Design of Polyproline-Based Catalysts for Ester Hydrolysis

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Supporting Information

ABSTRACT: A number of simple oligopeptides have been recently developed as minimalistic catalysts for mimicking the activity and selectivity of natural proteases. Although the arrangement of amino acid residues in natural enzymes provides a strategy for designing artificial enzymes, creating catalysts with efficient binding and catalytic activity is still challenging. In this study, we used the polyproline scaffold and designed a series of 13-residue peptides with a catalytic dyad or triad incorporated to serve as artificial enzymes. Their catalytic efficiency on ester hydrolysis was evaluated by ultraviolet−visible spectroscopy using the p-nitrophenyl acetate assay, and their secondary structures were also characterized by circular dichroism spectroscopy. The results indicate that a well-formed polyproline II structure may result in a much higher catalytic efficiency. This is the first report to show that a functional dyad or triad engineered into a polyproline helix framework can enhance the catalytic activity on ester hydrolysis. Our study has also revealed the necessity of maintaining an ordered structure and a well-organized catalytic site for effective biocatalysts.

INTRODUCTION

Natural enzymes, exhibiting powerful efficiency and remarkable selectivity because of their unique folded structures, are usually involved in biochemical reactions. They play a major part in maintaining the functions in organisms. For instance, proteases are capable of accelerating the digestion of proteins into shorter fragments by hydrolyzing the peptide bonds. However, restricted to their length and complexity, most enzymes are difficult to acquire and vulnerable to environmental conditions, such as pH and temperature. Thus, artificial enzymes have received much attention in recent years because of their great stability and easy accessibility. Baker et al. and Korendovych et al. explored the efficient catalysts with rigid protein scaffolds by computational design.1,2 Ulijn et al. conducted phage display as a discovery technique for selecting active sequences.3 To demonstrate more details on the importance of three-dimensional structures, many artificial enzymes using various scaffolds, such as α-helical-coiled coils and barrels, β-peptide bundles, short helices, and β-hairpins, were reported. Moreover, nanomaterials and self-assembled structures including membranes, amyloid fibers, and hydrogels were implemented for mimicking similar protein structures; yet, the activity of these supramolecular frameworks is still limited compared to natural enzymes.

In natural enzymes, their active site is frequently composed of several residues, taking responsibility for specific binding and chemical catalysis. For example, chymotrypsin, a serine protease, possesses a hydrophobic pocket for binding to aromatic substrates and a catalytic site composed of His57, Asp102, and Ser195 residues.19 It is generally accepted that more than two amino acid residues that operate together at the active site of enzymes are regarded as a catalytic dyad or triad. Mechanically, nucleophilic residues (cysteine and serine) play a role of attacking the substrate; histidine acts as a general base to deprotonate the nucleophile; and acidic residues (aspartic acid and glutamine acid) function to polarize and align the base (Figure 1A).20,21 Additionally, it is well-documented that the guanidyl group in the arginine residue22 and the backbone NH group23,24 can aid in stabilizing the transition state of the substrate. These functional residues were initially applied to the...
primary sequence design25 and subsequently considered in the coassembly system.15,16,22

Inspired by the previous reports, here we chose polyproline peptides as the scaffold and introduced a catalytic dyad or triad into the peptides. It was found that proline-rich sequences in proteins often form a polyproline II (PPII) structure, a left-handed helix with all trans peptide bonds, and three residues in each turn. On account of their structural stability and rigidity, oligopropyl peptides were widely used as rulers and scaffolds for various studies.26,27 Hence, we designed and synthesized a series of 13-residue polyproline peptides containing a histidine residue at the i position and other residues with a functional side chain, such as Asp, Ser, and Cys, at the i + 3 or i − 3 position, as minimalistic biocatalysts (Figure 1B). According to the structure of a PPII helix, these positions would be located on the same side, and we predicted that the imidazole side chain of His could place an appropriate orientation and form strong interactions with the functional groups at position i − 3 or i + 3 to form a dyad or triad active site. Additionally, (2S,4R)-hydroxyproline (Hyp) and (2S,4R)-methoxyproline (Mop), 4-substituted proline derivatives, were found to have a high propensity to form PPII conformation because of the stereoelectronic effects.28,29 Therefore, they were also used to replace the regular proline residues for understanding the correlation between PPII structure and enzyme activity. The results showed that the interactions between different functional groups significantly contributed to the catalytic ability and that a well-formed PPII structure could lead to a higher catalytic efficiency.

■ RESULTS AND DISCUSSION

To explore the activity of polyproline-based catalysts on ester hydrolysis, we chose (Pro)11 as the control peptide (free P11) and designed a series of peptides in which the specific position was substituted with His and the residues frequently found in the active sites of natural enzymes. For a single His-incorporated peptide (H6), the His residue was placed in the middle (position 6) of the peptide because the conformation of His might be fixed by the proline-rich sequence. In the multiple substitution peptides, His residues were incorporated into position 6, whereas nucleophilic (Cys, Ser, and His), neutral (Trp), and acidic (Asp) residues were placed at positions 3 and 9 to mimic the triad or dyad in natural enzymes. These peptides were designated as H3H6, W3H6, S3H6, S3H6D9, and C3H6D9. In addition, the peptides with Pro replaced by Hyp (Hyp-H3H6 and Hyp-C3H6D9) or Mop (Mop-H3H6) were applied to investigate the relationship between PPII-forming propensity and catalytic efficiency. A Gly–Tyr dipeptide was attached to the C-terminus of each peptide for concentration determination. Furthermore, all peptides except for free P11 have capped ends because a previous study found that the removal of the capping groups would reduce the catalytic activity.30 The sequences of designed peptides are shown in Table 1.

After synthesis of the peptides, circular dichroism (CD) spectroscopy was utilized to characterize their secondary structures. As shown in Figure 2, all peptides exhibit a similar far-UV spectrum with a positive band between 220 and 230 nm and a negative peak near 205 nm, indicating the presence of PPII conformation. We used the maximal molar ellipticity at the positive band to evaluate their PPII-forming propensity and content. As shown in Figure 2 and Table 2, the replacement of proline to nonproline residues causes the decrease in the PPII structure. Compared to free P11, all peptides with Pro replaced by other residues display a much weaker positive band, and the multiple substitutions impose an even greater effect on PPII conformation than does the single substitution. Although the peptides with double- and triple-substituted nonproline residues show a much weaker characteristic CD signal of the PPII structure, W3H6 displays a stronger positive band around 230 nm due to the electronic absorption of aromatic residues.30,31 Therefore, the PPII propensity of W3H6 could not be evaluated solely by its ellipticity at the positive band. As expected, Hyp-H3H6, Hyp-C3H6D9, and Mop-H3H6 form a relatively intense PPII structure, and their molar ellipticity in the positive band is close to or even higher than that of free P11 (Figure 2B and Table 2), which can be attributed to that Hyp and Mop prefer a C’-exo pucker and a trans peptide bond to enhance PPII-forming propensity.28,29

To evaluate the catalytic efficiency of our designed peptides and compare the results with the previous hydrolase designs, we chose p-nitrophenyl acetate (p-NPA) as a simple chromogenic substrate to monitor the catalytic reaction. The catalytic reaction is as follows:

The initial hydrolytic rate (V0) was determined by monitoring the production of 4-nitrophenol at 405 nm and pH 7.4 with ultraviolet–visible (UV–vis) spectroscopy. As shown in Figure 3A,B, the dependence of the initial hydrolytic rate on the substrate concentration for each peptide is consistent with the Michaelis–Menten model, and the kinetic parameters can be obtained and calculated by fitting the curves to the model. The determined parameters are listed in Table 3.

The reaction in the presence of free histidine residues was also monitored and used as a control. Figure S3 in the Supporting Information indicates that the rate of hydrolysis of p-NPA in the presence of free P11 was extremely low and close to that without any peptides or amino acids present in solution, suggesting that the polyproline backbone was unable to catalyze the reaction. This is likely attributed to the fact that free P11 does not contain nucleophiles to accelerate the reaction, and
the proline residues lack NH groups on the backbone to stabilize the oxyanion intermediate of ester hydrolysis as suggested by previous studies.\(^1,^{23,24}\)

To prevent the electrostatic repulsions and extra charges from affecting the catalytic efficiency, we introduced end caps by acetylating the N-terminus and amidating the C-terminus on the following peptides: H6, W3H6, S3H6, S3H6D9, and C3H6D9. Of the designed peptides, His, Trp, Ser, Asp, or Cys was incorporated in the multiple substitution peptides for the purpose of forming catalytic dyads or triads. As shown in Table 3, the catalytic efficiency of the H6 peptide (\(k_{\text{cat}}/K_M = 0.39 \text{ M}^{-1} \text{s}^{-1}\)) is lower than that of free His (\(k_{\text{cat}}/K_M = 0.45 \text{ M}^{-1} \text{s}^{-1}\)), indicating that the single His incorporation in a polyproline peptide could not induce an effective electron transfer to catalyze the reaction. By contrast, the polyproline peptides with double substitutions (H3H6, S3H6, and W3H6) are 1.5-fold more active in comparison with H6, suggesting that the residues (His, Ser, and Trp) at position 3 of H3H6, S3H6, and W3H6 could interact with His at position 6 to facilitate the electron transfer and enhance the catalytic properties. Therefore, it could be inferred that all peptides with double substitutions may form a catalytic dyad in their active site. As detailed in Table 3, W3H6 is more efficient than H3H6 and S3H6 and has a \(k_{\text{cat}}/K_M\) value of 0.60 M\(^{-1}\) s\(^{-1}\). It could be explained by an increased nucleophilicity of His because of the histidine–aromatic interaction provided by Trp at position 3, which was also observed in barnase, a natural hydrolyase.\(^{32}\)

To validate our assumption that the PPII structure can facilitate a proper orientation between the side chains of His and Trp and generate strong interactions to enhance the catalytic activity, we prepared a dipeptide Trp–His (WH) and measured its catalytic efficiency. As shown in Figure S4 of the Supporting Information, its catalytic efficiency (\(k_{\text{cat}}/K_M = 0.52 \text{ M}^{-1} \text{s}^{-1}\)) is lower than that of W3H6, providing a piece of evidence that the PPII framework does play a positive role to increase its catalytic activity. Surprisingly, the catalytic activity of the peptides with triple substitutions slightly decreased compared with those with double substitutions. Their catalytic activity of the hydrolyzing p-NPA is around 0.45 M\(^{-1}\) s\(^{-1}\). It seems that introducing more residues into the PPII structure could not always benefit their catalytic abilities. As shown in Table 2, the peptides with triple substitutions (S3H6D9 and C3H6D9) dramatically reduce the propensity of forming a PPII structure, which may lead to an increased distance between the i and i + 3 positions. Thus, it could be assumed that the catalytic triad may not be successfully formed in S3H6D9 and C3H6D9, and Asp cannot play a role as orienting the His residue and neutralizing the intermediates.

To demonstrate more structure–activity relationships, we replaced the proline residues of H3H6 and C3H6D9 with Hyp and used Mop to substitute for the proline of H3H6 as well to form a more robust PPII structure. Interestingly, as shown in Figure 3B, we found that both Hyp-H3H6 and Hyp-C3H6D9 remarkably accelerated the hydrolysis of p-NPA. An approximately threefold increase in catalytic efficiency was observed for Hyp-H3H6 compared with free His residue. Both of the Hyp-H3H6 (\(k_{\text{cat}}/K_M = 1.23 \text{ M}^{-1} \text{s}^{-1}\)) and Hyp-C3H6D9 (\(k_{\text{cat}}/K_M = 0.99 \text{ M}^{-1} \text{s}^{-1}\)) exhibited a twofold higher efficiency than that of their corresponding peptides H3H6 (\(k_{\text{cat}}/K_M = 0.56 \text{ M}^{-1} \text{s}^{-1}\)) and C3H6D9 (\(k_{\text{cat}}/K_M = 0.43 \text{ M}^{-1} \text{s}^{-1}\)), respectively. As shown in Table 3, the peptides with Hyp-rich sequences show a relatively stronger binding affinity (lower \(K_M\)) for the substrate than do their Pro-rich counterparts, leading to a much higher catalytic efficiency. It is likely that Hyp residues provide extra interactions and result in a stronger binding to the substrates. As reported in a few natural active sites, an oxyanion hole could stabilize the transition state of the substrate.\(^{1,23,24}\)

Therefore, we believe that the OH group on the Hyp side chain could potentially serve as a hydrogen bond donor to stabilize the reaction intermediate and increase the reaction rate. This argument may also be supported by the observation that Mop-H3H6 exhibits a slightly higher activity (\(k_{\text{cat}}/K_M = 0.62\)) than H3H6 but a much lower activity than Hyp-H3H6 (Figure 3C). Because the OMe group on the Mop side chain cannot be a hydrogen bond donor, Mop cannot form similar hydrogen

| peptide               | \(\lambda_{\text{max}}\) (nm) | \([\theta]_{\text{max}}\) (10\(^2\) deg cm\(^2\) dmol\(^{-1}\)) |
|----------------------|-----------------------------|--------------------------------------------------|
| free P11             | 228                         | 3.37                                             |
| H6                   | 228                         | 3.37                                             |
| W3H6                 | 229                         | 2.75                                             |
| H3H6                 | 229                         | 0.86                                             |
| S3H6                 | 229                         | 0.75                                             |
| S3H6D9               | 231                         | 0.43                                             |
| C3H6D9               | 231                         | -0.18                                            |
| Hyp-H3H6             | 226                         | 6.00                                             |
| Hyp-C3H6D9           | 226                         | 4.62                                             |
| Mop-H3H6             | 226                         | 3.12                                             |

Figure 2. Far-UV CD spectra for the designed peptides: (A) H6, W3H6, S3H6, and S3H6D9 and (B) H3H6, C3H6D9, Hyp-H3H6, Hyp-C3H6D9, and Mop-H3H6 at pH 7.4 and 25 °C. The spectrum of free P11 is included for comparison.
bonding interactions as Hyp does during the reaction, leading to a lower catalytic efficiency. In addition, the fact that Hyp-H3H6, Hyp-C3H6D9, and Mop-H3H6 have a higher catalytic efficiency than their corresponding peptides (H3H6 and C3H6D9) demonstrates the important role of a PPII structure.

As aforementioned, the peptides containing Hyp or Mop residues favor the formation of a stable PPII structure, which may bring about a closer distance between the \( i \) and \( i + 3 \) / \( i - 3 \) positions. As a result, it could be speculated that the PPII helix-forming propensity of our designed peptides also has a great impact on their catalytic activity. Although the efficiency of our designed peptides is not comparable to that of natural enzymes in catalyzing such an ester hydrolysis reaction, most of our designed polyproline-based peptides still exhibited a greater catalytic activity than some reported peptide-based nanostructures whose \( k_{cat}/K_M \) ranges from 0.09 to 0.62 M\(^{-1}\) s\(^{-1}\) at pH 7.5.\(^{12,15,16}\) Most notably, even as a short peptide, the activity of Hyp-H3H6 is better or comparable to the previously reported

| peptide | \( k_{cat} \) (10\(^{-3}\) s\(^{-1}\)) | \( K_M \) (mM) | \( k_{cat}/K_M \) (M\(^{-1}\) s\(^{-1}\)) |
|---------|-------------------------------|----------------|---------------------------------|
| free His (H-His-OH) | 0.78 ± 0.16 | 1.73 ± 0.49 | 0.45 ± 0.16 |
| free P11 | 0.28 ± 0.06 | 0.88 ± 0.32 | 0.31 ± 0.13 |
| H6 | 0.70 ± 0.05 | 1.81 ± 0.17 | 0.39 ± 0.05 |
| W3H6 | 1.15 ± 0.38 | 1.92 ± 0.85 | 0.60 ± 0.33 |
| H3H6 | 2.44 ± 0.73 | 4.38 ± 1.50 | 0.56 ± 0.25 |
| S3H6 | 0.86 ± 0.33 | 1.52 ± 0.82 | 0.56 ± 0.37 |
| S3H6D9 | 0.97 ± 0.24 | 2.12 ± 0.69 | 0.46 ± 0.19 |
| C3H6D9 | 1.05 ± 0.27 | 2.44 ± 0.78 | 0.43 ± 0.18 |
| Hyp-H3H6 | 1.76 ± 0.16 | 1.42 ± 0.19 | 1.23 ± 0.20 |
| Hyp-C3H6D9 | 1.73 ± 0.15 | 1.75 ± 0.20 | 0.99 ± 0.14 |
| Mop-H3H6 | 1.88 ± 0.67 | 3.02 ± 1.30 | 0.62 ± 0.35 |

Deviations of \( k_{cat} \) and \( K_M \) were the standard errors of fitting and were used to calculate the deviation of \( k_{cat}/K_M \) by the equation

\[
\sigma_{k_{cat}/K_M} = \left( \sigma_{k_{cat}/K_M} \right) \times \sqrt{\left( \sigma_{k_{cat}}^2 / k_{cat} \right)^2 + \left( \sigma_{K_M} / K_M \right)^2}.
\]

Figure 3. (A–C) Esterase activity for the designed peptides at pH 7.4 and 25 °C, with solid lines showing the fits to the Michaelis–Menten equation. (D) Esterase activity for the selected peptides under different pH conditions, with solid lines showing the fits to the modified Henderson–Hasselbalch equation (eq 1).

Table 4. Kinetic pK\(_a\) and Maximal Efficiency for p-NPA Hydrolysis by Selected Peptides

| peptide | pK\(_{cat}\) | pK\(_{cat}\) | \( k_{cat}/K_M(\text{max}) \) (M\(^{-1}\) s\(^{-1}\)) | \( k_{cat}/K_M(\text{max}) \) (M\(^{-1}\) s\(^{-1}\)) |
|---------|-------------|-------------|---------------------------------|---------------------------------|
| W3H6 | 6.73 ± 0.18 | 9.83 ± 0.01 | 0.50 ± 0.06 | 47.93 ± 0.49 |
| H3H6 | 6.30 ± 0.43 | 9.74 ± 0.01 | 0.33 ± 0.07 | 42.36 ± 0.49 |
| S3H6D9 | 6.27 ± 0.44 | 9.91 ± 0.03 | 0.29 ± 0.09 | 42.46 ± 1.39 |
| Hyp-H3H6 | 6.74 ± 0.93 | 9.92 ± 0.11 | 0.81 ± 0.46 | 53.78 ± 5.93 |
metal-based esterases ($k_{\text{cat}}/K_M$ is 0.33–1.38 M$^{-1}$ s$^{-1}$ at pH 7.5).\(^5\)

Because the protonated and deprotonated state of an active site would affect its catalytic activity, we investigated the hydrolysis reaction under different pH conditions for the selected peptides: W3H6, H3H6, S3H6D9, and Hyp-H3H6. As illustrated in Figure 3D, while the efficiency of these peptides remained low below pH 8.0, a noticeable increase could be observed as the pH values rose beyond 8.0. The catalytic efficiency ($k_{\text{cat}}/K_M$) of each selected peptide at pH 10.0 is in the range of 23–30 M$^{-1}$ s$^{-1}$, which is 30-fold greater than that at pH 7.4 (Table S3 in the Supporting Information). Using eq 1, a pair of kinetic pK$_a$ could be determined for each selected peptide (Table 4). As the typical pK$_a$ of a His imidazole ring is about 6, it is likely that the obtained pK$_a$ values around 6.5 represented deprotonation of a His side chain. Besides, we would suggest that the higher pK$_a$ reflected deprotonation of the Tyr side chain because its pK$_a$ is approximately 10.3 in the free form. We further used $^1$H NMR spectroscopy to measure the pK$_a$ of a His side chain in S3H6D9. A series of high-resolution one-dimensional ($^1$H) NMR spectra for S3H6D9 were acquired at pH values ranging from 4 to 8 (Figures 4A and S5 in the Supporting Information), whereas a titration curve was obtained by plotting the chemical shifts of His C$^\alpha$H versus pH (Figure 4B). By fitting the titration curve into the Henderson–Hasselbalch equation (eq 2), a pK$_a$ value of 6.64 and a Hill coefficient of 1.6 could be determined for S3H6D9. The pK$_a$ value measured by NMR is in good agreement with the pK$_a$ value derived by the kinetic hydrolysis assay, reflecting that the deprotonation of the His imidazole group could significantly increase the catalytic efficiency.

Furthermore, to have more insights into the structure of designed peptides, we conducted Hartree–Fock (HF) calculations on the conformation of oligopeptides. As it was found that Ac-(Pro)$_2$-OMe is the simplest peptide model to form a stable PPII structure,\(^3\) we used the seven-residue oligopeptide models, Ac-(Pro)$_2$Ser(Pro)$_2$HisPro-OMe, Ac-Cys(Pro)$_2$His(Pro)$_2$Asp-OMe, and Ac-(Pro)$_2$-OMe, to simplify the computational studies, in which Ac-(Pro)$_2$Ser(Pro)$_2$HisPro-OMe is utilized as a model to mimic S3H6, whereas Ac-Cys(Pro)$_2$His(Pro)$_2$Asp-OMe was used to mimic C3H6D9. In the energy-minimized structure, the dihedral angles of Ac-(Pro)$_2$Ser(Pro)$_2$HisPro-OMe and Ac-Cys(Pro)$_2$His(Pro)$_2$Asp-OMe are similar to those of Ac-(Pro)$_2$-OMe and an idealized PPII helix, indicating that this peptide could form a PPII-like helix (Table S4 in the Supporting Information). For an ideal PPII helix, every third residue is about 9 Å apart, suggesting that the distance between the two side chains at positions $i$ and $i + 3$ should be less than 9 Å to form possible interactions. As shown in Figure S6 of the Supporting Information, the distance between the side chains of Ser and His in the energy-minimized structure of Ac-(Pro)$_2$Ser(Pro)$_2$HisPro-OMe is 5.4 Å, which is significantly shorter than 9 Å, suggesting that the functional groups of Ser and His might interact and form a reaction dyad to catalyze ester hydrolysis. This finding may in part explain why S3H6 exhibits a better activity than H6. For the optimized structure of Ac-Cys(Pro)$_2$HisPro-OMe (Figure 5), the distance between Cys and His is 2.90 Å, whereas the distance between His and Asp is up to 6.7 Å. In comparison, the distances within the catalytic triad of chymotrypsin, a serine protease, are shorter than 3 Å,\(^19\) which facilitates the proton transfer between them. It could be inferred that the functional groups in the triple substitution peptide, C3H6D9, might not form a catalytic triad because the long distance between His and Asp could hinder them from forming an appropriate orientation between the functional groups. Therefore, the observation in the computational structure may rationalize why the triple substitution peptides exhibited a less catalytic efficiency than did the double substitution peptides.

## CONCLUSIONS

In the current work, a series of polyproline peptides were designed and prepared to form PPII helices and serve as a scaffold to catalyze ester hydrolysis. We found that introducing a pair of residues containing His into the PPII structure could successfully imitate a catalytic dyad and improve the catalytic efficiency. However, introducing more than two nonproline residues could dramatically perturb the PPII structure and failed to form a catalytic triad in the polyproline-based scaffold. Surprisingly, the replacement of Pro to Hyp in the peptide not only favors forming a stable PPII helix but also provides additional interactions to accelerate the hydrolysis of ester. In addition, the deprotonation of histidine side chains could enhance the catalytic activity of peptides. Our results provide compelling lines of evidence that the secondary structure of the peptide-based enzymes could have substantial impacts on their catalytic activity.
chlorotrityl resin was used to produce a free C-terminus, whereas the use of the Rink amide resin generated an amidated C-terminus upon cleavage. Cleavage of the peptides from the resin and side-chain deprotection was performed using a solution of 95% trifluoroacetic acid (TFA)/2.5% triisopropylsilylamine (TIS)/2.5% H₂O (v/v) or 94% TFA/1% TIS/2.5% H₂O/2.5% 1,2-ethanedithiol (v/v) to treat the peptide-resin product for 3 h at ambient temperature. The filtrate was then precipitated and washed with ice-cold methyl t-butyl ether and purified by reverse-phase HPLC with a semipreparative C18 column. H₂O/acetonitrile gradients with 0.1% (w/v) TFA were used as the eluting solvent system to purify the peptides. The purified products were identified by MALDI-TOF-mass spectrometry (Table S1 and Figure S2 in the Supporting Information). All peptides were more than 90% pure according to the HPLC analysis as shown in Figure S1 of the Supporting Information.

**Preparation of Peptide Stock Solutions.** Purified and lyophilized peptides were dissolved in 25 mM buffer solution [MES (pH 6.0–6.5), HEPES (pH 7.0), Tris (pH 7.4–9.0), and CAPS (pH 10.0)] to make a 0.5 mM peptide stock solution. The concentration of peptides was determined by the UV absorbance at 280 nm in 6 M guanidine hydrochloride at pH 6.5, using an extinction coefficient of 1420 M⁻¹ cm⁻¹ for Tyr and 5500 M⁻¹ cm⁻¹ for Trp.

**CD Spectroscopy.** CD spectra were recorded on an Aviv model 410 CD spectrometer with a 1 mm path-length-quartz cuvette. The measurements were performed in pH 7.4 and 25 mM Tris buffer with a peptide concentration of 100 μM. The far-UV CD spectra were taken from 190 to 260 nm with an averaging time of 10.0 s and a wavelength step of 1 nm at 25 °C.

**Kinetic Assays.** The catalytic activities of the peptides were determined using p-NPA as the substrate. The kinetic measurements were performed on a JASCO V-630 spectrophotometer associated with an STR-773 water thermostat cell holder and stirrer by monitoring the absorbance of the hydrolysis product (p-nitrophenol) at 348 nm or 405 nm at 25 °C. A volume of 100 μL of the freshly prepared peptide stock solution was added to 1900 μL of buffer solution. After having recorded the initial absorbance (blank), a volume of x μL of 20 mM p-NPA stock in acetonitrile was subsequently added to obtain a final p-NPA concentration of 100–1600 μM (the final acetonitrile content was less than 5% in all reaction mixtures). The reaction mixture was stirred for 60 s before collecting the data, and the absorbance was recorded every 10 s for 150–1200 s (as shown in Table S2 of the Supporting Information). Initial rates were determined from linear fits of the plots of absorbance versus time, and the values were from averaging duplicate measurements. The extinction coefficients of the hydrolysis product at different pH values were experimentally determined by measuring the absorbance of p-nitrophenol in the specified buffers and by taking the average of at least three repeats. The reported kinetic parameters (kcat and KM) were derived by fitting the data to the Michaelis–Menten equation, and the pH-dependent curves were fit to the following modified Henderson–Hasselbalch equation

\[
\frac{k_{cat}}{K_M} = \frac{k_{cat}}{k_{cat}} = \frac{1}{1 + 10^{pK_{A}^{-pH}}} + \frac{k_{cat}}{k_{cat}} = \frac{1}{1 + 10^{pK_{A}^{-pH}}}
\]
Determination of pKₐ by NMR. A series of S3H6D9 (1.2 mM) peptide samples were prepared in 90% H₂O/10% D₂O-containing sodium 3-(trimethylsilyl)-1-propanesulfonate in solution as the chemical shift reference. Their pH values in the range of 4–8 were adjusted by adding NaOD or DCI directly into the samples. High-resolution 1D ¹H NMR spectra were acquired at 25 °C using a Varian VNMRS-700 NMR spectrometer (Varian, Inc.) at National Tsing Hua University Instrumentation Center. The spectra were analyzed using Bruker Topspin (v.2.1) software, and the pKₐ values were determined by fitting a plot of the chemical shift (δ) of His δH versus pH into the modified Henderson–Hasselbalch equation

\[ \delta = \frac{\delta_1 + \delta_0 10^{\delta(pK_a - pH)}}{1 + 10^{\delta(pK_a - pH)}} \]  

(2)

where δ is the observed chemical shift, δ₁ is the chemical shift for the fully deprotonated species, δ₀ is the chemical shift for the fully protonated species, and n is the Hill coefficient, which is 1 for a residue that follows ideal Henderson–Hasselbalch behaviors.

Structure Modeling. Geometry optimization for the model peptide in the aqueous phase was carried out using the HF/6-31+g(d) and CPCM-SCRF solvation method, conducted by the Gaussian 09 software. The initial structure was generated with idealized torsion angles for the PPII backbones based on the reported structural data.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00928.

Measured molecular weights of the peptides, detailed experimental conditions for pH-dependent kinetic assays, pH-dependent kinetic parameters for ester hydrolysis, dihedral angles of energy-minimized structural models, HPLC chromatograms, MALDI-TOF spectra, UV–vis-monitored ester hydrolysis reaction curves, additional esterase activity plots, additional pH-dependent ¹H NMR spectra, and energy-minimized structure model for Ac-(Pro)₂Ser(Pro)₂His-Pro-OMe (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Richter, F.; Blomberg, R.; Khare, S. D.; Kiss, G.; Kuzin, A. P.; Smith, A. J. T.; Gallaher, J.; Planowski, Z.; Helgeson, R. C.; Grijanow, A.; Xiao, R.; Seetharaman, J.; Su, M.; Vorobiev, S.; Lew, S.; Forouhar, F.; Kornhaber, G. J.; Hunt, J. F.; Montellone, G. T.; Tong, L.; Houk, K. N.; Hilvert, D.; Baker, D. Computational design of catalytic dyads and oxyanion holes for ester hydrolysis. J. Am. Chem. Soc. 2012, 134, 16197–16206.

(2) Moroz, Y. S.; Dunston, T. T.; Makhluyents, O. V.; Moroz, O. V.; Wu, Y.; Yoon, J. H.; Olsen, A. B.; McLaughlin, J. M.; Mack, K. L.; Gosavi, P. M.; van Nuland, N. A. J.; Korendovych, I. V. New tricks for old proteins: Single mutations in a nonenzymatic protein give rise to various enzymatic activities. J. Am. Chem. Soc. 2015, 137, 14905–14911.

(3) Maeda, Y.; Javid, N.; Duncan, K.; Birchall, L.; Gibson, K. F.; Cannon, D.; Kanetsuki, Y.; Knapp, C.; Tuttle, T.; Ulijn, R. V.; Matsui, H. Discovery of catalytic phages by biocatalytic self-assembly. J. Am. Chem. Soc. 2014, 136, 15893–15896.

(4) Burton, A. J.; Thomson, A. R.; Dawson, W. M.; Brady, R. L.; Woolfson, D. N. Installing hydrolytic activity into a completely de novo protein framework. Nat. Chem. 2016, 8, 837–844.

(5) Zastrow, M. L.; Pecoraro, V. L. Influence of active site location on catalytic activity in de novo-designed zinc metalloenzymes. J. Am. Chem. Soc. 2013, 135, 5895–5903.

(6) Zastrow, M. L.; Peacock, A. F. A.; Stuckey, J. A.; Pecoraro, V. L. Hydrolytic catalysis and structural stabilization in a designed metalloprotein. Nat. Chem. 2011, 4, 118–123.

(7) Wang, P. S. P.; Nguyen, J. B.; Schapertz, A. Design and high-resolution structure of a β-hpeptide bundle catalyst. J. Am. Chem. Soc. 2014, 136, 6810–6813.

(8) Bezer, S.; Matsumoto, M.; Lodewyk, M. W.; Lee, S. J.; Tantillo, D. J.; Gagné, M. R.; Waters, M. L. Identification and optimization of short helical peptides with novel reactive functionality as catalysts for acyl transfer by reactive tagging. Org. Biomol. Chem. 2014, 12, 1488–1494.

(9) Matsumoto, M.; Lee, S. J.; Gagné, M. R.; Waters, M. L. Cross-strand histidine-aromatic interactions enhance acyl-transfer rates in beta-hairpin peptide catalysts. Org. Biomol. Chem. 2014, 12, 8711–8718.

(10) Matsumoto, M.; Lee, S. J.; Waters, M. L.; Gagné, M. R. A catalyst selection protocol that identifies biomimetic motifs from β-hairpin libraries. J. Am. Chem. Soc. 2014, 136, 15817–15820.

(11) Zaramella, D.; Scrimin, P.; Prins, L. J. Self-assembly of a catalytic multivalent peptide-nanoparticle complex. J. Am. Chem. Soc. 2012, 134, 8396–8399.

(12) Zhang, Q.; He, X.; Han, A.; Tu, Q.; Fang, G.; Liu, J.; Wang, S.; Li, H. Artificial hydrolyase based on carbon nanotubes conjugated with peptides. Nanoscale 2016, 8, 16851–16856.

(13) Poznik, M.; König, B. Cooperative hydrolysis of aryl esters on functionalized membrane surfaces and in micellar solutions. Org. Biomol. Chem. 2014, 12, 3175–3180.

(14) Rufo, C. M.; Moroz, Y. S.; Moroz, O. V.; Stöhr, J.; Smith, T. A.; Hu, X.; DeGrado, W. F.; Korendovych, I. V. Short peptides self-assemble to produce catalytic amylloids. Nat. Chem. 2014, 6, 303–309.

(15) Zhang, C.; Xue, X.; Luo, Q.; Li, Y.; Yang, K.; Zhuang, X.; Jiang, Y.; Zhang, C.; Liu, J.; Zou, G.; Liang, X.-J. Self-assembled peptide nanofibers designed as biological enzymes for catalyzing ester hydrolysis. ACS Nano 2014, 8, 11715–11723.

(16) Wang, M.; Lv, Y.; Liu, X.; Qi, W.; Su, R.; He, Z. Enhancing the activity of peptide-based artificial hydrolyase with catalytic Ser/His/Asp triad and molecular imprinting. ACS Appl. Mater. Interfaces 2016, 8, 14133–14141.

(17) Wong, Y.-M.; Masunaga, H.; Chuah, J.-A.; Sudes, K.; Numata, K. Enzyme-mimic peptide assembly to achieve amidolytic activity. Biomacromolecules 2016, 17, 3375–3385.

(18) Singh, N.; Conte, M. P.; Ulijn, R. V.; Miravet, J. F.; Escuder, B. Insight into the esterase like activity demonstrated by an imidazole appended self-assembling hydrogelator. Chem. Commun. 2015, 51, 13213–13216.
(19) Blow, D. M.; Birktoft, J. J.; Hartley, B. S. Role of a buried acid group in the mechanism of action of chymotrypsin. *Nature* 1969, 221, 337—340.

(20) Brady, L.; Brzozowski, A. M.; Derewenda, Z. S.; Dodson, E.; Dodson, G.; Tolley, S. P.; Turkenburg, J. P.; Christiansen, L.; Hug-Jensen, B.; Norskov, L., et al. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* 1990, 343, 767—770.

(21) Dodson, G. Catalytic triads and their relatives. *Trends Biochem. Sci.* 1998, 23, 347—352.

(22) Huang, Z.; Guan, S.; Wang, Y.; Shi, G.; Cao, L.; Gao, Y.; Dong, Z.; Xu, J.; Luo, Q.; Liu, J. Self-assembly of amphiphilic peptides into bio-functionalized nanotubes: a novel hydrolyase model. *J. Mater. Chem.* B 2013, 1, 2297—2304.

(23) Kamerlin, S. C. L.; Chu, Z. T.; Warshel, A. On catalytic preorganization in oxyanion holes: highlighting the problems with the gas-phase modeling of oxyanion holes and illustrating the need for complete enzyme models. *J. Org. Chem.* 2010, 75, 6391—6401.

(24) Simón, L.; Goodman, J. M. Enzyme catalysis by hydrogen bonds: the balance between transition state binding and substrate binding in oxyanion holes. *J. Org. Chem.* 2000, 8, 2675—2680.

(25) Arora, P. S.; Ansari, A. Z.; Best, T. P.; Ptashne, M.; Dervan, P. B. Design of artificial transcriptional activators with rigid poly-L-proline linkers. *J. Am. Chem. Soc.* 2002, 124, 13067—13071.

(26) Bonger, K. M.; Kapoerchan, V. V.; Grotenbreg, G. M.; van Koppen, C. J.; Timmers, C. M.; van der Marel, G. A.; Overkleeft, H. S. Oligoproline helices as structurally defined scaffolds for oligomeric G protein-coupled receptor ligands. *Org. Biomol. Chem.* 2010, 8, 1881—1884.

(27) Horng, J.-C.; Raines, R. T. Stereoelectronic effects on polyproline conformation. *Protein Sci.* 2006, 15, 74—83.

(28) Chiang, Y.-C.; Lin, Y.-J.; Horng, J.-C. Stereoelectronic effects on the transition barrier of polyproline conformational interconversion. *Protein Sci.* 2009, 18, 1967—1977.

(29) Chakrabartty, A.; Kortemme, T.; Padmanabhan, S.; Baldwin, R. L. Aromatic side-chain contribution to far-ultraviolet circular dichroism of helical peptides and its effect on measurement of helix propensities. *Biochemistry* 1993, 32, 5560—5565.

(30) Lin, Y.-J.; Chu, L.-K.; Horng, J.-C. Effects of the terminal aromatic residues on polyproline conformation: Thermodynamic and kinetic studies. *J. Phys. Chem. B* 2015, 119, 15796—15806.

(31) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G. A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Casselton, L. A.; Morokuma, K.; Raghavachari, K.; Al-Laham, M. A.; Cui, W.; Baboul, A. G.; Farkas, O.; Tomasi, J.; Francl, M.; Marzari, N.; Foresman, J. B.; Ochterski, J. W.; Petrone, A.; Henderson, D.; Raghavachari, K.; Piskorz, P.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, O.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J.

*Gaussian 09*, Revision B.01; Gaussian, Inc.: Wallingford, CT, 2009.

(32) Cowan, P. M.; McGavin, S. Structure of poly-L-proline. *Nature* 1955, 176, 501—503.