A chimaeric glutamyl:glutaminyl-tRNA synthetase: implications for evolution

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aaRSs (aminoacyl-tRNA synthetases) are multi-domain proteins that have evolved by domain acquisition. The anti-codon binding domain was added to the more ancient catalytic domain during aaRS evolution. Unlike in eukaryotes, the anti-codon binding domains of GluRS (glutamyl-tRNA synthetase) and GlnRS (glutaminyl-tRNA synthetase) in bacteria are structurally distinct. This originates from the unique evolutionary history of GlnRSs. Starting from the catalytic domain, eukaryotic GlnRS evolved by acquiring the archaeal/eukaryote-specific anticodon binding domain after branching away from the eubacteria family. Subsequently, eukaryotic GlnRS evolved from GluRS by gene duplication and horizontally transferred to bacteria. In order to study the properties of the putative ancestral GluRS in eukaryotes, formed immediately after acquiring the anticodon binding domain, we have designed and constructed a chimaeric protein, cGluGlnRS, consisting of the catalytic domain, Ec GlnRS (Escherichia coli GlnRS), and the anticodon binding domain of EcGlnRS (E. coli GlnRS). In contrast to the isolated EcN-GluRS, cGluGlnRS showed detectable activity of glutamylation of E. coli tRNA\(^{\text{ts}}\) and was capable of complementing an E. coli ts (temperature-sensitive)-GluRS strain at non-permissive temperatures. Both cGluGlnRS and EcN-GluRS were found to bind E. coli tRNA\(^{\text{ts}}\) with native EcGluRS-like affinity, suggesting that the anticodon-binding domain in cGluGlnRS enhances \(k_{\text{cat}}\) for glutamylation. This was further confirmed from similar experiments with a chimaera between EcN-GluRS and the substrate-binding domain of EcDnaK (E. coli DnaK). We also show that an extended loop, present in the anticodon-binding domains of GlnRSs, is absent in archaeal GlnRS, suggesting that the loop was a later addition, generating additional anti-codon discrimination capability in GlnRS as it evolved from GluRS in eukaryotes.

Key words: chimaera, complementation, evolution, specificity, synthetase, tRNA.

INTRODUCTION

A major mechanism of generation of protein diversity appears to be by shuffling and acquisition of domains followed by mutational changes [1–4]. Although this principle appears to be well accepted, many unresolved questions remain. Chimaeric proteins that initially form from such recombination events are not found, as expected, in extant organisms because of further mutational changes. A better understanding of the nature of such chimaeric proteins, formed immediately after domain shuffling and/or acquisition, may offer a deeper appreciation of selective advantages of such events as well as biochemical mechanisms of further evolution. In addition, principles learnt from such studies may provide us with a better way to evolve proteins in vitro.

aaRSs (aminoacyl-tRNA synthetases) are multi-domain proteins [5–7]. It is believed that the catalytic, class-defining domain of aaRS may be the most ancient [8]. It was suggested that other domains, such as the anti-codon binding and the editing domains of aaRS, were added later to augment the specificity of aminoacylation for enhanced adaptability [5]. One such domain-acquisition event probably occurred during the evolution of GluRS (glutamyl-tRNA synthetase) and GlnRS (glutaminyl-tRNA synthetase) after divergence of the archaeal and eubacterial kingdoms from the last common universal ancestor [9]. One possible scenario for evolution of GluRS and GlnRS across all kingdoms of life has been outlined by Mirande and co-workers [9]. They have argued that GluRS in the last common universal ancestor consisted of only the class-defining catalytic domain. After eubacteria and archaea/eukaryotes split, the two lineages independently acquired two structurally distinct anti-codon binding domains: (1) the bacterial kingdom acquired the helical-cage anti-codon binding domains found in extant eubacterial GluRS; (2) archaea/eukaryotes acquired the β-barrel anti-codon binding domains found in all GlnRSs and in all known archaeal and eukaryotic GlnRSs. The initial event towards the development of a GlnRS may have been the creation of a chimaera of the class-defining catalytic domain of GluRS and the β-barrel anti-codon binding domain in the archaea/eukaryote lineage. Although this putative chimaeric protein has overall domain structure similar to extant GlnRSs and GluRSs of archaeal/eukaryotic origin, the domain interfaces may be substantially different. The extant aaRSs have undergone further evolution and optimization of the domain interfaces as well as acquisition of additional structural elements, such as loops. Thus extant aaRSs are not good models for this putative evolutionary intermediate protein.

To recapitulate such a possible evolutionary process and to examine the properties of the putative chimaeric protein, a chimaeric protein (from now on called cGluGlnRS, for chimaeric glutamyl-glutaminyl tRNA synthetase) to distinguish it from other cases where the nomenclature GluGlnRS was used in the literature) was created by joining residues 1–314 of cEgGlnRS, cGluGlnRS showed detectable activity of glutamylation of E. coli tRNA\(^{\text{ts}}\) and was capable of complementing an E. coli ts (temperature-sensitive)-GluRS strain at non-permissive temperatures. Both cGluGlnRS and EcN-GluRS were found to bind E. coli tRNA\(^{\text{ts}}\) with native EcGluRS-like affinity, suggesting that the anticodon-binding domain in cGluGlnRS enhances \(k_{\text{cat}}\) for glutamylation. This was further confirmed from similar experiments with a chimaera between EcN-GluRS and the substrate-binding domain of EcDnaK (E. coli DnaK). We also show that an extended loop, present in the anticodon-binding domains of GlnRSs, is absent in archaeal GlnRS, suggesting that the loop was a later addition, generating additional anti-codon discrimination capability in GlnRS as it evolved from GluRS in eukaryotes.

Key words: chimaera, complementation, evolution, specificity, synthetase, tRNA.

Abbreviations used: aaRS, aminoacyl-tRNA synthetase; EcDnaK, Escherichia coli DnaK; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; EcGluRS, Escherichia coli GluRS; EcGlnRS, Escherichia coli GlnRS; EcN-GluRS, Escherichia coli N-terminal GluRS; EcC-GlnRS, Escherichia coli C-terminal GlnRS; cGluGlnRS, chimaeric GluRS (EcN-GluRS–EcC-GlnRS); ts, temperature-sensitive; TthGluRS, Thermus thermophilus GluRS.

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GlnRS) with two extra amino acids in between. cGluGlnRS created this way has the same overall domain structure as that of the extant GlnRSs and GluRSs of archaeal/eukaryotic origin. However, the anti-codon/catalytic domain interface is likely to be non-optimized. A long loop that originates from the β-barrel anti-codon binding domain and interacts with the catalytic domain in GlnRS, may not interact with the catalytic domain of cGluGlnRS as the interaction interface is quite different (see below). Thus, cGluGlnRS may be a more realistic model for the putative evolutionary intermediate chimaeric protein. This article reports the properties of this chimaeric protein and its significance in the evolution of GlnRS from GluRS.

EXPERIMENTAL

Materials

E. coli tRNA<sub>glu</sub> was purchased from Sigma Chemical Company and dialysed extensively against sterile water before use. All other materials were of analytical grade.

Design and construction of cGluGlnRS

In the absence of an available crystal structure of EcGluRS, residues spanning the catalytic domain of EcGluRS were determined indirectly from a close sequence homologue with known structure, TthGluRS ( Thermus thermophilus GluRS). By visual inspection of the TthGluRS crystal structure [10], we deduce that the N-terminal catalytic domain spans from residue 1 to 322 (Figure 1, top panel). Although the N-terminal half of GluRS or GlnRS may consist of more than one domain as defined by standard domain definition, we will define residues 1–322 in TthGluRS or equivalent residues in other GluRSs or GlnRSs as the N-terminal domain. Similar nomenclature was used for the C-terminal half. The end residue of the N-terminal domain is 322 in TthGluRS, which is equivalent to residue 314 in EcGluRS (Figure 1, bottom panel; marked cyan). Thus, residues 1–314 of EcGluRS were chosen as the N-terminal domain (EcN-GluRS) to be incorporated in cGluGlnRS. In order to design the restriction sites needed to fuse the two fragments and keep mutations to a minimum, we decided to join residues 1–314 of EcGluRS with residues 335–554 in EcGlnRS (EcN-GlnRS) with two additional residues in between [11]. The construction of the cGluGlnRS gene was performed by amplifying EcGluRS (1–314) and EcGlnRS (335–554) genes by PCR. The primers were chosen in a manner that HindIII sites occurs in-frame at the end and the beginning of the two respective fragments. The two amplified fragments, after appropriate restriction digestions, were first cloned separately in pUC18 (EcN-GluRS in EcoRI and HindIII site and EcC-GlnRS in HindIII and BamHI site respectively). Then the plasmids were purified, digested with appropriate restriction enzymes, gel purified, ligated through their HindIII sites and cloned in pBR322 to generate the cGluGlnRS gene. [cGluGlnRS carried a two amino acid insertion, underlined; C-terminal end of the EcGluRS (1–314) in italics and N-terminal end of EcGlnRS (335–554) in bold italics; KLLWLNHHYINAL KLPAPRAMAVIDPV]. The resultant EcGluRS (1–314)-KL-EcGlnRS (335–554) gene (EcGluRS) was sequenced and sub-cloned into the pET28a vector with an N-terminal His-tag. Similar protocols were followed for construction of EcGluRS-DnaK. Site-directed mutagenesis was performed by the overlap extension procedure [12].

Purification of enzymes

pET28A plasmid containing the cGluGlnRS gene was transformed into either the E. coli strain BL21(DE3) or the ts JP1449 (DE3). From the transformed plate, a single colony was picked and allowed to grow in LB (Luria–Bertani) medium at 37°C containing 35 µg/ml of kanamycin until it attained an attenuation of 0.2. For the JP1449(DE3) host strain, the initial growth temperature was 32°C. Then the cells were transferred to 16°C and kept there for approx. 30 min without shaking to allow...
the cells to come to thermal equilibrium. After 30 min, 0.5 mM IPTG (isopropyl β-D-thiogalactoside) was added and shaking was resumed at 16°C overnight. Cells were then harvested and the cell pellet was resuspended and sonicated in lysis buffer (50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 1 mM 2-mercaptoethanol, 20% glycerol, PMSF and 5 mM imidazole). The sonicated cells were spun at 21,400 g. The resultant supernatant was loaded slowly on to a Ni-NTA agarose resin equilibrated with the lysis buffer but without 2-mercaptoethanol. After the loading was complete, the column was washed with 10 column volumes of lysis buffer, followed by 5 column volumes of lysis buffer without 2-mercaptoethanol, but containing 20 mM imidazole. The protein was eluted by lysis buffer without 2-mercaptoethanol but containing 500 mM imidazole. The eluted protein was analysed on SDS/PAGE and the major fractions were pooled and then dialysed in 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 20% glycerol. Purification of EcN-GluRS was performed in the same way. Purification of GluRS was performed as described before [13,14].

Fluorescence methods

Steady state fluorescence was measured in a Hitachi F3010 spectrofluorimeter. The experiments were performed at 25°C. The excitation and emission bandpasses were 5 nm each, unless stated otherwise. Spectra of appropriate buffers were subtracted from the fluorescence spectra. Binding of E. coli tRNA<sub>thr</sub> to cGluGlnRS, EcGluRS and EcN-GluRS was performed in 20 mM Hepes buffer, pH 7.2, containing 20% glycerol and 5 mM MgCl<sub>2</sub>. The excitation wavelength was 295 nm and emission wavelength was 340 nm. Each point was performed separately as described before [15]. After transferring the protein from a stock solution to the cuvette, the fluorescence intensity was measured. A predetermined concentration of E. coli tRNA<sub>thr</sub> was then added and after 1 min the fluorescence was determined again. The inner filter effect correction was done using the formula:

\[ F_{\text{corrected}} = F_{\text{observed}} \times \left( \frac{(A_{ex} + A_{em})}{2} \right) \]

where \( F \) is the fluorescence intensity and \( A \) is the absorbance. The ratios of corrected fluorescence intensity at 340 nm were used to measure the quenching.

Determination of enzyme activity in vitro

In all in vitro assays, enzymes were prepared from strain JP1449 (DE3) bearing appropriate plasmids. The enzyme assays were performed as described before, except the assay temperature was 42°C to remove the endogenous EcGluRS [16]. In the assays, the concentrations of cGluGlnRS and EcN-GluRS were each kept at 1 μg per assay point, whereas EcGluRS was kept at 1 ng per point.

Determination of \( K_m \) of cGluGlnRS with respect to E. coli tRNA<sub>thr</sub>

The assay of cGluGlnRS was performed as described by Bhattacharyya et al. [16]. The changes that were made to calculate the \( K_m \) were as follows: the enzyme concentration was kept at 1.2 μg per assay point; the concentration of L-glutamate was kept at 500 μM; the concentration of E. coli tRNA<sub>thr</sub> was increased from 0 to 10 μM; the slope obtained in each case was plotted against the respective E. coli tRNA<sub>thr</sub> concentration and the required \( K_m \) was obtained by curve fitting. The assay temperature was 37°C.

Results

One of the ways to judge as to how effective a protein is for the survival of an organism is through complementation assays. Whether cGluGlnRS is capable of effectively transferring L-glutamate in vivo to E. coli tRNA<sub>thr</sub> was judged by in vivo complementation assay. The host strain JP1449 (DE3), carrying a ts (temperature-sensitive) mutation in the chromosomal copy of the EcGluRS, does not grow at 42°C [17]. pET28a vectors carrying different genes were used to transform the strain and the transformed strains were grown at 42°C. Figure 2 shows the growth curve of JP1449 (DE3) transformed with plasmids bearing different genes at the non-permissive temperature. JP1449 (DE3) transformed with plasmid bearing the wild-type EcGluRS gene grew normally, whereas JP1449 (DE3), complemented with the gene for cGluGlnRS, grew slower but reasonably well. pET28a bearing JP1449 (DE3) did not grow at all. The support of EcN-GluRS was performed in the same way. Purification of GluRS was performed as described before [13,14].

In order to make sure that the growth of JP1449 (DE3), complemented by the cGluGlnRS gene, is due to the glutamyl- 
tation activity of cGluGlnRS, mutations in the conserved HIGH signature sequence were introduced in the cGluGlnRS gene (H16A mutation) [19]. The H16AcGluGlnRS gene was incapable of complementing the ts-EcGluRS at the non-permissive temperature, indicating that glutamyl- 
tation activity of cGluGlnRS was the actual source of growth at the non-permissive temperature. In order to see if the C-terminal anti-codon binding domain played any role in the glutamyl- 
tation activity, a plasmid bearing the N-terminal domain of EcGluRS (1–314) gene, EcN-GluRS, was introduced in JP1449 (DE3). This transformed strain was also unable to grow at the non-permissive temperature indicating that the C-terminal anti-codon binding domain of EcGluRS plays a crucial role in augmenting the glutamyl- 
tation activity of cGluGlnRS. One possible role of the anti-codon binding domain may be a general conformational stabilization of the N-terminal domain bearing the active site. To find out whether the role of the C-terminal anti-codon binding domain is to interact specifically with the anti-codon loop of E. coli tRNA\textsubscript{glu} or to induce mere non-specific conformational stabilization, we have created another chimaeric protein in which the N-terminal do- 
main of EcGluRS (1–314) was fused with the substrate-binding domain of EcDnaK (E. coli DnaK) (384–638) (a domain of similar size; the construction was similar to cGluGlnRS and contained a KL residue between the two domains). The plasmid bearing the gene of the resultant EcGluRS (1–314)-KL-EcDnaK (384–638) chimera (from now on called GluRS-DnaK) was transformed into JP1449 (DE3) and a transformant colony was grown at the non-permissive temperature. The GluRS-DnaK was also unable to support growth at 42 °C, indicating that the role of the C-terminal domain of EcGluRS in the cGluGlnRS is specific for this domain. The anti-codon of E. coli tRNA\textsuperscript{gln} differs by a single base from E. coli tRNA\textsuperscript{gln} at position 36. Substitution of E. coli tRNA\textsuperscript{gln} at this position reduces \( k_{cat} \) and modestly increases \( K_m \) for EcGluRS, but retains decent overall activity [20]. This may indicate that the \( \beta \)-barrel domain may be capable of recognizing the anti-codon loop of E. coli tRNA\textsuperscript{gln} in the context of cGluGlnRS and may interact with the anti-codon nucleotides of E. coli tRNA\textsuperscript{gln}.

In order to better understand the role played by the C-terminal anti-codon binding domain in cGluGlnRS, we have puri- 
fied cGluGlnRS and the corresponding control protein EcN- 
GluRS (cGluGlnRS without the C-terminal domain) and 
determined their catalytic and substrate-binding properties. The His-tagged cGluGlnRS from JP1449 (DE3) was purified by Ni-NTA affinity chromatography to homogeneity. The tertiary structure of the protein was evaluated by fluorescence spectroscopy. Figure 3 shows the fluorescence emission spectrum of cGluGlnRS. The observed emission maximum was 339.4 nm, indicating solvent-buried tryptophan side chains characteristic of a folded protein. The emission maximum was very close to that of wild-type EcGluRS, which was found to be 337 nm. For comparison, the emission maximum of EcN-GluRS was measured, and remained unchanged at 340 nm. The CD spectra of the EcGluRS and EcN-GluRS were also typical of proteins with large \( \alpha \)-helical content, characterized by two distinct minima around 208 and 222 nm (results not shown).

cGluGlnRS, purified from JP1449 (DE3) background, was assayed for E. coli tRNA\textsuperscript{gln} glutamylation activity at 42°C to avoid any possible contamination from wild-type GluRS activity. Figure 4(A) shows the glutamyl- 
tation activity of the cGluGlnRS, EcGluRS and EcN-GluRS. Clearly, cGluGlnRS has

Figure 3  Fluorescence emission spectrum of cGluGlnRS

The excitation wavelength was 295 nm. The buffer was 50 mM Tris/HCl pH 7.8, containing 100 mM KCl, 10 mM 2-mercaptoethanol and 20% glycerol. Temperature was 25 ± 1 °C.

Figure 4  In vitro aminoacylation assay at 42 °C

(A) Aminoacylation assay of cGluGlnRS ( ), EcN-GluRS ( ) and EcGluRS ( ) performed with E. coli tRNA\textsuperscript{gln} and L-glutamate at 42 °C. For cGluGlnRS and EcN-GluRS, the amount of enzyme added per assay point was 1 ng. In the case of EcGluRS, the amount of protein added per point was 1 μg. (B) Determination of \( K_m \) of cGluGlnRS. cGluGlnRS (1.2 ng per point) was assayed with increasing concentrations of E. coli tRNA\textsuperscript{gln}. Aminoacylation assays are described in Bhattacharyya et al. [16].
DISCUSSION

It is generally believed that all GluRSs evolved from an ancestral GluRS gene [23,24]. One possible clue as to how such an event took place lies in the fact that the anti-codon binding domains of bacterial GluRS and GlnRS are structurally completely different: an α-cage in GluRS and a β-barrel in GlnRS [11,25]. Homology studies of C-terminal domains of extant bacterial GluRS and GlnRS from different organisms led to the conclusion that archaical and eukaryotic C-terminal domains of GluRS are structurally similar to the β-barrel domain of GlnRS [9,23], whereas bacterial GluRS anti-codon domains are all α-helical, similar to that seen in the TthGluRS crystal structure [10]. Based on this observation, Mirande and co-workers have proposed that, before the divergence of bacteria and archaea/eukaryotes, GluRS consisted of only the class-defining catalytic domain that also glutamylated tRNA^\text{glu}\) and was able to incorporate glutamine through the transamidation pathway [9]. After the divergence of eubacteria and archaea/eukaryotes, the former acquired the α-helical anti-codon binding domain, whereas the latter acquired the β-barrel domain. Subsequent gene duplication in eukaryotes gave rise to GlnRS from GluRS. The GlnRS gene was then transmitted to certain species of the bacterial kingdom [23].

An important question in this regard is how GlnRS evolved in the eukaryotic branch and not in any other kingdoms of life. An interesting difference between the archaical GluRS and eukaryotic GluRS and GlnRS appears to be the presence of the long loop (474–495 in EcGluRS) that extends from the β-barrel anti-codon binding domain to the class-defining catalytic domain (Figure 6). This loop appears to be crucial for specificity of this synthetase [20]. We have attempted to find out the existence of this loop in different kingdoms of life by multiple sequence alignment of GlnRS and GluRS from different sources. We examined 28 archaical GluRS sequences and found the loop to be absent in all (Figure 6). In contrast, bacterial and eukaryotic GlnRS, represented by EcGluRS and a eukaryotic GlnRS (Saccharomyces cerevisiae), have this loop (Figure 6). In fact, mutagenesis of this loop resulted in somewhat relaxed anti-codon specificity in EcGluRS [26]. It may be postulated (Figure 7) that insertion of this loop occurred in the eukaryotic branch after divergence of eukaryotes and archaea and may have played a crucial role towards the development of sufficient anti-codon discrimination ability to develop GluRS and GlnRS [23]. We created cGluGlnRS to study the properties of the putative chimera protein produced by the postulated recombination with the isolated N-terminal class-defining domain in the early eukaryote/archaea branch. This early chimaeric enzyme may have either lacked the loop, spanning the anti-codon binding domain and the class-defining domain containing the active site, or was characterized by a reduced coupling between the N-
cGluGlnRS may represent the early chimaeric GluRS in the archaea/eukaryote branch after the separation of the bacterial kingdom and before the development of GlnRS. The chimaeric enzyme constructed here thus structurally resembles the putative GluRS of early archaea/eukaryotes.

The N-terminal domain of EcGluRS, Ec-N-GluRS, showed negligible activity at best; insufficient to complement a ts defect in the EcGluRS gene at non-permissive temperatures. In contrast, cGluGlnRS is at least several-fold more active in vitro and was able to complement a ts defect in the EcGluRS gene at non-permissive temperatures. Clearly, the presence of the β-barrel domain increases the catalytic activity of the active site of EcGluRS (in the context of cGluGlnRS) situated several tens of angstroms away. For Ec-N-GluRS, the binding affinity of E. coli tRNA\(^{\text{Glu}}\) was almost Ec-GluRS-like, indicating that the anticodon binding domain in EcGluRS might primarily contribute to the enhancement of \(k_{\text{cat}}\). Similarly, the E. coli tRNA\(^{\text{Gln}}\) binding affinity to cGluGlnRS is also almost EcGluRS-like. As stated before, due to similarity of the anti-codon binding loops of the E. coli tRNA\(^{\text{Glu}}\) and tRNA\(^{\text{Gln}}\), it is likely that the β-barrel domain, in the context of cGluGlnRS, interacts with the anti-codon loop of E. coli tRNA\(^{\text{Glu}}\). Thus, the additional postulated interaction energy of the β-barrel domain in the context of cGluGlnRS may be used to cause a conformational change that increases the \(k_{\text{cat}}\). Taken together, all of this evidence suggests that the addition of a β-barrel anti-codon binding domain may have created an enzyme with a better \(k_{\text{cat}}\). A question then is how this anti-codon binding information is transmitted to the distant active site in the context of cGluGlnRS?

It has been shown that mutation of identity elements in the anti-codon loop of E. coli tRNA\(^{\text{Glu}}\) predominantly reduces \(k_{\text{cat}}\) in EcGluRS [27]. This suggests existence of pathways that transmit this binding information to the active site. Similar, but more detailed, conclusions have been drawn for EcGlnRS. Recent work on EcGlnRS suggests that there may be two pathways for the transmission of the correct anti-codon binding information to the active site. One involves the residues that are responsible for interacting with the inside of the L-shaped tRNA [28,29]. The other involves the loop (474–495 in EcGlnRS) mentioned above [30,31]. However, these extant enzymes are evolutionarily optimized, whereas cGluGlnRS is not. Recognition of correct tRNA itself is a complex process involving many identity elements and coupled conformational changes, neither of which is well understood. In several cases it has been demonstrated that the binding of a cognate tRNA leads to the ordering of the active site, including the associated water molecules [32]. As far as it is known today, protein-mediated pathways play important roles in transmitting the effect of distant identity elements to the active site [28]. tRNA-mediated pathways, if any, may be unimportant [33]. It is likely that the \(k_{\text{cat}}\) effect seen in cGluGlnRS may derive from a non-optimized protein-mediated rudimentary anti-codon binding information transmission pathway. Existence of such rudimentary transmission pathways after a domain-shuffling event or insertion of loops may create proteins which may confer some evolutionary advantages followed by further optimization.

Other chimaeric enzymes have also been created by domain exchange [34,35]. When the domain exchange involves related proteins, the resultant chimera tends to have significant activity [36]. This is probably a result of divergence of domain interfaces from the optimized one. During evolution, many domains undergo recombination processes to generate new domain combinations. However, not all combinations are found in extant organisms. The basis of such selectivity is unclear. One possibility that emerges from this study is that only those chimaeric proteins that exhibit enhanced activity, perhaps due to some degree of complementary
interface, prove advantageous for the organism. This may be followed by further evolution to a more efficient entity.

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