PERSPECTIVE

Challenges in the design of self replicating peptides

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Introduction

The design of self replicating molecules had its genesis in the ground breaking work of von Kiedrowski[1] and Orgel and Zielinski.[2] Using palendromic oligonucleotides as a starting point, these researchers designed a means by which the product of a reaction between two smaller oligonucleotides could act as the template to promote this reaction. Hence the product could be formed in an autocatalytic or self replicating fashion. These initial experiments have spurred interest in a wide range of self replicating molecules including other oligonucleotides,[3] nucleotide–imide conjugates,[4] micelles,[5] Troger's base[6] and peptides.[7] Recent design aspects of self replicating peptides will be reviewed here.

The self replicating peptides designed to date have employed a common structural motif: the coiled coil.[8] Coiled coils are composed of helical peptides that may associate to form a wide range of oligomeric assemblies.[8] The common feature of the coiled coil is the presence of a hydrophobic interface that is organized along a heptad repeat. The initial self replicating peptide was based on the coiled coil of the GCN4 transcription factor (Fig. 1).[9] Whereas the oligonucleotide self replicating systems relied on hydrogen bonding for precise templating, the recognition surface for template binding in self replicating coiled coil peptides occurs within the hydrophobic heptad repeat, along with electrostatic contributions from residues at the **e** and **g** positions of the heptad (Fig. 1).

Experiment emulating prebiotic conditions have often been found to yield short peptides as products. The discovery that a peptide-based biopolymer could promote its own synthesis led to very interesting speculations on the molecular origin of life. With this in mind self replicating peptides have been designed with many of the fundamental properties of living systems, including dynamic error correction,[10] chiroselectivity[11] and hypercycle catalytic networks.[12] Although great progress has been made, intrinsic problems with self replicating peptides, and other self replicating molecules, still need to be addressed. A major challenge to be confronted is that of poor catalytic efficiencies due to product inhibition. An essential feature of efficient self replication is the adequate dissociation of the product–template complex (B, Fig. 1). Another challenge is the design of a mechanism to control the replication process. This control feature will be essential if self replicating peptides are to be used for novel applications. Herein we review state of the art research in progress which addresses these challenges.

Challenge I: efficiency of self replication

Using the minimal replicator theory of von Kiedrowski, there are two means by which the success of a self replicating molecule may be measured: catalytic efficiency and reaction order.[13] Catalytic efficiency is defined as the rate of the autocatalytic reaction of the termolecular complex T, over the rate of the background reaction. The background reaction results from the reaction of non-templated peptide fragments; an increase in the background rate results in a decrease in catalytic efficiency. The initial GCN4 self replicating peptide, for instance, had a catalytic efficiency of 500.[9] Improvements in catalytic efficiency may be made if the template is more available to accelerate the reaction. A decrease in product inhibition would, therefore, result in a more efficient self replicating peptide. Catalytic efficiency may also be improved by a decrease in the background reaction, a process that is often controlled by interactions between the peptide fragments.

The reaction order for a self replicating process is a value that indicates how close the reaction is to exponential growth and the extent of product inhibition.[13] For a dimeric self replicating peptide with no product inhibition and unlimited starting materials, for instance, exponential growth would be observed, and a reaction order of 1.0 would be obtained.[13] When product inhibition is a significant problem, however, parabolic growth and a reaction order of 0.5 would be observed (Fig. 2). The trimeric GCN4 self replicating reaction, for example, was found to have a reaction order of 0.63, indicating parabolic growth; a minimum reaction order of 0.67 would occur for product inhibition with a trimeric system.[9] The difference between exponential and parabolic growth has a profound consequence on product selection profiles – selection of the most efficient replicators necessitates exponential growth. Hence the desire to obtain exponential, highly efficient self replicating systems.

There is, however, an intrinsic difficulty in overcoming product inhibition: efforts to weaken the bimolecular complex B (Fig. 1) will also most likely weaken the termolecular complex T, leading to less effective templation. Therefore, a delicate

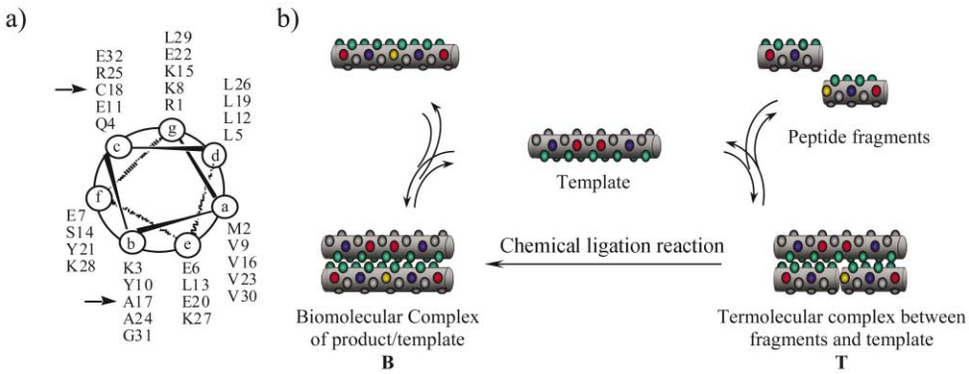

Fig. 1 (a) A helical wheel diagram depicting the sequence of the self replicating GCN4 peptide. The arrows designate the ligation positions. (b) An overall schematic for a self replicating process.

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balance that must be achieved between the dissociation constants for B and T and the catalytic rate constant. Kiedrowski and coworkers devised an innovative, solid-supported system for self replicating oligonucleotides which alleviates the problem of product inhibition.[14] More recently, modifications have been introduced into self replicating peptides to improve catalytic efficiency and move closer towards exponential amplification.

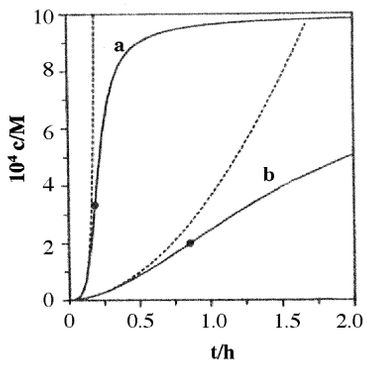
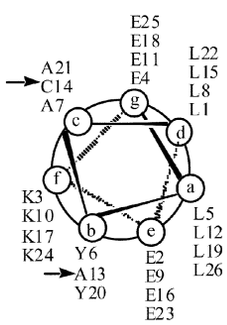


Fig. 2 A self replicating reaction based on a dimeric template displaying (a) exponential (p = 1) or (b) parabolic (p = 0.5) growth curves. The dashed curves represent the case of unlimited growth. Figure derived from von Kiedrowski.[13]

In an effort to improve the catalytic efficiency of the self replicating peptide E1E2,[15] for instance, we sought to destabilize its coiled coil structure. Fairman and coworkers achieved dramatic decreases in stability for tetrameric coiled coil peptides by shortening the chain lengths,[16] and Hodges and coworkers found similar effects with dimeric coiled coil peptides.[17] With these precedents as a basis, a peptide was designed for self replication, RI-26.[18] This peptide contains 3 full heptad repeats within the coiled coil, one shorter than the original E1E2 sequence. RI-26a and RI-26b, therefore, correspond to the two fragments of RI-26 that would undergo thioester mediated chemical ligation to produce RI-26 (Fig. 3). The full length



RI-26: Ac-LEKELYALEKELACLEKELYALEKEL-CONH$_2$
RI-26a: Ac-LEKELYALEKELA-COSR
RI-26b: CLEKELYALEKEL-CONH$_2$

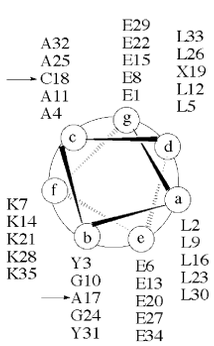
Fig. 3 Helical wheel diagram and sequence of the tetrameric self replicating peptide RI-26 and its fragments.

template, RI-26, was found to adopt a helical conformation and to exist as a tetramer. E1E2 by contrast exists as a dimer under similar conditions.[18] As is indicative of an autocatalytic system, adding increasing amounts of the template RI-26 led to a dramatic acceleration in product formation with an apparent catalytic rate constant, k_a, of 50.6 $M^{-1.91}s^{-1}$ and non-catalytic rate constant, k_b, of 5.04 × 10^{-4} $M^{-1}s^{-1}$ with a catalytic efficiency ($\varepsilon = k_a/k_b$) of 1.0 × 10^5.[15] This is a remarkably efficient system when compared to other self replicating molecules; self replicating peptides and oligonucleotides have displayed catalytic efficiencies in the range of 24 to 3700.[9,15,19]

The efficiency observed with RI-26 is comparable to that observed for some enzymatic systems, such as glutathione transferases.[20] The uninstructed non-catalytic or background reaction, presumably a result of the association between the two fragments, is also much slower in this peptide system than any of the other reported peptide self replication systems (90-fold slower than with the starting E1E2). This is most likely due to the presence of fewer leucine residues in the shorter fragments, thereby reducing the hydrophobic interactions between them.

The order of the self replicating reaction with RI-26 was found to be 0.91.[18] For a self replicating tetramer such as RI-26 the reaction order (p) would be expected to be 0.75 if the system exhibited product inhibition.[13] For RI-26 a significantly higher reaction order was observed, thereby classifying this replicating system as weakly exponential ($0.75 < p < 1$).[13] This was the first self replicating system that attained a reaction order higher than the product inhibited order. One potential cause of the reduction in product inhibition, was the observed reduction in the stability of the tetrameric product of RI-26 as compared to the dimeric product of E1E2 self replication.

An alternative pathway to reduce the stability of coiled coils is to modify the residues present at the hydrophobic interface. With this in mind, we have recently studied E1E2 peptides with Pro, Ala and Gly replacements for Leu19 (Fig. 4).[21] Because of their small side chain, locating Ala or Gly at the hydrophobic core would reduce the stability of the coiled coil peptide. For instance, Tanaka and coworkers destabilized a triple-stranded coiled coil peptide, IZ, to an almost random coil structure by replacing a Leu at the hydrophobic core with an Ala.[22] We employed this replacement strategy to decrease the stability of the self replicating peptide E1E2 as the change was in the center of the template. In the overall design, it was envisioned that this modification would have less effect on the stability of the ternary complex of template and peptide fragments because the mutation position was near the N-terminus of E2. The Pro modification located in the hydrophobic core would not only change the hydrophobicity in this position, but it should also destabilize the coiled coil through the incorporation of a bend in the helix.



E1E2-X: Ac-ELYALEKELGALEKELACLXKELGALEKELYALEK-CONH$_2$
E1: Ac-ELYALEKELGALEKELA-COSR
E2-X: CXEKELGALEKELYALEK-CONH$_2$
X = L (E1E2), P (E1E2-P), G (E1E2-G), A (E1E2-A)

Fig. 4 Helical wheel diagram and sequence of peptides E1E2(X), where X = L, P, G, A. Peptide fragments used in the replication reactions are shown.

With E1E2(P), no obvious acceleration in product formation was observed after adding increasing amounts of template.[21] This may be caused by a modification in the structure next to the cysteine residue in the fragment E2(P) that changed the proximity between the side chain of Cys and the thioester in fragment E1. Both E1E2(A) and E1E2(G), however, showed a significantly higher catalytic efficiency (2400 and 2800, respectively) as compared to E1E2 (120).[21] Comparing the data obtained for E1E2, this improved catalytic efficiency was mainly achieved by a decrease in the background reaction rate.

The non-catalytic rate constants for E1E2(A), E1E2(G) were 50- to 75-fold slower than that for E1E2, with rates of 8.9×10^{-4} M^{-1} s^{-1}, 6.0×10^{-4} M^{-1} s^{-1}, respectively. Similarly to RI-26, this difference is also most likely due to the presence of fewer hydrophobic interactions between the two fragments that resulted in reduced binding between the two fragments.

Challenge II: controlling self replication

There have been many peptides that have been designed for novel biomaterial applications, such as hydrogels,[23] tapes,[24] and self healing materials.[23b,25] Self replication has great potential to make unique contributions to these areas. However, there is an underlying need to control the replication process, such that self replication may be achieved on demand for technological applications. With this in mind a range of environmental conditions have been exploited for control of peptide self replication, including pH and salt changes.

For instance, a peptide sequence, E1E2, was designed that was capable of self-replication from two peptide fragments (E1 and E2) in a pH-dependent manner (Fig. 5).[15] E1E2 was based on the EE peptide of Zhou *et al.*[26] Like EE, E1E2 formed a dimeric coiled coil under acidic conditions due to protonation of Glu side-chains at the **e** and **g** positions of the helical heptad repeats. Under neutral conditions, however, the negatively-charged side-chains of Glu were found to destabilize the coiled coil, and E1E2 adopted a random coil conformation. As predicted, under acidic conditions the coupling between E1 and E2 to form E1E2 proceeded *via* an autocatalytic pathway, whereby the coupled product, E1E2, acted as a template to organize the two subunits and accelerate their condensation (Fig. 5). At neutral pH, however, E1E2 formation proceeded mainly by a noncatalytic pathway due to the lack of structure in the E1E2 template. 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 resulted in uncoiling of E1E2 and led to a diminished templating of E1 and E2 by E1E2. The addition of denaturants and trifluorethanol was found to significantly inhibit the self replicating reaction as well, even at low pH.

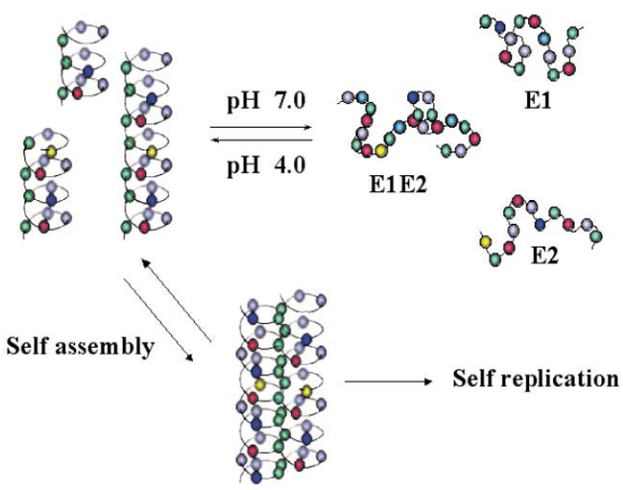


Fig. 5 The effect of pH on the self replicating reaction involving E1E2.

Autocatalysis in the formation of E1E2 from E1 and E2 at a pH of 4.0 was unambiguously established by performing the reaction in the presence of differing amounts of E1E2.[15] The experimental data were analyzed to provide a catalytic rate constant of 5.48 $M^{-2/3}$ s^{-1} and a background rate of 0.045 M^{-1} s^{-1} with an autocatalytic efficiency of 120. This study led to the successful design of a pH-modulated, self-replicating peptide which promotes its own production under acidic conditions. At neutral pH, however, self replication was suppressed and the reaction with added template was indistinguishable from the background reaction. This was the first demonstration of self-replication combined with environmental control within the peptide autocatalysis regime. Although the E1E2 system had a low catalytic efficiency, modifications to its overall sequence, as with RI-26 and E1E2(G), led to great increases in catalytic efficiency while maintaining the pH switch that was the original hallmark of the E1E2 design.

A modification to this overall strategy was investigated to identify other self-replicating peptides that might respond to environmental conditions. To this end the peptide K1K2 was designed for self-replication from two peptide fragments (K1 and K2) in a salt-dependent manner (Fig. 6).[19e] K1K2 was designed based on the sequence of the KK peptide of Zhou *et al.*[26] The K1K2 peptide contains Lys residues at the **e** and **g** positions of the leucine repeat, thus preventing stable coiled coil formation at acidic and neutral pH due to electrostatic repulsion. Under highly basic conditions or neutral conditions with addition of high concentrations of shielding counterions, the repulsive forces would be minimized, thus allowing appropriate dimerization of the coiled coil peptide.

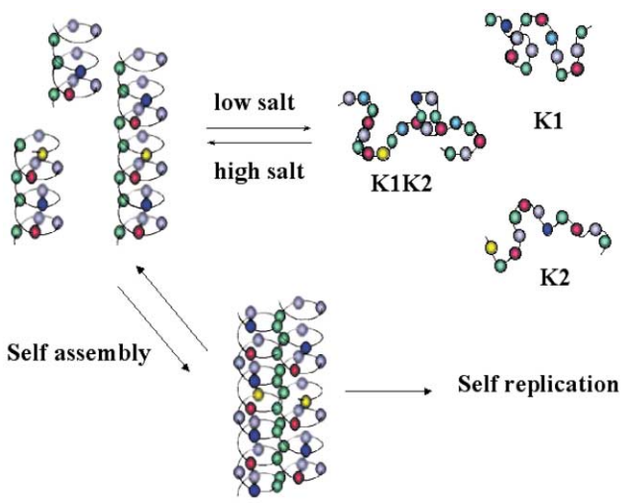


Fig. 6 High salt concentration is essential for self replication with the highly cationic peptide K1K2.

As anticipated, the coupling between K1 and K2 to form K1K2 under acidic or neutral conditions proceeded *via* a non-autocatalytic pathway, as K1K2 existed mostly in a random coil, non-templating conformation.[19e] The addition of certain salts, such as $NaClO_4$ has been shown to enhance coiled coil formation with peptides containing Lys residues in the **e** and **g** positions due to reduced electrostatic repulsion.[26] These conditions were found to promote the templating ability of K1K2 for K1 and K2, leading to autocatalytic formation of K1K2 at neutral pH. As the concentration of $NaClO_4$ in the reaction mixture was decreased from 2.0 M there was a concomitant decrease in the formation of K1K2. The significantly higher rate of K1K2 formation at 1.0 and 2.0 M $NaClO_4$ as compared to other salt concentrations is presumably due to the coiled coil and templating ability of the product K1K2 under these conditions. The autocatalytic formation of K1K2 at pH 7.5 in the presence of $NaClO_4$ was unambiguously established by performing the coupling reaction between K1 and K2 at two different concentrations of $NaClO_4$ and in the presence of differing amounts of K1K2 as a template. The experimental results were analyzed and provided a catalytic rate of 24.6 $M^{-3/2}$ s^{-1} and a background rate of 0.087 M^{-1} s^{-1} with a catalytic efficiency of approximately 280. Presumably the efficiency of

this reaction could also be improved using similar strategies as employed with E1E2.

Depending on the application, it is easy to envision numerous other switches that could be used to promote self replication. This approach would be an exciting addition to the design of novel biomaterials that respond and replicate as a function of the environmental conditions. Conditional selection could also be used to turn on one self replicating process at a time as environmental conditions change.[12b]

Conclusions

Two essential challenges that face self replicating molecules have been described in the context of peptide replicators: how to avoid product inhibition thereby promoting catalytic efficiency, and how to obtain self replication on demand through environmental control. By fine tuning the properties of the coiled coil motif, namely aggregation states, dissociation constants, hydrophobic interfaces, and electrostatic repulsion at the hydrophilic interface, a deeper understanding has been achieved of the factors that influence self replication. Ideas have also been presented to promote the use of self replicating peptides in novel biomaterial and biomedical applications. At both a fundamental and applied level there will be many new and exciting challenges that face the future of peptides with self replicating properties.

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