Peptide Catalysts

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Allosterically Regulated Phosphatase Activity from Peptide–PNA Conjugates Folded Through Hybridization

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Abstract: *The importance of spatial organization in short peptide catalysts is well recognized. We synthesized and screened a library of peptides flanked by peptide nucleic acids (PNAs) such that the peptide would be constrained in a hairpin loop upon hybridization. A screen for phosphatase activity led to the discovery of a catalyst with >25-fold rate acceleration over the linear peptide. We demonstrated that the hybridization-enforced folding of the peptide is necessary for activity, and designed a catalyst that is allosterically controlled using a complementary PNA sequence.*

Catalysis in living systems is generally achieved by proteins that fold in unique three-dimensional structures. However, only a few residues generally participate in catalysis, as exemplified by the catalytic triad of proteases (Figure 1).[1] The relatively large size of enzymes (>20 kDa) is required to achieve a stable, folded conformation. The prospect of recapitulating a given catalytic reaction using minimal peptides or a rigid scaffold to position the required functionalities has attracted significant attention, and catalysis with oligopeptides has delivered ground-breaking results.[2,3] For catalysis with short peptides, select amino acids are frequently included to create a conformational bias (for instance, Pro-Aib- or Cα-tetrasubstituted amino acids have been used to induce a β-turn)[3a] and position the required side chains in close proximity. Oligonucleotides adopt stable tertiary structures with relatively short sequences. However, relatively few reactions are efficiently catalyzed by structured oligonucleotides, phosphodiester cleavage by the hammerhead ribozyme[4] being one example (Figure 1). Clearly, the larger pallet of functional groups present on amino acid side chains endows proteins with a broader scope of chemistries. We reasoned that short peptides conjugated to flanking oligonucleotides should allow for structuring through hybridization, resulting in a productive disposition of amino acid side chains for catalysis (Figure 1). For ease of conjugate synthesis, peptide nucleic acids (PNA)[5] were used because the oligomer could be synthesized by standard solid-phase peptide synthesis (SPPS). Hybridization of PNA–peptide conjugates has already been used to modulate the conformation of peptides involved in biologically relevant peptide-receptor interactions;[6] however, this approach has not been used in the context of catalysis.

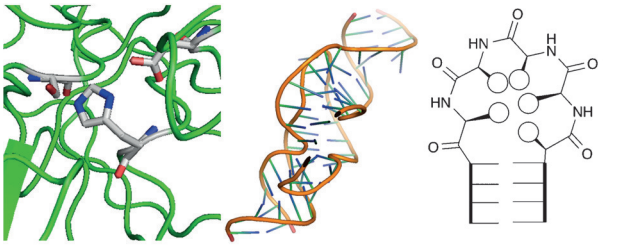

Figure 1. Representation of catalysts arising from controlled positioning of key functionalities in space: left, catalytic triad of trypsin (PDB ID:2PTC); middle, hammerhead ribozyme (PDB ID:3ZD5); right, a peptide–oligonucleotide structure folded through hybridization.

The library was designed to contain a bias for residues frequently found in hydrolytic enzymes (His, Ser, Asp, Lys) in a hairpin loop resulting from a constant CGGC sequence prior to the peptide and a complementary GCCG sequence at the end of the peptide. A preliminary experiment showed that a tetraglycine hairpin with this sequence had a T_m of 70 °C, ensuring a predictable folding. A library of 1000 PNA-peptide-PNA adducts was prepared by split-and-mix synthesis[7] using 3×10 sets of peptide fragments with variable length (0- to 4-mer; Figure 2). Aliquots of each synthetic pool were kept for eventual deconvolution and the pools from the third peptide fragment synthesis were kept as discrete entities for screening, thus affording the library as 10 pools of 100 putative catalysts with a hairpin composed of 4–10mer peptides. The synthesis made use of an orthogonal linker triggered by the reduction of the *para*-azidobenzyloxy moiety and 1,6-elimination upon azide reduction (Figure 2). We have previously shown that this type of elimination proceeds with a half-life of approximately 20 min ($k = 0.035\ \text{min}^{-1}$).[8] To prevent acid-catalyzed cleavage of the benzyloxy moiety during peptide deprotection, a carboxamide was introduced *ortho* to the benzyloxy group. Cleavage of the present linker with 2 mM PMe_3 in THF/H_2O had a comparable half-life of 30 min.

Several methods have been reported to screen libraries of catalysts on beads.[3b,d,9,10] We opted to screen for the hydrolysis of a phosphate ester based on the fact that the background hydrolysis is very slow and cleavage can be monitored using a fluorogenic precipitating dye (quinazolinone precipitating dye: QPD; Figure 3)[11] which is routinely used to image alkaline phosphatase activity.[12,13]

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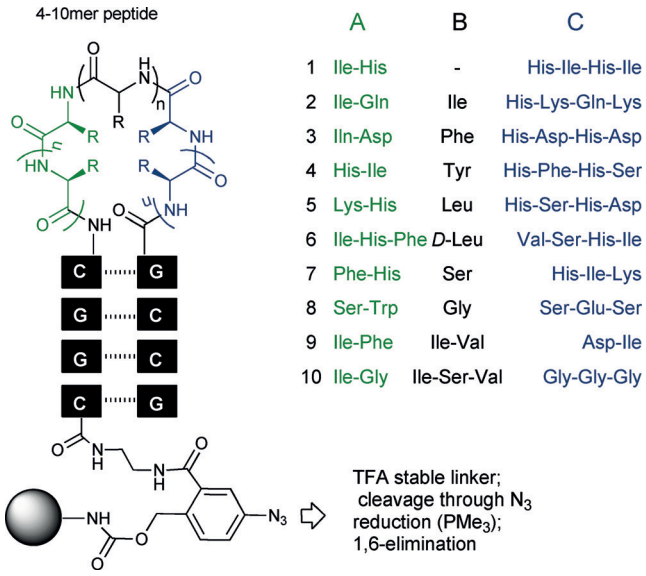

Figure 2. General structure of the library composed of 4- to 10-mer peptides constrained by a hairpin structure of self-complementary PNAs (left). The library was prepared by three rounds of split-and-mix combinatorial synthesis. The peptide fragments used in each consecutive round are shown in green, black, and blue, respectively. The sequence used for each pool is shown on the right (A_{1-10}: green; B_{1-10}: black; C_{1-10}: blue).

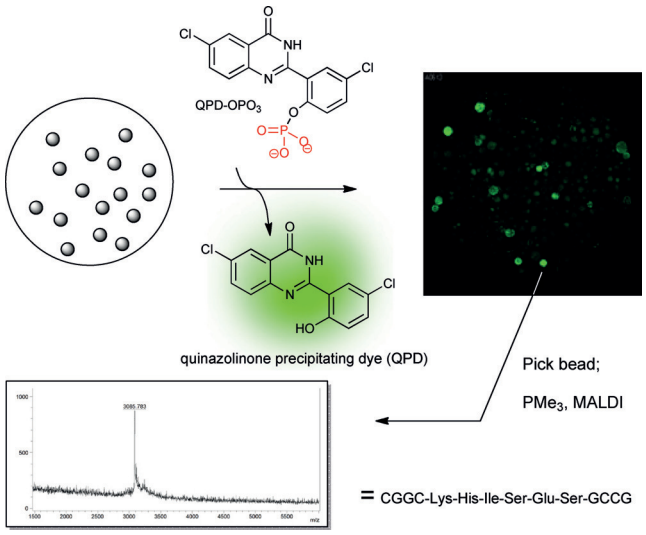

Figure 3. Screening of the library for phosphatase activity using a fluorogenic substrate (QPD-$OPO3^{-2}$) yielding a green quinazolinone-based precipitating fluorophore.

The library was screened by exposing 400–700 beads of each pool (i.e. 4–7 copies of each library member) to the fluorogenic precipitating dye substrate (2 mM) in PBS buffer (pH 8.0). The development of fluorescence was monitored as a function of time by fluorescence microscopy using a high-content screening microscope at 37 °C over a period of 5 h (Figure 3; see Figure S1 for images of all pools as a function of time and Figure S2 for quantitative analysis). A clear discrepancy in the number of highly fluorescent beads was discernable amongst the 10 pools, with the pool corresponding to the fragment C8 showing some of the brightest beads. The brightest beads were removed with a pipette tip, distributed on a MALDI plate and treated with 2 μL of a 2 mM PMe_3 solution followed by matrix for direct MALDI analysis. Three of the beads had the mass corresponding to the sequence Lys-His-Ile-Ser-Glu-Ser (A5-B2-C8), with a mass redundancy between pool B2, B5, and B6. Based on this redundancy, the three pools corresponding to redundant masses (A5-Bx-C8) were remade from the aliquot of resin and rescreened according to the same procedure (Figure S3). On the basis of this comparison, the sequence Lys-His-Ile-Ser-Glu-Ser was selected for further studies.

To validate this result, the sequence was resynthesized on a Rink amide resin to obtain the product in solution following TFA cleavage. To ascertain the contribution of PNA hybridization, control sequences lacking the PNA (**1**; Figure 4), with

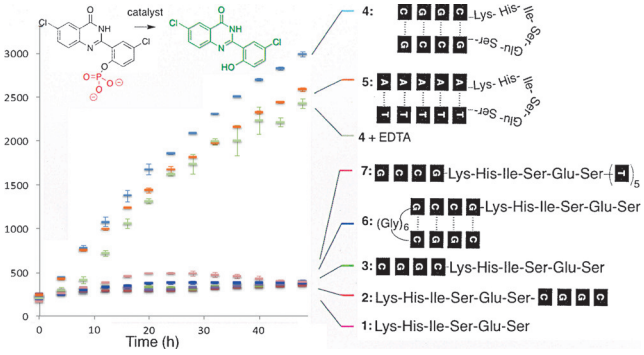

Figure 4. Catalytic activity of identified catalyst and control sequences measured in PBS (25 mM, pH 8.0) at 37 °C. The fluorescence was measured at $\lambda_{ex} = 345$ nm and $\lambda_{em} = 530$ nm (Y-axis shows arbitrary fluorescent units). Each data point is the average of 3 experiments.

only one side of the PNA (at the N- and C-terminus, **2** and **3** respectively; Figure 4) or a different hairpin forming sequence (CGGC-peptide-GCCG and AAAAA-peptide-TTTTT: **4** and **5**; Figure 4) were prepared. As a further controls, a sequence containing the linear peptide Lys-His-Ile-Ser-Glu-Ser emanating from a hexaglycine hairpin duplex (**6**) and a conjugate that cannot fold (**7**) were also prepared. Analysis of the phosphate hydrolysis using the fluorogenic substrate (100 μM catalyst, 2 mM substrate, 25 mM PBS buffer, pH 8) confirmed the activity of the selected peptide (**4**; Figure 4), and showed that the hybridization-enforced hairpin was necessary for catalysis. The peptide alone (**1**), the peptide with only one side of the PNA (**2**,**3**), the PNA-hairpin conjugate to the linear peptide (**6**), and unfolded PNA–peptide adduct (**7**) showed dramatically less activity (Figure 4). Furthermore, specific nucleobases are unlikely to be involved in the catalysis since comparable phosphate hydrolysis activity was obtained for the GC-based hairpin as with the AT-based hairpin (**4** vs. **5**). Comparing the slope of the reaction for compound **4** vs. **6** indicates a rate acceleration of > 25-fold upon hairpin formation. Performing the reaction with 2 mM EDTA did not alter the rate of reaction, suggesting metal chelation is not involved.

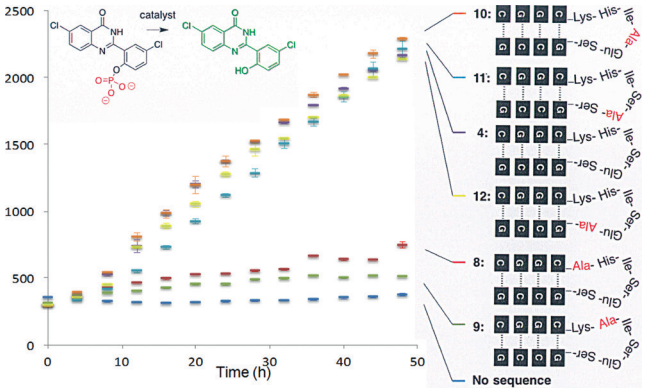

Figure 5. Alanine-scan of the catalyst measured in PBS (25 mM, pH 8.0) at 37 °C. The fluorescence was measured at $\lambda_{ex} = 345$ nm and $\lambda_{em} = 530$ nm (Y-axis shows arbitrary fluorescent units). Each data point is the average of 3 experiments.

We next investigated the relative contribution of each residue with a heteroatom using an Ala scan of the sequence. As shown in Figure 5, Lys or His substitution (compounds **8** and **9**) led to loss of activity, whereas the other positions (compounds **10**–**12**) yielded products with comparable activity. It should be noted that compound **4** failed to cleave other aryl phosphates (phosphorylated form of 4-nitrophenol, 2-nitrophenol, 7-hydroxycoumarin, and salicylamide), suggesting some substrate specificity (for prior example of substrates specificity with peptide catalysts[14]). Taken together, these results support the hypothesis that the observed activity results from catalysis mediated by a constrained conformation of the Lys-His dipeptide within a hairpin loop that provides a favorable interaction with the substrate.

Based on the impact of the hairpin on catalysis, we next asked whether allosteric control of activity could be engineered by toehold strand displacement switching the hairpin configuration to an open configuration.[15] To this end, compound **13** (Figure 6) with a hairpin made up of a 3-mer PNA with a 3-mer overhang was prepared. It is interesting to note that at 37 °C compound **13** had 50 % of the activity of compound **4**. However, at 25 °C, both compounds showed comparable activity. This is consistent with the measured T_m of the hairpin (75 °C for **4** and 45 °C for **13**; Figure S4); at 25 °C, both **4** and **13** are predominantly in the hairpin configuration, whereas at 37 °C a significantly larger portion of compound **13** is in the non-productive open form than compound **4**. As shown in Figure 6, the catalytic activity of compound **13** was indeed dramatically attenuated in the presence of the complementary PNA (ATAGCG) that opens the hairpin, demonstrating that a programmable allosteric control is possible. Concurringly, the activity of **13** could also be attenuated during the course of the reaction by the addition of the complementary PNA (ATAGCG).

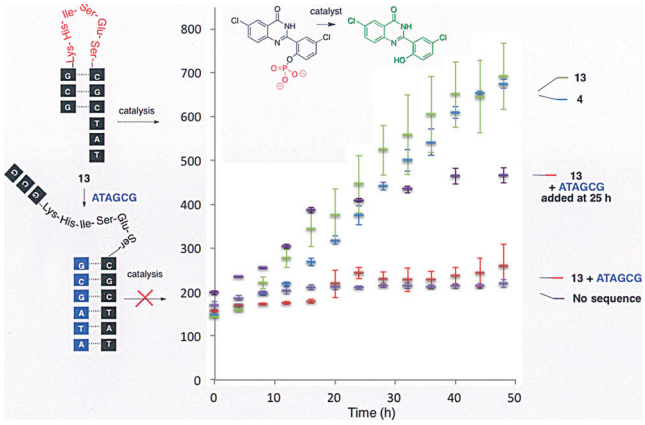

Figure 6. Allosteric switch of catalytic activity of 13 measured in PBS (25 mM, pH 8.0) at 25 °C. The fluorescence was measured at $\lambda_{ex} = 345$ nm and $\lambda_{em} = 530$ nm (Y-axis shows arbitrary fluorescent units). Each data point is the average of 3 experiments.

In conclusion, we have identified a peptide sequence that undergoes a dramatic gain of activity (>25-fold) for phosphate hydrolysis when folded into a hairpin loop through hybridization. While this peptide was identified from a relatively small library (10^3), its discovery supports the notion that hybridization can be used to induce and interrogate different conformations within a peptide library. The fact that the activity can be modulated by oligonucleotides that compete for hybridization should make this design applicable in responsive systems for nucleotide sensing.[16] The present demonstration that the hybridization of a peptide–PNA conjugate confers a protein-like function with allosteric regulation should also find application in the design of chemical networks that respond to environmental cues. Finally, the methodology presented herein complements alternative library design or screening methods based on phage display[17] and catalytic dendrimers.[18] The advent of DNA-templated synthesis[19] or the use of hybrid structures with aptamers would also potentially benefit from iterative cycles of selection/diversification.

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