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A pH-Modulated, Self-Replicating Peptide

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Replication of self is a fundamental aspect of life that scientists have only begun to emulate in the laboratory on a molecular level. Examples of self-replicating systems include nucleotide-based oligomers,[1−3] adenine−Kemp's triacid conjugates,[4] peptides,[5] and micelles.[6] The development of molecular systems that link self-replication and natural selection has been a more elusive target.[1d,2c] Recent work of Lee *et al.* demonstrated that peptides from the GCN4 leucine zipper domain self-replicate in an autocatalytic cycle.[5] We sought a peptidic self-replicating system that would be sensitive to environmental conditions and reproduce only under extreme conditions. We now disclose a peptide sequence capable of replication of self from two peptide fragments in an autocatalytic, pH-dependent manner.

We designed a peptide, E1E2 (Figure 1), based on the peptide of Zhou *et al.* (EE).[7] Like EE, E1E2 was designed to form a coiled-coil under acidic conditions due to protonation of Glu side chains at the **e** and **g** positions of the helical heptad repeats, which should relieve electrostatic repulsion in the coiled-coil. Under physiological conditions, however, the negatively-charged side chains of Glu should destabilize the coiled-coil, and E1E2 should adopt a random coil conformation. Two fragments of E1E2,[8] the electrophilic thioester-containing fragment E1 and the nucleophilic fragment E2 containing a free cysteine at its N-terminus, were designed to produce E1E2 on the basis of the thioester-promoted, peptide bond formation strategy developed by Kent.[9] We predicted that under acidic conditions the coupling between E1 and E2 to form E1E2 should proceed via an autocatalytic pathway, whereby the coupled product, E1E2, would act as a template to organize the two subunits and accelerate their condensation. At neutral pH, however, E1E2 formation should proceed by a noncatalytic pathway due to the lack of structure in E1E2, thereby removing its templating abilities.

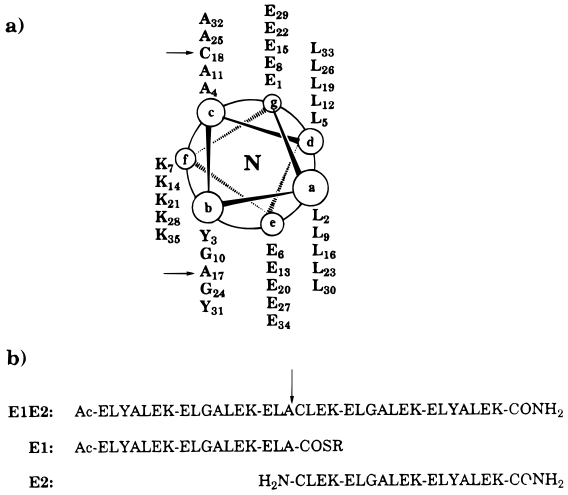

Figure 1. (a) Helical wheel diagram of E1E2 showing the positions of the coiled-coil, heptad repeat (**a**−**g**). (b) Peptide sequences employed in the study [R = $(CH_2)_2CONH_2$]. Ligation residues Ala and Cys are located at the solvent exposed **b** and **c** positions.

Circular dichroism (CD) spectroscopy was used to assess the helical content of the designed peptides.[10] It was found that the peptides adopted a helical conformation in a pH-dependent manner.[11] The helical content of E1E2 reached a maximum of 86% at pH 4.0, presumably due to formation of a coiled-coil upon protonation of the Glu residues as was observed with EE.[7] The ratio of $\theta_{220nm}/\theta_{207nm}$ has been shown to correlate with the presence of a coiled-coil structure, and a value of 1.07 was obtained for E1E2 at pH 4.0 which is in good agreement with those of other coiled-coil peptides.[12] Addition of 50% trifluoroethanol (TFE), a solvent reported to disrupt interhelical interactions, to E1E2 at pH 4.0 lowered the $\theta_{220nm}/\theta_{207nm}$ ratio to 0.9, a value indicative of a single-stranded α-helix.[12] Size exclusion chromatography also confirmed that E1E2 is in an aggregated state at pH 4.0; an apparent molecular weight of approximately 14 000 was obtained which corresponds to a trimer at E1E2 concentrations as low as 50 μM.

The secondary structure of the shorter peptides, E1 and E2, was also pH dependent with maximum helical contents of 18 and 22%, respectively, at pH 4.0. The CD spectra of E1 and E2 in the presence of E1E2 at pH 4.0 showed an increase in the helical content of E1 and E2 to 31 and 44%, respectively, confirming the role of E1E2 as a template for the smaller peptides. To ascertain whether E1 and E2 would associate at pH 4.0, CD spectra of equimolar concentrations of a mixture of E1 and E2 were compared to the addition spectra of E1 and E2. No increase in helical content was observed with the mixture of peptides over that obtained with the individual peptides alone, suggesting a lack of association between E1 and E2.

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(8) Peptides E1E2 and E2 were synthesized by solid phase peptide synthesis methodology on the Rink resin[17] using Fmoc-based chemistry.[18] Peptide E1 was synthesized with a MBHA resin functionalized with Boc-Ala-S$(CH_2)_2CO_2H$[14,19] using Boc-based chemistry with the Kent *in situ* neutralization method.[9c] Cleavage of the peptide from the solid support with HF/anisole provided the thioester-containing peptide E1 as shown in Figure 1b. All peptides were purified to homogeneity by reverse phase HPLC and characterized by mass spectrometry (plasma desorption) and amino acid analysis.

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(11) Conditions for CD: 100 μM solutions of each peptide in 50 mM phosphate, 100 mM NaCl at pH 4.0 and 26 °C. To ensure that no reaction took place during the course of the studies, a variant of the E2 peptide was used in all CD studies which contained an Ala residue in place of the Cys residue.

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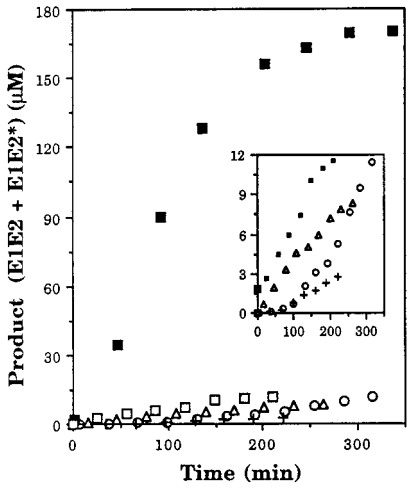

Figure 2. Product formation as a function of time at different pH: (○) 3.0, (■) 4.0, (+) 5.3, (△) 6.6, (□) 7.5 (E1E2 and E1E2* were both considered product in this study for a more accurate comparison). (inset) Expanded region emphasizing the 0−12 μM portion of the graph.

Since the conformation of E1E2 could be controlled by pH, the production of E1E2 from E1 and E2 was next investigated over a pH range of 3.0−7.5. As the pH of the reaction was decreased from 7.5, there was a concomitant decrease in the formation of E1E2 with the exception of pH values of 4.0 and 3.0 (Figure 2 and inset). The significantly higher rate of E1E2 formation at pH 4.0 as compared to all other pH values is presumably due to the coiled-coil and templating ability of the product E1E2 under these conditions and a reasonably high rate of reaction between the coupling partners. As the pH of the reaction was raised the deprotonation of the Glu residues would result in uncoiling of E1E2 and lead to a diminished templating of E1 and E2 by E1E2. At a pH of 3.0, however, the rate of E1E2 formation was significantly lower than at 4.0 presumably due to the lower reactivity of the condensation reagents, although the distinct sigmoidal growth in E1E2 product formation confirms the autocatalytic nature of the reaction under these conditions as well (Figure 2, inset).[1,2,4,5]

Autocatalysis in the formation of E1E2 from E1 and E2 at a pH of 4.0 was unambiguously established when the reaction was performed in the presence of differing amounts of E1E2 (Figure 3a).[13] The reaction was accelerated by the presence of template: increasing the amount of E1E2 in the reaction mixture increased the initial rate of E1E2 formation. The initial rate of product formation was found to be linearly proportional to the square root of the template concentration (Figure 3b), a phenomenon that is commonly observed in autocatalytic reactions.[1f,2,4,5] Furthermore, addition of E1E2 to the pH 7.5 reaction mixture led to no increase in product formation, confirming the lack of autocatalysis under these conditions. Interestingly, we were able to observe the reaction intermediate, the thioester ligation product (E1E2*), during the course of the formation of E1E2. The thiolactone intermediate has been observed previously,[9d,14] and under our reaction conditions (pH = 4), the free amino terminus of E2 would be in the protonated form and less likely to undergo facile reaction with the thioester.

The experimental data were analyzed using the program SimFit[1d,e] based on the empirical equations for autocatalysis

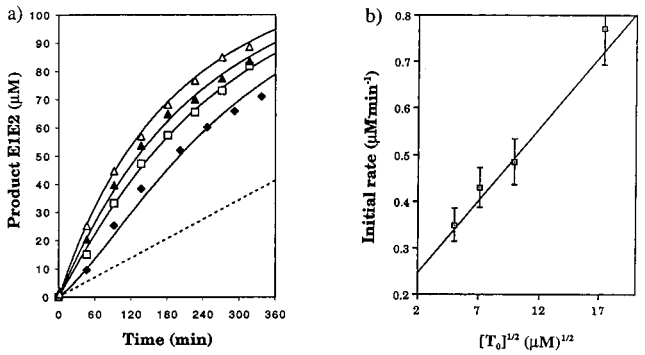

Figure 3. (a) E1E2 production as a function of time for reaction mixtures of E1 and E2 containing different initial concentrations of E1E2 as the template at pH 4.0: (■) no E1E2, (□) 25 μM E1E2, (▲) 50 μM E1E2, (△) 100 μM E1E2. Curves were generated from the program SimFit.[1d,e] The dashed line represents the calculated production of E1E2 in the absence of autocatalysis. (b) Initial rate of E1E2 formation as a function of the square root of the initial template concentration (T_0).

derived by von Kiedrowski.[15] This analysis provided an apparent autocatalytic rate constant of $k_a = 24.4\ M^{-3/2}\ s^{-1}$ and a noncatalytic rate constant of $k_b = 0.0265\ M^{-1}\ s^{-1}$ with an autocatalytic efficiency ($\epsilon = k_a/k_b$) of approximately 900 ($M^{-1/2}$). There was also a noticeable sigmoidal growth in E1E2 production when no template was added, providing further evidence for autocatalysis under these conditions, and significant inhibition of the reaction upon addition of 50% trifluoroethanol, an agent which is known to inhibit coiled-coil formation,[12,16] providing evidence for the role of E1E2 as a template for the reaction of E1 with E2. Since E1E2 exists as a trimer at concentrations greater than 50 μM, it is possible that the templating species is a dimeric or trimeric coiled-coil, although we cannot rule out the potential for a monomeric template at the low, initial concentrations of E1E2 during the early stages of the reaction.

In conclusion, we have successfully designed a pH-modulated, self-replicating peptide which promotes its own production under acidic conditions. At neutral pH, however, autocatalysis is suppressed and the reaction with added template is indistinguishable from the background reaction. This self-replication demonstrates the first application of environmental control within the peptide autocatalysis regime. We are applying this strategy to design self-replicating systems where cross-catalysis is possible under controlled conditions.

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Supporting Information Available: Characterization, CD spectra and helical contents as a function of pH for peptides, data for E1E2 production as a function of time and pH, HPLC profile for the production of E1E2 at pH 4.0, and SimFit files (14 pages). See any current masthead page for ordering and Internet access instructions.

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(13) Reaction conditions: mixtures of E1 (240 μM) and E2 (300 μM) were incubated at 23 °C in 250 mM MOPS (1% (v/v) 3-mercaptopropionic acid) adjusted to the desired pH with NaOH. At the indicated time interval, 50 μL of the reaction mixture was evaluated by reverse phase HPLC (for conditions, see the Supporting Information). The reaction products were identified by direct isolation followed by characterization by mass spectrometry or by HPLC coinjection with authentic samples. Concentrations of product were determined by interpolation of peak area from a standard curve. In reactions with template E1E2, E2 and E1E2 were premixed in the desired buffer before E1 was added.

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(15) We assumed the following reaction equations for data analysis with SimFit: (a) E1 + E2 → E1E2 with a rate constant of k_b; (b) E1 + E2 + 0.5 E1E2 → 1.5 E1E2 with a rate constant of k_a. Formation of thiolactone intermediate E1E2* was regarded as a reversible process,[9d,14] and the subsequent amide bond-forming reaction was considered to be the irreversible, rate-determining step under our reaction conditions. Therefore, the whole ligation process could be regarded as a one-step, rate-determining reaction without taking E1E2* into account.[1c,f]

(16) Less than 1% of the possible production of E1E2 was observed after 5 h with the addition of 50% TFE to the reaction mixture.

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