Can ligating homochiral polypeptides explain the origin of homochiral biomolecules?

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In a series of papers, the α-helical coiled coil protein folding motif was generated by ligating 15- and 17-residue peptide fragments. This formed a 32-residue template able to mould additional fragments of the same chirality autocatalytically. Autocatalysis in a racemic mixture of fragments generated almost only all D or all L 32-residue peptides. It has been claimed that this may explain the origin of homochiral biomolecules. However, providing a steady supply of large already homochiral fragments having the necessary primary sequences to the carefully designed system will not occur naturally. Furthermore, the system only works because the end of one of the two fragments was first chemically activated to a thiobenzyl ester.

A distinctive feature of biology is the autonomous synthesis of multiple copies of enantiomerically pure biomolecules. For over a century immense effort has been devoted world-wide to finding naturalistic mechanisms able to generate polypeptides consisting of only L-amino acids and nucleotides containing only D-sugars.

A few examples of self-replicating molecule schemes have been devised.[1–3] Most rely on hydrogen-bond donor and acceptor interactions of nucleic acid base-pairing. Since RNA is a complicated molecule, many organic chemists do not believe the enantiopure nucleotides could have been made reliably and in the huge amounts necessary in a plausible prebiotic context.[4–6] But one encounters claims in the literature that short D- or L-peptide chains favour further chain extensions leading to enantiomerically pure polymers. Professor Ghadiri at the Scripps Research Institute is the key figure associated with these experiments. He has designed a peptide-based auto-catalytic replicator based on residues of the same chirality.[4,7]

Siegel advertises a key publication by Ghadiri in *Nature* rather dramatically claiming that "Ghadiri *et al.* use a peptide system to demonstrate how 'homochirality', or single-handedness, may have evolved in biological molecules."[8] He did not write that this research provided some insights or possible analogies but that this may indeed be how homochirality arose naturally.

Many others have also referred to Ghadiri's work as significant in explaining the origin of homochiral biomolecules. Cintas wrote that "In a recent study, Ghadiri *et al.* suggest that peptides consisting exclusively of D or L amino acids will only replicate on templates of the same handedness."[4] Statements like these lead to the impression that individual D-enantiomer amino acids or small peptides will be selectively added to peptide chains once a small homochiral template chain is available.

Ghadiri and his colleagues have candidly admitted:

> "The origin of homochirality in living systems is often attributed to the generation of enantiomeric differences in a pool of chiral prebiotic molecules, but none of the possible physiochemical processes considered can produce the significant imbalance required if homochiral biopolymers are to result from simple coupling of suitable precursor molecules."[7]

I thoroughly agreed with this assessment. But has a breakthrough now been discovered by them after over a century of fruitless attempts?

α-helical coiled coil peptides

The experiment design of Dr Ghadiri's work was based on a well-known protein folding motif, the α-helical coiled coil (figure 1).[9–12] In this structure two or more α-helices intertwine using non-covalent interactions.

About 10% of all biological proteins display this motif.[13] Coiled coils can have parallel or antiparallel arrangement, and they can be formed by intrachain interaction of the same subunit, or by interchain bonds between distinct polypeptide chains.[14] α-helical coiled coils range in length over two orders of magnitude. They are known to form rods, segmented ropes, barrels, funnels, sheets, spirals, and rings, using anywhere from two to more than 20 helices in parallel or antiparallel orientation.[15] The simplest version consists of an identical pair of parallel α-helical peptides that wrap around each other with a slightly left-handed superhelical twist.

A distinguishing feature of α-coiled coil peptides is the heptad repeat motif $(abcdefg)_n$. Looking down the α-coil structure reveals a series of vertically aligned amino acid residues, figure 2.

The *a* and *d* residues comprise a hydrophobic core surface between the complementary α-coils, forming a knobs-into-holes type packing.

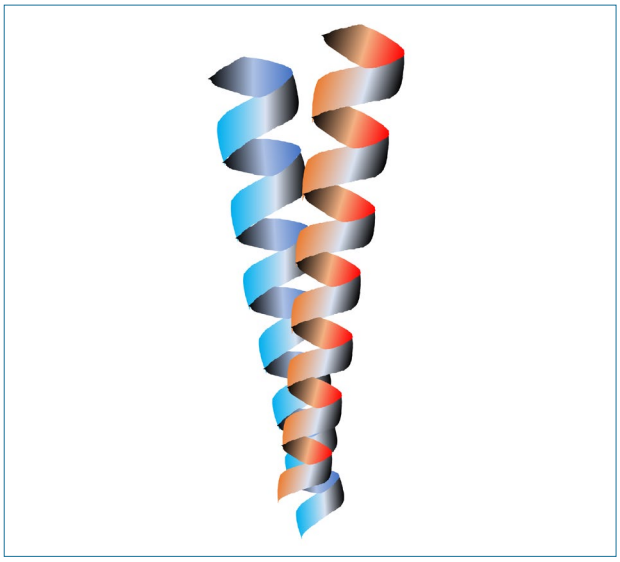

Figure 1. The α-helical coiled coil motif consists of two or more intertwined α-helices.

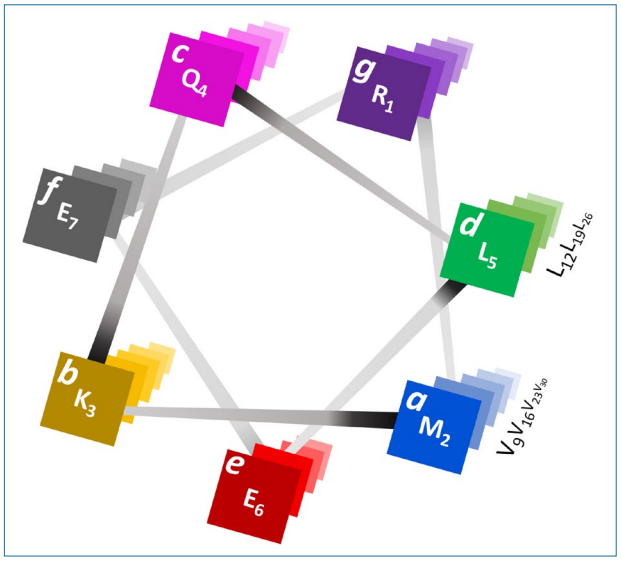

Figure 2. One member of an α-helical coiled coil dimer, which join at the *a* and *d* positions. Viewed from above, with three to four residues aligned beneath each heptamer position. Capitalized are standard 1-letter amino acid abbreviations; subscripts are their position in the primary sequence of the peptide. Figure based on work reported in reference 16.

Design of the autocatalytic peptide system

In these studies, the 32-residue templating peptide was based on the leucine-zipper domain of the yeast transcription factor GCN4.[17] The individual monomeric peptides are typically random coils in water, but form α-helices when aggregated. The researchers reasoned that if a 32-residue peptide were split into two shorter fragments these could be chemically ligated into a full-length intact peptide which could act as an organizing template for additional copies of the two shorter fragments.[17]

Ghadiri's studies used the primary sequence shown in [1] or sometimes a slight modification of it.[16]

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Sequence: RMKQLEEKVYELLSKVACLEYEVARLKKLVGE[1]
Heptad:   gabcdefgabcdefgabcdefgabcdefgabc
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The sequence [1] differs from GCN4 in six residues. Two tyrosine residues were placed to facilitate spectroscopic quantitation using HPLC-UV, and alanine and cysteine residues were placed at the ligation site on the solvent-exposed surface. An asparagine was replaced with valine (N16V) to allow autocatalysis through one- and/or two-stranded α-helical template structure(s). The ligation site was astutely designed to lie on the solvent-exposed surface of the α-helical structure to avoid interference with the hydrophobic recognition surface.[17]

The interhelical recognition surface between the two peptide replicators is dominated by both hydrophobic packing interactions and electrostatic interactions (especially between the residues E_6 and R_1).[17] The residues K_3, Q_4, and E_7 are exposed to the solvent and should be more tolerant to substitutions.[16]

One full-length α-helical monomer serves as a template to mould the shape and location of both peptide subunits.[17] Optimal placement of each peptide end which is to be ligated thus accelerates amide bond formation. One peptide is a 17-residue fragment, E, with the electrophilic end preactivated as a thiobenzyl ester (figure 3). The sulfhydryl of the cysteine is more nucleophilic at neutral pH than all other side chain moieties, ensuring that the correct reaction occurs.

The 15-residue fragment N contains an N-terminal cysteine which serves as the nucleophilic partner. The experiments were carried out in dilute neutral aqueous solutions: 100 mM 3-(N-morpholino)-propanesulphonic acid (MOPS), 2M NaCl, 1% v/v BnSH, at pH 7.5.[7,17]

Both the E and N fragments consisted entirely of homochiral amino acids. The L-enantiomer chains were labelled E^L and N^L; the D-enantiomer chains, E^D and N^D. When equimolar proportions of these four peptides were condensed by forming an amide bond, four templates (T) were generated: either homochiral (T^{LL} and T^{DD}) or heterochiral (T^{LD} and T^{DL}) (figure 4). T^{LL} and T^{DD} were generated preferentially. T^{LL} templates autocatalytically accelerated the reaction of E^L with N^L faster than the heterochiral (T^{LD} and T^{DL}) templates do.[7] As further evidence of the selective templating effect, E^L, E^D, N^L, and N^D were added to a high concentration of pure T^{LL}, which led to a high predominance of T^{LL}.

Fragment E: Ar-RMKQLEEKVYELLSKVA-COSBn
Fragment N: CLEYEVARLKKLVGE-$CONH_2$
Fragment T: RMKQLEEKVYELLSKVACLEYEVARLKKLVGE

Error correction is claimed

Experiments showed that a single residue change from D→L or from L→D can prevent self-replication, since this disrupts the perfect α-helical structure, probably causing steric interference in the residue side chains.[18] This was demonstrated as follows.[7]

Three nucleophilic peptide versions of N^L were synthesized, each having just one L-residue replaced by its enantiomer: N^L_{dLeu26}, N^L_{dArg25}, and N^L_{dCys18}. The first two 'mutants' were designed to test the effects of one residue with the wrong chirality within the informational complementary hydrophobic recognition interface (N^L_{dLeu26}) and the non-informational solvent-exposed helical surface (N^L_{dArg25}). Reaction mixtures using equimolar amounts of E^L and either of these two mutants produced only background rates of the corresponding fragment condensation products T^{LL}_{dLeu26} and T^{LL}_{dArg25}, i.e. with no autocatalytic effect.

The third 'mutant' N^{LL}_{dCys18} was expected to have a minimal effect on the stability of the developing helical fragment or on its interactions with the template. Reacting equimolar amounts of E^L and N^L_{dCys18} displayed only a small rate enhancement in the formation of product T^{LL}_{dCys18} (1.3 times over the background rate).

Unexpectedly, all three of the template mutants accelerated production of the homochiral replicator T^{LL} when 'non-mutated' E^L and N^L were combined.[7]

This was presented as an example of error correction[19] leading to reliable replication, but a better insight is that only the enantiopure E^{LL} template works properly. A more amenable finding for evolutionary purposes would have been a series of peptide sequences displaying increasing rate enhancement with ever fewer disruptive residues of the wrong chirality. That would have offered a more plausible path for chance to produce the optimal templating sequences, starting with slightly functional sequences having mixed D and L residues.

In another experiment, a very conservative chirality-conserving alteration was made. Alanine was substituted for a leucine at position 26 in the nucleophilic peptide fragment, which prevented self-replication.[17]

Another study showed that electrostatic substitutions at the solvent-exposed position could decrease the initial rate of peptide fragment condensation by more than 3 orders of magnitude.[20] This further emphasizes the limited number of acceptable alternative residues per position competent to produce α-helical coiled coils.

Autocatalytic peptide network stabilized against errors

Ghadiri and colleagues have devoted much effort attempting to show that their carefully designed system has prebiotic relevance. We are informed that

> "The peptide-based systems described are the first examples of self-organized chemical networks that display characteristics essential for evolution: sequence-selective reproduction and dynamic error correction."[19]

The authors reiterated their claim that loss of autocatalysis due to single residue replacement represents 'error correction'.[19] Two additional series of experiments were performed, involving four peptide fragments: the native electrophilic E and nucleophilic N fragments, which when ligated produce the optimized template T; and fragments E_{9A} and N_{26A} having one alanine of the same chirality replaced. Four potential templates can form:

$$E + N \rightarrow T \quad [2]$$
$$E + N_{26A} \rightarrow T_{26A} \quad [3]$$
$$E_{9A} + N \rightarrow T_{9A} \quad [4]$$
$$E_{9A} + N_{26A} \rightarrow T_{9/26A} \quad [5].$$

The results of experiments to identify catalytic and autocatalytic effects are summarized in figure 5.

The doubly modified product $T_{9/26A}$ is catalytically inactive. Mutant templates T_{26A} [3] and T_{9A} [4] do not auto-catalyze their constituent peptide fragments,[19] and the intact template T never catalyzes ligation involving a modified peptide.

Furthermore, the single-residue-modified templates T_{26A} and T_{9A} catalyze the ligation of E with N, generating additional T, which further autocatalyzes ligation of E with N.[19]

All these reactions accelerate ligation of E with N, and none of the modified peptides. The authors claim this leads to error-correcting self-organized autocatalytic cycles, which collaborate to produce the native sequence T.[19] They reference key publications by Eigen on his notions of hypercyclic networks.[21–23]

Ghadiri *et al.* discovered by accident a scheme which contradicts the 'error-correcting' effect. E with a mutant N_1 produced a replicator R_1, and E with N_2 produced R_2, which cross-catalyzed each other. By judiciously adjusting the initial concentrations of R_1 and R_2, it is possible to set up a 'hypercyclic network' where R_1 and R_2 can coexist, at least for some undetermined number of cycles.[24]

The oscillating effect in relative concentrations occurred between two 32-residue peptides merely differing at one position, but the authors refer to it as a hypercycle. In genetic systems complex subsystems of completely unrelated biomolecules are indeed linked; for example, the production of mature t-RNAs and aminoacyl-tRNAs-synthetases. A true prebiotic hypercycle must produce raw materials autocatalytically and feed these into consuming autocatalytic reactions. Nothing like that was demonstrated in this work.[24] Peptides E and N were not generated through an integrated process but were simply made available as needed by an intelligent chemist.

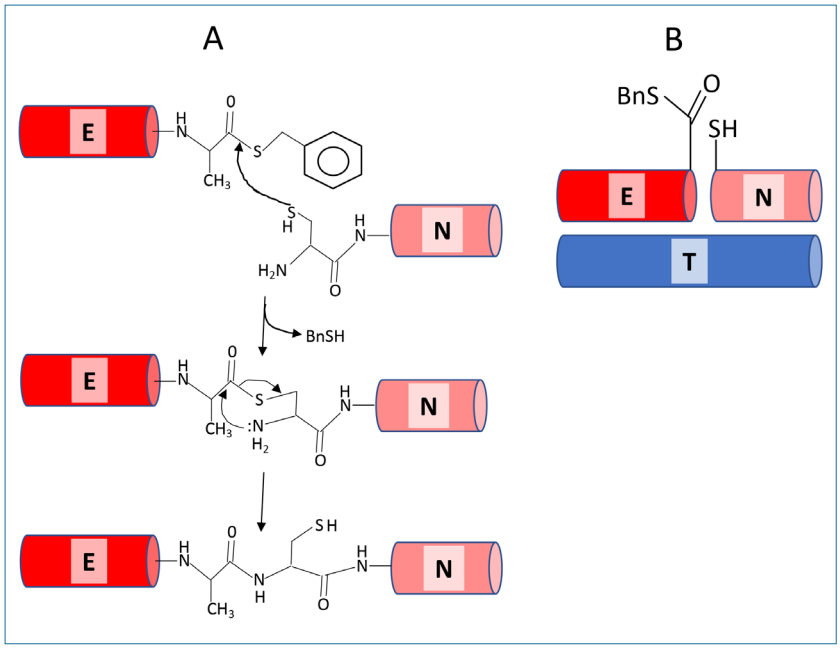

Figure 3. Ligation mechanism between the activated *C*-terminus of the electrophilic peptide fragment E and cysteine of the nucleophilic peptide fragment N (Bn means benzyl). A) Full mechanistic details. The intermediate thioester rearranges rapidly to produce the native amide bond. B) Simplified view of the ligation of E with N. Modification of figures found in reference 16.

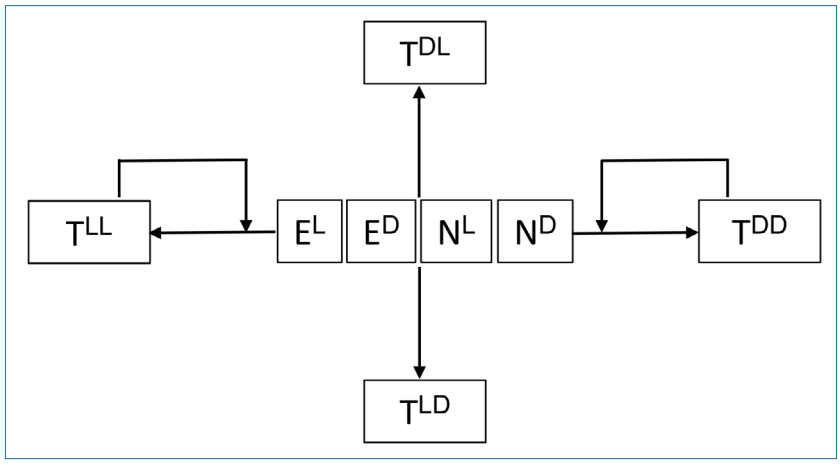

Figure 4. Condensation of enantiomerically pure L- or D-peptide fragments E with L- or D-peptide fragments N produces primarily the homochiral templating products T^{LL} or T^{DD} and very little of the mixed version T^{DL} or T^{LD}. Modification of a figure found in reference 16.

Usage of 'errors' misleading

What do Ghadiri *et al.* mean by 'errors'? Errors in biological genetic systems are clearly understood, referring to mutational defects which ultimately damage the host organism. This occurs because an important multicomponent process is damaged. But the researchers wrote, "Here we describe autocatalytic peptide networks that stabilize themselves against errors by subjugating the mutant population for the synthesis of the wild-type peptide."[19] Here 'error' simply means a modification in peptide sequence leading to slower autocatalysis. Is this correct biologically or for abiogenesis purposes? Is everything which accelerates a process, e.g. cancerous cell growth, beneficial for living systems and to be selected for?

Biological systems are all about regulation, starting and stopping enzymatically catalyzed biochemical reactions at the right time and place, generating products in the appropriate concentration range. A runaway uncontrolled process leading to a single chemical compound is the opposite of what is needed.

"Subjugating the mutant population" invokes Darwinian thinking but has no relevance to the rich variety of DNA-, RNA-, and protein-error-correcting molecular machines which are encoded on genomes. These correct specific flaws in the individual biomolecules, such as replacing mismatched nucleotides on DNA or chemically tagging a misfolded protein to be enzymatically degraded. The 'correction' claimed merely reflects faster reproduction of one sequence vs. slower ones, although there is no reason to assume that the faster one is what would be needed along a random naturalistic trajectory leading to living systems.

Autocatalysis made possible through a designed system

In a key 1997 paper, Ghadiri and colleagues emphasized the word design multiple times when discussing these experiments and never referred to evolution. When alluding to the purpose and value of their research, they state correctly that applications in the future "would first require the ability to rationally design informational self-reproducing and self-replicating molecular systems."[16]

They should have emphasized the need for intelligent conception by pointing to facts their work revealed. Only the use of enantiopure fragments and the resulting templates provided reliable peptide catalytic cycles. The findings an evolutionist would have hoped for were not obtained, namely a wide variety of functional sequence alternatives linking non-functional to ever better autocatalytic sequences.

Despite having used a highly effective templating sequence, after 170 minutes almost 15% side products were obtained.[16] If this kind of system were to arise naturally it would operate for only a few iterations and then discontinue like the other experiments based on RNA or DNA Ghadiri and his colleagues criticized.[7]

The templates did not facilitate addition of only individual D or only L amino acids, nor were the E and N sequences selected from among a vast pool of alternatives present. The homochiral E and N peptides which were made available must have been large to have molded with a specific template and would have been provided in the same molar proportion. No effort was invested to extrapolate to very dilute unstirred concentrations of E and N having a relative proportion of, for example, <1:10,000, which is very plausible given the extreme sequence specificity of the two fragments. Autocatalysis would have been insignificant and have soon consumed the less available fragment.

Likelihood of a random sequence leading to an α-helical coiled coil

In free nature a large concentration of 32-residue homochiral peptides with the correct primary sequence will not arise at the same time and location. Under plausible aqueous natural conditions, peptides 15 to 17 residues long having any specified sequence would be present in at best negligible concentrations.[25]

Suppose somehow only the 20 proteinogenic amino acids were present in only the L-enantiomer form. We will ignore the >99% other potential contaminants. Only a tiny subset of all $20^{32} \approx 4.3 \times 10^{43}$ possible sequences based on twenty proteinogenic amino acids would serve the intended templating purpose. The coiled coil dimers must pack together in a specific knobs-into-holes manner.[26] Every seventh residue must be aligned vertically along the α-coil to produce a suitable hydrophobic surface at which to dimerize, figure 2. Each turn will have approximately 3.5 residues, with the heptad positions *a* and *d* forming a hydrophobic surface on one side of the helix.[27]

As a minimal requirement the canonical seven-residue repeat must contain hydrophobic (H) apolar residues at positions *a* and *d* and polar (P) residues elsewhere, generating the pattern (H•P•P•H•P•P•P).[28] But this pattern can lead to dimeric, trimeric, or anti-parallel tetrameric conformations. The parallel, dimeric coiled coils exhibit strong preferences for specific hydrophobic amino acids at the five *a* and four *d* positions.[27] Loss of function for coiled coils has been shown to occur often even when similar hydrophobic residues are used.[29,30]

The primary sequence is very constrained, since coiled coils are stabilized by both intrachain and interchain interactions. A leucine repeat is often found at the *d* positions and a preponderance of β-branched amino acids (valine, isoleucine, or leucine) at the *a* positions since this favours dimer formation due to packing considerations.[27] Asparagine is often also found at the *a* positions, which directs dimerization by forming buried hydrogen bonds.[27]

In addition, whether hydrophobic residues will support the coil structure depends on the other hydrophobic residues they interact with on the same or the partner molecule. Sauer *et al.* found that many combinations of hydrophobic residues were nonfunctional,[31] and Ransone *et al.* reported that mutating two or more leucine residues prevented formation of the heterodimer.[30] Indeed, it has been demonstrated that disruptions to the coiled coil structure occur irrespective of whether the mutated residue lay in an inner *a*/*d*, adjacent *e*/*g*, or outer *b*/*c*/*f* position.[28]

Hodges *et al.* have demonstrated that leucine residues at the *a* and *d* positions contribute more than other hydrophobic residues tested (Ile, Val, Phe, Tyr, and Ala) to the stability of the coiled coil.[32] Replacing a single Leu by the very similar residue Ala at *a* positions decreases stabilization of the dimer on average by about 3.3 kcal/mol and 2.0 kcal/mol at *d* positions.[33] In general, mutations using less hydrophobic residues tend to be more deleterious for protein stability at more buried positions.[34–37]

Thus, not every combination of eight possible hydrophobic residues (alanine (A), valine (V), leucine (L), isoleucine (I), proline (P), phenylalanine (F), methionine (M), and tryptophan (W)) can be used at the *a* or *d* positions. For example, multiple bond-bending proline residues or bulky phenylalanines or tryptophans would prevent effective packing. Therefore, we estimate that positions *a* and *d* should have no better than about 3/20 probability per residue position of having an acceptable residue by chance in the E or N sequences.

For the non *a* and *d* heptad positions, theoretically around 5/20 of the residues might work in some positions, being polar and non-charged (serine (S), threonine (T), tyrosine (Y), asparagine (Asn), glutamine (Q)). (Chemists don't all agree on how to classify cysteine (C), and histidine (H) is polar and often charged). This is surely an overestimate, since multiple bulky side chains could interfere with packing.

Salt bridges formed by the interaction between positively (lysine, K or arginine, R) and negatively (aspartate, D or glutamate, R) charged amino acid side chains can help hold an α-loop together, but if in the wrong positions would twist the coil out of register. Interaction of residues having the same charge would destabilize the structure. Interhelical salt bridges between residues, especially at the *e* and *g* positions, may be necessary to stabilize the structure in some sequence variants.[15,38] However, at the wrong locations covalent disulfide bonds could hinder forming the coiled structured by affecting how the side chains interact. Similar considerations apply to histidine, tyrosine, phenylalanine and tryptophan, which can form weak hydrogen bonds.

Formation of a covalent disulfide bond between two cysteine residues can ensure or hinder that the two α-helices

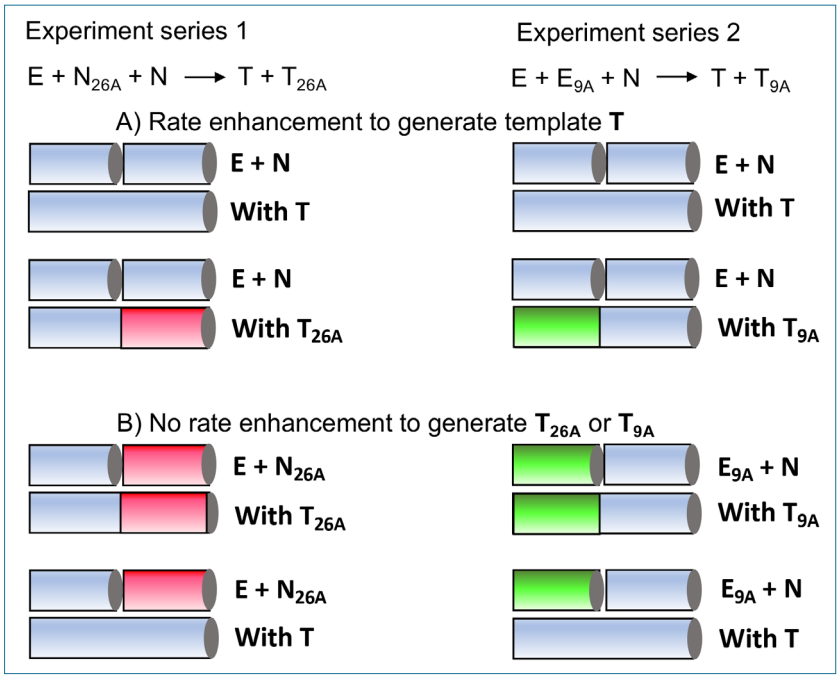

Figure 5. Catalytic effects of template variants. The optimized sequence is shown in blue, variants in other colours. See main text for discussion. Modification of a figure found in reference 19.

are in a parallel and in-register alignment to produce the coiled-coil structure. The amount of stabilization of protein structure contributed by a disulfide bond is around 1 kcal/mol but, depending on distance and angle, can be higher.[39]

Summary of the hurdles to produce the system naturally

1. Only a miniscule number of linear peptides large enough to produce this autocatalytic system would have existed in water, with two having to be exactly 15 and 17 residues long.[25] Call the number of candidate peptides with suitable length r_{15} and r_{17}.
2. r_{15} and r_{17} must consist of only L-residues (p = ½) and have suitable primary sequences (p ≈ ¼). The joint probabilities would be roughly (½ x ¼)^{15} and ~ (½ x ¼)^{17}, respectively. A template cannot form unless both fragments exist. This demands that $r_{15} > 1/(⅛)^{15} \approx 3.5 \times 10^{13}$ and $r_{17} > 1/(⅛)^{17} \approx 2.3 \times 10^{15}$ copies of peptides of correct lengths must be formed.
   Under plausible prebiotic scenarios this seems unlikely. But including the next constraint removes all doubt.
3. The electrophilic end of the r_{17} must be preactivated, for example as a thiobenzyl ester, but not the other peptides, which could react with the N fragment. The probability is for all practical purposes zero.

Constraint (2) does not consider the relative proportions of amino acids present when proposing a factor of ¼ (i.e. 5/20) per residue position, but seems reasonable. Valine and leucine would have indeed been more plentiful, facilitating formation of the hydrophobic surface. However, they would then also be more likely to be found at positions where hydrophobic residues are not desired. The most plentiful amino acid claimed to be formed by natural processes, glycine, is rarely used by an α-helical coiled coil. And non-proteinogenic α, β, γ, δ, and ε amino acids can also form naturally and are delivered to Earth by meteorites; so far more than 20 alternatives would compete at each residue position. We also neglected side chain reactions involving their amino and carboxylic acid groups, intramolecular ring formation, and all oxidation and other possible destructive reactions.

It is true that the templating effect produces more T^{LL} and T^{DD} than T^{DL} and T^{LD}, and perhaps sufficient enantiopure E and N variants had been generated. Suppose all the amino acids used at each position had an L/D abiotic ratio of 1/0.55, an absurd assumption. Then only 1 out of 7,844 of the N would be enantiopure ($1/0.55^{15}$), and 1 out of 25,931 of the E sequences ($1/0.55^{17}$). The more numerous activated 'wrong' E would still react together, with or without partial templating enhancement.

Suppose, hypothetically, that one template (T) appeared somewhere during tens of millions of years despite factors (1) – (3). Now an additional pristine E and N fragment must not only overcome these overwhelming constraints, but this time in addition must do so right next to T before it degrades. A flood of interfering racemic peptides with incorrect sequences of various lengths, not just fifteen and seventeen long, would surround these new correct E and N peptides. Furthermore, isolated single-stranded helices like T are unstable in aqueous solutions.[40,41] Therefore, very little time would be available before T, E, and N would both hydrolyze and racemize.

Expecting this first T to autocatalyze a copy of itself is unrealistic. But to be relevant for origin-of-life purposes, this would have to occur countless times at the exact same location!

Significance for extant life

Variants of the 32-residue sequence do exist within some biological proteins, encoded for on DNA. The researchers made no attempt, however, to explain how their peptide autocatalytic system could have converted into a genetic system, with the α-helical coiled coil motif now found in many proteins with unrelated primary sequences.

The fascinating experiments were inspired by existing biological protein structures so cleverly designed that no

one would have thought up the autocatalytic scheme without them. But ligation of E with N only occurs thanks to first converting to a highly reactive thiobenzyl ester, so the autocatalysis could *not* have arisen naturally. Templating merely accelerates the process by optimally moulding the reactants together. Essentially the authors demonstrated that if optically pure E and N were mixed with no contaminants present, properly activated in a laboratory, a chemical bond would form. Nothing resembling a solution to the origin of homochirality in a genetic system has been demonstrated.

The researchers did demonstrate that L- or D-only residue sequences interact more effectively with a peptide template of the same chirality, an unsurprising result, but the peptides were already homochiral. The unstated implication is that obtaining homochiral peptides E and N would be more likely than a full homochiral 32-residue peptide by chance. But E and N must be present at the exact same location concurrently, and only the homochiral version of E must be preactivated.

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