Design and High-Resolution Structure of a $\beta^3$-Peptide Bundle Catalyst

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Supporting Information

ABSTRACT: Despite the widespread exploration of $\alpha$-peptides as catalysts, there are few examples of $\beta$-peptides that alter the course of a chemical transformation. Our previous work demonstrated that a special class of $\beta^3$-peptides spontaneously self-assembles in water into discrete protein-like bundles possessing unique quaternary structures and exceptional thermodynamic stability. Here we describe a series of $\beta^3$-peptide bundles capable of both substrate binding and chemical catalysis—ester hydrolysis. A combination of kinetic and high-resolution structural analysis suggests an active site triad composed of residues from at least two strands of the octameric bundle structure.

Peptides embody two molecular properties that engender chemical catalysis. The propensity of a polyamide backbone, even a short one, to occupy a restricted conformational space facilitates the judicious placement of potential catalytic, recognition, or stabilizing groups, while the chirality of amino acid monomers and the structures they form can impart intrinsic selectivity. Despite the widespread exploration of $\alpha$-peptides as catalysts for numerous reactions, there are only two reported examples of $\beta$-peptides that alter the course of a chemical transformation. $\beta$-Peptides are polymers of $\beta$-amino acids, which differ from natural $\alpha$-amino acids by the addition of a single backbone methylene unit per residue; the additional methylene unit imparts structural and metabolic stability.

Our previous work demonstrated that a special class of $\beta^3$-peptides spontaneously self-assembles in water into discrete helical bundles possessing a protein-like tertiary fold and exceptionally high thermodynamic stability. Here we report a series of $\beta^3$-peptide bundles capable of both substrate binding and chemical catalysis—ester hydrolysis. A combination of kinetic and high-resolution structural analysis suggests an esterase active site composed of three functional groups positioned on separate strands of the octameric bundle structure.

Our design began with the structure of Zwit-EYYK, the most thermally and kinetically stable $\beta^3$-peptide bundle characterized to date. The Zwit-EYYK bundle folds cooperatively ($T_M = 78$ °C at 25 $\mu$M) and is >90% octameric at this concentration. As a model reaction, we chose the hydrolysis of 8-acetoxypyrene-1,3,6-trisulfonate (1), which releases the fluorescent product pyranine (2) upon ester hydrolysis (Figure 1A). Previous work has shown that arginine side chains in natural enzymes can interact favorably with sulfonate groups; the binding of coenzyme M to hydrogenases is one example of such an interaction. Previous work has also shown that histidine side chains are used extensively within the active sites of natural esterases, either as nucleophiles or, more frequently, as general acids/bases. There is also an extensive biomimetic chemistry literature to support combining binding and catalytic groups to facilitate chemical reactions in aqueous solution. We sought to test the hypothesis that a $\beta^3$-peptide bundle endowed with judiciously positioned arginine and histidine side chains would catalyze the hydrolysis of 1.

To test this hypothesis, we modified the sequence of the Zwit-EYYK monomer to electrostatically guide substrate 1 into the proximity of a single histidine side chain while minimally perturbing the bundle structure. Substrate 1 is planar, with three sulfonate groups whose structural relationship mimics that between side chains at positions 3 and 9 of Zwit-EYYK (U and Z in Figure 1B) would facilitate electrostatic guidance to a histidine side chain at interaction.

Figure 1. (A) Reactions evaluated in the presence or absence of the $\beta^3$-peptides shown in panels B–D. Not all sequences assemble into $\beta^3$-peptide bundles (see text). (B–D) Helical net diagrams of $\beta^3$-peptides studied herein.

Received: February 9, 2014
Published: May 6, 2014
position 1 (X in Figure 1B) without severely compromising bundle stability. A similar logic has been previously applied to design a cyclic peptide catalyst for an analogous ester substrate. Based on this design rationale, we synthesized three variants of Zwit-EYYKY carrying a single α-histidine (αH) at position 1 and one or two β-homoarginine (βR) residues at positions 3 and 9 (Figure 1B). Preliminary data showed that all three of these first-generation peptides—βEst-1, βEst-2, and βEst-3—catalyzed the hydrolysis of 1 mM substrate 1 at a catalyst loading of 10 mol% in a solution buffered at pH 6, increasing the background reaction rate by a factor of 20–30. Zwit-EYYK, as expected, was inactive, while free histidine at 10 mol% enhanced the reaction rate by <5-fold (Figure S1).

We next performed steady-state measurements to characterize the reaction kinetics in greater detail. Incubation of 25 μM β345, and 487 μM concentration in the absence or presence of the indicated 5.5.7e In a similar way, a designed 4-helix groups on the substrate. Relative to the backbone of guanidinium groups on the peptide and sulfonate which substrate binding is mediated by electrostatic interactions between the βM re Fl for substrate catalysis and catalytic activity. While βEst-2/βH was more structured than βEst-2 (almost 80% bundle at 25 μM), its esterase activity was compromised, with kcat/KM = 23 M⁻¹·min⁻¹ (Figure S3). In a similar way, βEst-2N, βEst-2C, and βEst-28 all exhibited higher degrees of association (>80% bundle at 25 μM) than βEst-2 (Figure S4). However, the catalytic activities of these peptides varied drastically: βEst-2C was more efficient than βEst-2, βEst-2N was virtually inactive, and βEst-28 displayed very rapid initial rates but did not obey Michaelis–Menten kinetics (Figure 2B). The dependence of catalytic activity on the relative positions of αH and βR residues implies that peptide–substrate interactions are highly specific. The nearly 2-fold increase in the catalytic efficiency of βEst-2C (kcat/KM = 98 M⁻¹·min⁻¹) over βEst-2 (kcat/KM = 54 M⁻¹·min⁻¹) is a result of its improved affinity for the substrate (Kd = 147 μM) and perhaps the enhanced helicity

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<th>Table 1. Kinetic Constants Characterizing the Hydrolysis of 1 by β1-Peptide Catalysts</th>
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Values of 0.013, 0.018, and 0.019 min⁻¹ and Kd values of 447, 345, and 487 μM for βEst-1, βEst-2, and βEst-3, respectively, βEst-2N, containing two βR residues, displayed a more favorable Kd and the highest specificity (kcat/KM = 54 M⁻¹·min⁻¹). Since Kd reflects binding affinity, this observation supports a model in which substrate binding is mediated by electrostatic interactions between guanidinium groups on the peptide and sulfonate groups on the substrate. Relative to the buffer reaction, βEst-1, βEst-2, and βEst-3 enhanced the rate of ester hydrolysis (kcat/Kuncat) by factors of 413, 588, and 612. These kinetic parameters are comparable to those of a similarly sized dendrit peptide, RM-G2, which catalyzes the hydrolysis of 1 with a rate enhancement of 340 and kcat/KM = 120 M⁻¹·min⁻¹ at pH 5.5. In a similar way, a designed 4-helix α-peptide bundle, MNKR, catalyzes p-nitrophenyl fumarate ester hydrolysis with kcat/KM = 10.2 M⁻¹·min⁻¹ at pH 5.5. The only other 14-helical β-peptide catalyst reported, whose structure is unknown, catalyzes the retroaldol cleavage of a β-hydroxyketone with kcat/KM = 26 M⁻¹·min⁻¹ despite a rate acceleration of kcat/Kuncat = 3000. The favorable kinetic constants notwithstanding, subsequent circular dichroism experiments revealed that while βEst-1, βEst-2, and βEst-3 assembled into bundles at high concentration, they were primarily monomeric at 25 μM, the concentration chosen for steady-state kinetics (Figure S2). Compared to Zwit-EYYK, which was >90% octameric at 25 μM, βEst-1, βEst-2, and βEst-3 were <30% assembled at this concentration. The Zwit-EYYK X-ray structure revealed that the βE at position 1 (X in Figure 1B) is involved in an interhelical salt-bridge interaction. This side chain is substituted by αH in βEst-1/βEst-3, suggesting that the observed destabilization could be due to loss of this acidic side chain or inclusion of an α-amino acid at this position, or a combination of these effects.

We pursued three strategies to recover bundle stability. To evaluate whether the decrease in stability resulted from the presence of an α-amino acid (αH) at position 1, we synthesized a variant of βEst-2 that contained βH at this position (βEst-2/βH in Figure 1B). To evaluate whether the decrease was due to loss of βE at position 1, we restored this residue and appended the αH to either the N- or C-terminus as a 13th residue (βEst-2N and βEst-2C in Figure 1C). Finally, to evaluate whether entropic effects could be harnessed to improve bundle stability, we synthesized a covalent dimer containing two βEst-2 monomers joined with a tetra-β-homoglycine (βG) linker, an analogue of the highly stable Z28 bundle reported previously (βEst-28 in Figure 1D).

The kinetic constants in Table 1 reveal dimerization as the most effective strategy to regain bundle structure and improve catalytic activity.
of βEst-2C at 25 μM. The nearly 100-fold increase in the catalytic efficiency of βEst-28 is also the result of a greatly improved substrate affinity ($K_M = 4 \mu M$).

We designed βEst-28 using a previously reported strategy that recapitulates the characteristic β-peptide octamer fold with 4 subunits instead of 8. Although the relative positions of the αH and the βR residues in βEst-28 mimic those in βEst-2, the kinetic profile of βEst-28 was entirely unanticipated. Instead of initial velocity ($V$) increasing as a function of substrate concentration, $V$ reached a maximum at $[\text{S}] = 200 \mu M$ and then steadily descended toward an asymptote. This behavior is diagnostic of substrate inhibition, a well-known phenomenon that occurs in ∼20% of natural enzymes, often to avoid excessive production or degradation of important metabolic intermediates. We used the Haldane equation—a modified version of the Michaelis–Menten equation that includes an additional equilibrium constant, $K_f$, to fit the hydrolysis kinetics observed in the presence of βEst-28. The Haldane model calculates $k_{cat}/K_m = 5102 \text{ M}^{-1} \text{ min}^{-1}$ for βEst-28, almost 2 orders of magnitude greater than that for βEst-2. βEst-28 is 85% bundle at 25 μM concentration, emphasizing the benefit of a catalyst possessing higher order structure and multiple potential catalytic sites.

To provide additional support for the substrate inhibition model, we conducted kinetic measurements under pre-steady-state conditions. These measurements were performed using excess βEst-28 (5- to 40-fold over substrate 1), which allowed us to monitor a single substrate turnover. Because the substrate was present in such small amounts relative to βEst-28, the possibility of substrate inhibition was effectively excluded. The observed rate constants, $k_{obs}$, were extracted from fits of the data at each catalyst concentration to single-exponential curves (Figure S5). A plot of $k_{obs}$ against catalyst concentration (Figure 2C) was then fit to a hyperbolic function to obtain the rate constant for the chemical step, $k_{chem}$ (the horizontal asymptote), and the apparent $K_{i}$ (the peptide concentration corresponding to half of the asymptote). The kinetic parameters obtained from pre-steady-state studies of βEst-28 ($k_{chem} = 0.083 \text{ min}^{-1}$; $k_{app} = 14 \mu M$) agreed well with those obtained from steady-state measurements ($k_{cat} = 0.020 \text{ min}^{-1}$; $K_M = 4 \mu M$), providing support for the substrate inhibition model. $k_{chem}$ is expected to be equal to or greater than $k_{cat}$ since the latter is reflective of the rate-limiting step of the reaction. On the other hand, $k_{app}$ should closely match $K_M$, since both reflect the affinity of the catalyst for the substrate.

One explanation for the substrate inhibition observed with βEst-28 is that, as a covalently linked dimer of βEst-2, it contains 4 βR and 2 αH residues, increasing the likelihood of alternative, nonproductive catalyst–substrate interactions. To investigate this possibility, we synthesized two βEst-28 variants, one containing a single αH per βEst-28 monomer and another containing a single pair of βR residues per βEst-28 monomer (βEst-28-2R and βEst-28-2R in Figure 1D, respectively). βEst-28-1H was 5-fold less active than βEst-28, with changes in both $k_{cat}$ and $K_M$. Surprisingly, βEst-28-2R is (slightly) more active than βEst-28. Substrate inhibition was observed in both cases (Figure S6), suggesting that further studies will be necessary to completely understand the origins of this effect. Pre-steady-state analyses reveal, nevertheless, that the additional αH and βR residues enhance catalyst efficiency in a single substrate turnover; as assessed by the metrics $k_{chem}$ and $k_{app}$, neither of the two 28-mer variants was as active as the parent βEst-28 (Figure 2C).

We next investigated the dependence of catalytic activity on β-peptide bundle stoichiometry. As previously reported, there exists a direct relationship between bundle stoichiometry and β-peptide sequence; specifically, β-peptides with βL residues at positions i, i+3, i+6, and i+9 assemble into octamers, those with βV or βI at these positions assemble into tetramers, and those with βA at these positions are constitutively monomeric. Based on this relationship, we synthesized two stoichiometric variants of βEst-2C, one containing an all-valine face and another containing an all-alanine face (βEst-2C-V and βEst-2C-A in Figure 1C, respectively). Steady-state kinetics measurements revealed that the monomeric βEst-2C was the least effective catalyst of this series (Figure 2D), while βEst-2C and βEst-2C-V (80% and 73% bundle at 25 μM) exhibited similar levels of activity. These results support the conclusions that bundle formation contributes to catalysis and that octamers and tetramers, but not monomers, can assemble a functional esterolytic active site.

To understand the differences in activity between bundle-forming and monomeric β-peptide catalysts, we obtained a high-resolution X-ray structure of βEst-2C. As predicted, βEst-2C self-assembles into a quaternary assembly whose backbone skeleton is virtually superimposable with that of the Zwit-EYYK octamer (RMSD = 0.171; SDM at 5.0 Å cutoff = 3.425; Q-score = 0.920) (Figure 3A). The refined model of the βEst-2C bundle at 1.81 Å resolution (R/$R_{free}$ = 21.1/24.6%) consists of eight 14-helices (B–D) Representative interhelical active sites at the (B) parallel, (C) antiparallel, and (D) tetramer–tetramer interfaces. Distances between histidine and arginine side chains are highlighted in red.
But how many potential active sites does the βEst-2C bundle contain? The dimensions of substrate 1 (7.4 Å long, 5.4 Å wide) suggest that an active site on the βEst-2C bundle would be characterized by one αH and two βR residues located within roughly 15–20 Å. Taken with the structure, this analysis suggests that the βEst-2C bundle contains three fundamentally different active sites. The first occurs at the parallel interhelical interface and consists of a βRαH13 side-chain pair from one helix and a βRβRαH13 side-chain from an adjacent helix (Figure 3B). The second potential active site, which occurs at an antiparallel interhelical interface, contains the same βRαH13 pair but includes βRβRαH13 from the neighboring helix (Figure 3C). The third potential active site is located at the tetramer–tetramer interface, consisting of αH13 from one helix and a βRαH13 pair from another (Figure 3D). Considering that each bundle comprises 4 parallel, 2 antiparallel, and 4 tetramer–tetramer helical contacts, and there are 2 active sites per interhelical interface, there are theoretically 20 intermolecular active sites per bundle. This analysis could explain the observation that bundle assembly enhances catalysis, even assuming low active-site occupancy.

Finally, we asked whether intrinsic chirality would endow β3-peptide bundles with the ability to effect enantioselective catalysis. Indeed, βEst-2C exhibited significant chiral discrimination between the enantiomers of the 2-phenylpropionate ester substrate (R)-3 and (S)-3, catalyzing the hydrolysis of (R)-3 4 times faster than that of (S)-3 at 10 mol% catalyst loading (Figure S7). Although the selectivity of βEst-2C is modest in comparison to that of natural enzymes, it compares favorably with other synthetic esterases demonstrating activity on similar substrates. One dendritic peptide, for example, favors the enantiomer (S)-3 with an enantiomeric ratio E = 2.8 76. This result, together with the kinetic parameters of the peptides we evaluated, suggests that β3-peptide bundles are no less desirable than α-peptides as scaffolds for catalyst development and may have unique advantages due to the combined attributes of structural predictability, stability, and metabolic orthogonality.

In summary, here we describe a structurally characterized β3-peptide bundle possessing measurable catalytic function. Unlike two previously reported β3-peptide catalysts, the molecules described here self-assemble into discrete, unique, thermostable quaternary structures and are capable of both substrate recognition and chemical catalysis. The dependence of catalytic activity on the geometric arrangement of histidine and arginine residues, as well as bundle assembly, points to the existence of substrate-specific active sites that could be optimized using structure-guided design.

■ ASSOCIATED CONTENT

Supporting Information
Detailed descriptions of peptide synthesis and characterization, kinetics, CD, and structure determination. Coordinates of the βEst-2C bundle have been deposited in the Cambridge Crystallographic Data Centre as entry CCDC 1000723. These data can be obtained free of charge at www.ccdc.cam.ac.uk/data_request/cif. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to the W. M. Keck Foundation for support of this work and to Professor Scott Miller, Professor Anna Marie Pyle, and Dr. Clarissa Melo Czekster for helpful discussions. We are especially thankful to Professor Richard Baxter for providing laboratory space for peptide crystallization.

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