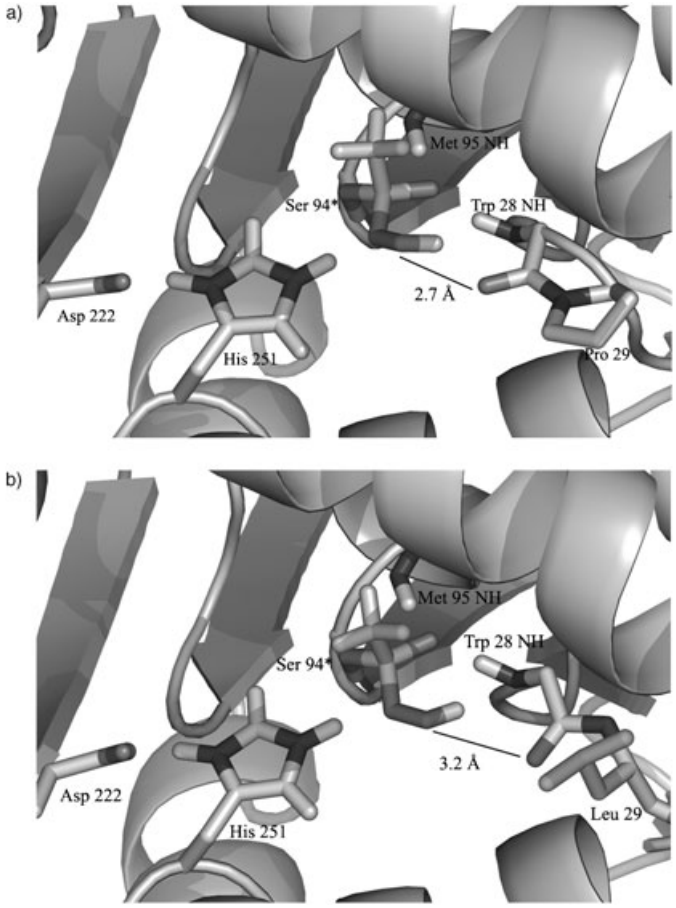

Figure 2. Models of the second tetrahedral intermediates of peracetic acid formation: a) Leu 29 Pro PFE has increased perhydrolase activity and a hydrogen bond between the backbone carbonyl oxygen atom of Trp 28 and the peroxide substrate hydroxy group (O–O = 2.7 Å, O···H–O angle = 133°); b) wild-type PFE has low perhydrolase activity and a weak hydrogen bond in the same location (O–O = 3.2 Å, O···H–O angle = 114°). Both structures show strong hydrogen bonds from the substrate to the catalytic histidine and in the oxyanion hole. The turn of residues 223–225, the side chains of residues 28 and 95, and the main chains of residues 29, 94, 222, and 251 are omitted for clarity.

of residue 28 and the hydroxy intermediate with hydrogen bonds, and thus lower the transition-state energy to facilitate deacylation. In Leu 29 Pro, the analogous space is insufficient for access of a water molecule and such stabilization is therefore missing (Supporting Information). As a result, the esterase activity for Leu 29 Pro was low for *p*NPAc because this change slowed the presumed rate-limiting deacylation step. Modeling suggests that the first tetrahedral intermediate for *p*NPAc showed no differences that could explain the large decrease in rate. The *p*-nitrophenyl moiety does adopt different configurations in wild-type and Leu 29 Pro PFE, but neither structure encounters significant steric strain.

In summary, the substitution of a single amino acid was sufficient to shift the hydrolase activity of PFE to make perhydrolysis the preferred reaction in aqueous solution. The catalytic activity is similar to that of naturally occurring perhydrolases. A molecular basis for the increase in perhydrolase activity is the presence of a carbonyl group in the vicinity of the active site that serves as a means to stabilize hydrogen peroxide attack on a putative acyl-enzyme intermediate (Figure 3). Modeling also shows that subtilisin, which also has a Ser–His–Asp catalytic triad but a different protein fold, lacks perhydrolase activity likely

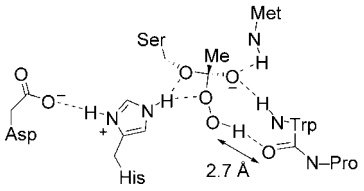

Figure 3. Proposed molecular basis of perhydrolase activity in an esterase from *P. fluorescens*: the formation of the second tetrahedral intermediate (after nucleophilic attack by the substrate peroxide) is facilitated and subsequently stabilized by a key hydrogen bond in the Leu 29 Pro mutant.

because the perhydrolysis tetrahedral intermediate lacks key hydrogen bonds (Supporting Information). A similar approach may be used to increase the perhydrolase activity of other serine hydrolases or to alter the preferred nucleophile to substrates other than water or hydrogen peroxide.

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