Totally *in vitro* protein selection using mRNA–protein fusions and ribosome display

Richard W Roberts

Both chemists and biochemists have great interest in creating peptides and proteins with desired structure, recognition and catalytic properties. Recently, two techniques have been developed that afford functional selection of proteins entirely *in vitro*: mRNA–protein fusions, and ribosome display. Improvements in both methods have allowed peptide and protein libraries of unprecedented size (10^{11}–10^{13} different members) to be generated and screened for function.

Addresses
Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA; e-mail: rroberts@its.caltech.edu

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Abbreviation
scFv single-chain antibody

Introduction

In the past decade, a number of laboratories have worked to generate peptides and proteins with desired functions through combinatorial chemistry. These experiments provide an 'irrational' or 'semi-rational' solution to the protein design problem in that no explicit knowledge of how sequence changes affect function is required to find a solution. The underlying principles of these methods are simple — a collection of protein sequences (a library or pool) is first generated and then sieved to isolate members that possess a particular function (e.g. binding or catalysis).

In general, the chance of finding a particular activity within a library is proportional to the size of the library that can be screened. Until recently, most protein selection schemes contained an obligate *in vivo* step, which limited the maximum library size to the transfection limit of the organism involved. Thus, libraries constructed in bacteria using the phage display system are limited to $1–10 \times 10^9$ different members [1]. Similarly, libraries constructed in yeast using the two-hybrid [2,3], three-hybrid [4,5] or yeast display techniques [6] must be commensurately smaller because of their much lower transfection efficiency compared with bacteria. In addition to limiting the library size, the *in vivo* step may be considered undesirable in that it provides selection pressure that cannot be controlled by the experimenter. On the other hand, *in vivo* techniques do provide the ability to select for protein function in the complex environment of the cell and may be applied to select modified (e.g. glycosylated) proteins or complex phenotypes involving an entire pathway.

Recently, two methods have been developed that provide for totally *in vitro* synthesis and selection of proteins — mRNA–protein fusions and ribosome display (for a recent review of ribosome display and translation, see [7•]). These systems shall be discussed within this review and open the possibility of peptide and protein selections containing more than 10^{13} different sequences in the total absence of a living cell. For combinatorial libraries to be useful, it must be possible to isolate functional sequences and characterize their properties. Synthesis and characterization provide rigid boundary on the maximum complexity that can be screened. In practice, the upper boundary on library size is limited by both the ability to create diversity and the ability to deconvolute the functional sequences after the selective step has occurred. Genetic molecules such as RNA and DNA provide an elegant pathway around these barriers. Chemical synthesis and enzymatic mutagenesis provide simple methods to generate libraries with arbitrarily high complexities. The functional sequences present can be recovered because these molecules can be copied and amplified by known enzymes. Thus, a selection cycle can be designed containing multiple rounds of enrichment and amplification. By combining multiple modest enrichments (100–1000 fold) in series, even vast libraries (10^{12}–10^{16} members) can be screened over several rounds to find a single active molecule present in the original pool [8].

The lure of larger libraries

While libraries currently used for protein selection may seem large (10^5–10^9 members), they pale in comparison with the libraries used to perform RNA and DNA selection experiments. Because all steps in a selection cycle can be performed *in vitro*, library sizes of $\geq 10^{15}$ members can be readily generated. The upper boundary on pool complexity with these libraries results from limits on the amount of extendible template that can be synthesized chemically or enzymatically (typically 5–50 nmol) and the maximum volume of PCR that can be reasonably performed to generate the library (0.1–1.0 L). It therefore follows that if methods could be found to perform protein selection entirely *in vitro*, the transfection limitation would be surmounted, and protein libraries as large as ~10^{15} sequences (a 10^6-fold increase in size) might be possible.

Proteins unfortunately are not genetic molecules and cannot be copied by any known enzymatic activity. Therefore, to construct an *in vitro* protein selection cycle, the genetic information (in the form of RNA or DNA) must be kept topologically linked to the functional information (protein) for copying and amplification after enrichment. The protein synthesis machinery uses complexes containing the two components necessary to perform this selection,

Figure 1

Formation of a mRNA–protein fusion. **(a)** The ribosome binds and transits a mRNA–puromycin conjugate. **(b)** Upon reaching the RNA/DNA junction, the ribosome pauses, allowing entry of the puromycin (shown as a circled P) into the peptidyl transferase center (P) to form **(c)** the completed mRNA–protein fusion product. A, aminoacyl-tRNA-binding site of the ribosome; aa, amino acid; N, amino terminus of the protein; P, peptidyl-tRNA-binding site of the ribosome; UTR, untranslated region of mRNA. The straight arrow in (a) indicates movement of the ribosome along the mRNA strand.

mRNA and the nascent protein it codes for. Unfortunately, these complexes also contain the ribosome, a macromolecular conglomerate weighing in at more than 2.5×10^6 D. Nevertheless, the high concentration of ribosomes in translation systems (e.g. 0.1–0.2 μM in reticulocyte lysate [9]) provides an entrée to synthesizing extraordinarily large protein libraries in easily tractable volumes.

Two approaches have emerged to take advantage of protein synthesis to generate large combinatorial libraries. Ribosome display utilizes stalled, noncovalent ribosome–mRNA–protein complexes to perform protein selection, while mRNA–protein fusions utilize a covalent mRNA–protein complex.

mRNA–protein fusions

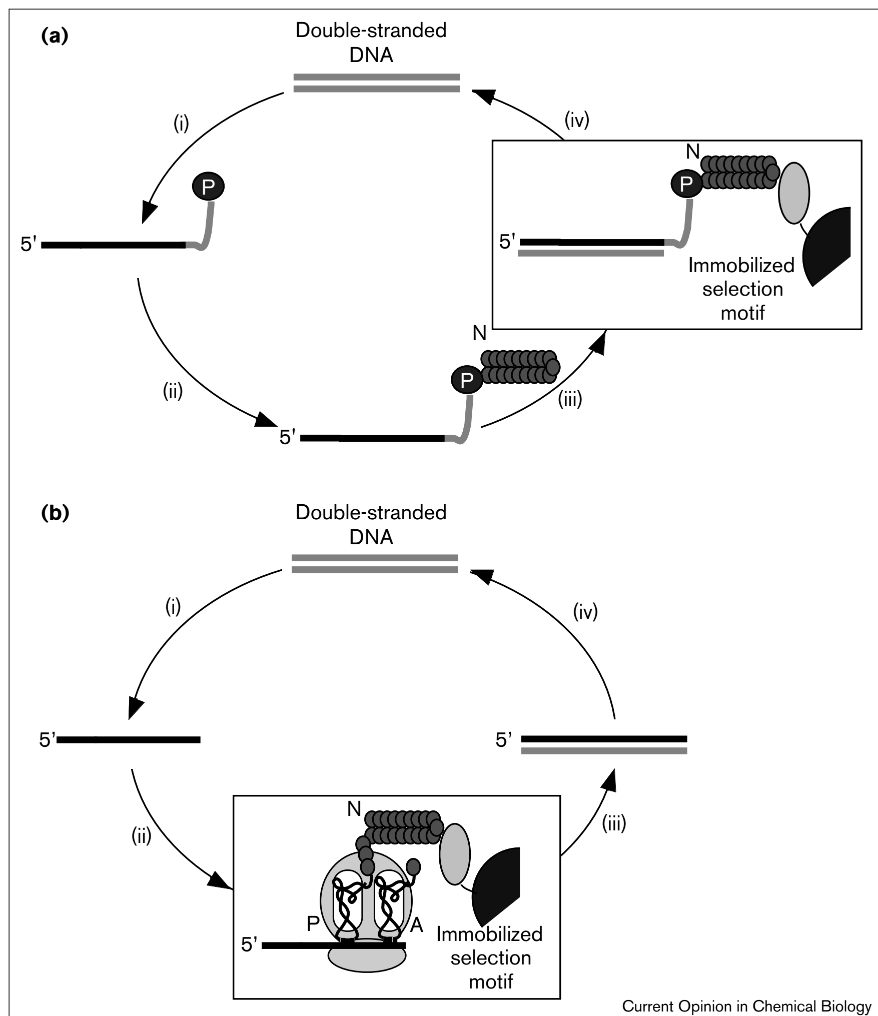
A mRNA–protein fusion comprises a protein sequence covalently linked via its carboxyl terminus to the 3′ end of its own mRNA (Figure 1c). In order to synthesize a mRNA–protein fusion, mRNA molecules are generated that contain the drug puromycin at their 3′ end. Initially, the ribosome binds and transits the template normally in a 5′→3′ direction (Figure 1a). When the ribosome reaches the 3′ end of the message conjugate, puromycin enters the peptidyl transferase site to form a covalent mRNA–protein fusion (Figures 1b,c). Thus, these mRNA–puromycin conjugates are able to perform two functions simultaneously, acting as both template and peptide acceptor on the surface of the ribosome [10••].

Formation of the fusion in *cis* (within one mRNA–peptide–ribosome complex) preserves the informational linkage between the protein and the mRNA it is covalently attached to. Therefore, an *in vitro* cycle similar to the one used for RNA and DNA selection can be constructed to enable repeated cycles of enrichment and amplification based on protein function (Figure 2a). Experimentally, the selective step can be performed either before or after cDNA synthesis using reverse transcriptase. Prior to cDNA synthesis, libraries of fusions with ‘naked’ mRNA allow isolation of either RNA aptamers present in the mRNA sequence library, functional protein sequences, or complexes where the mRNA and protein collaborate. cDNA–mRNA hybrid fusions should be devoid of any functional RNA structures, thereby greatly enhancing the isolation of only functional proteins.

The rudiments of selection

In 1997, Roberts and Szostak [10••] demonstrated that each of the steps needed for a selection cycle (synthesis, enrichment and amplification) could be performed using the fusion system (see Figure 2a). The work began by testing whether peptides containing the Myc epitope tag (EQKLISEEDL, single letter amino acid code) could be covalently attached to templates containing a 3′ puromycin molecule using *in vitro* translation extracts. A standard linker (i.e. an oligonucleotide 30 nucleotides in length, containing a 3′ puromycin [P], 5′-A_{27}dCdCP) was generated using a protected form of puromycin attached to an

Figure 2



Schematics of *in vitro* protein selection experiments. The selective step in each case is shown boxed. **(a)** mRNA–protein fusions. (i) A double-stranded DNA is used to generate a mRNA template (black) for translation that contains 3′ puromycin (circled P). (ii) The mRNA–puromycin conjugate is translated *in vitro* to generate the mRNA–protein fusion (amino acids are shown as dark gray circles; N, amino terminus). (iii) After cDNA synthesis, the construct is then screened for function using an immobilized selection motif. (iv) PCR is then used to generate double-stranded DNA enriched in functional sequences. **(b)** Ribosome display. (i) A double-stranded DNA is used to generate a mRNA translation template. (ii) After a brief incubation, translation is halted by addition of Mg^{2+} and incubation at low temperature or addition of translation inhibitors. The ribosome complexes are then screened for function using an immobilized selection motif. (iii) The mRNA is then used (either purified or directly) to construct cDNA. (iv) PCR is then used to generate double-stranded DNA as described above. A, aminoacyl-tRNA-binding site of the ribosome; P, peptidyl-tRNA-binding site.

oligonucleotide synthesis resin. Templates were then generated with open reading frames of 1, 12 and 33 amino acids attached to this standard. The templates were translated *in vitro* using reticulocyte lysate [9], isolated and loaded on denaturing urea polyacrylamide gel electrophoresis to assay fusion formation. In all cases, [^{35}S]-methionine was incorporated, indicating fusion formation with little degradation of the mRNA. Translation of these same templates in wheat germ extract also produced fusions but with an unacceptably high level of mRNA degradation (RW Roberts and JW Szostak, unpublished data), probably because of a higher level of ribonuclease activity. The nature of the peptides incorporated was assayed by removing the RNA portion of the template using RNase A. The mobility of linker–peptide products produced indicated both that the length of template was proportional to the peptide attached, and that the size of the peptides was quite homogeneous. Disulfide-bond chromatography of the fusion products demonstrated that the ribosome read to at least the penultimate codon prior to fusion formation and confirmed the homogeneity of the peptide lengths [10••].

A relatively small percentage (0.2–1.0%) of the input template was converted to mRNA–protein fusion in the initial experiments. While this yield corresponded to synthesis of more than 10^{12} fusions per ml of translation, it presented a technical hurdle for selection experiments due to the high background from unfused templates. Two purification steps (T_{25} [i.e. an oligonucleotide 25 nucleotides long containing all thymidines] agarose to purify all template, followed by disulfide-bond chromatography to purify all fusions) were necessary to purify fusion products prior to the selective step, an immunoprecipitation using an anti-Myc antibody. Following this scheme, the Myc epitope sequence could be enriched from a vast excess of random peptide sequences.

An alternative approach?

A similar scheme for generating readable protein sequences, termed '*in vitro* virus', was also put forward by Yanagawa and coworkers [11]. The construction was markedly similar to that of Roberts and Szostak; a mRNA lacking a stop codon was attached to a DNA linker containing puromycin at the 3′ end. The much longer linker chosen by Yanagawa and co-workers

[11], 132 nucleotides, represents one notable difference between the two systems. When puromycin is omitted from the 3′ end of the linker, the chimeric RNA/DNA template efficiently stalls the ribosome in a puromycin-reactive state, as evinced by incorporation of [^{32}P]-labeled rCP (i.e. a dinucleotide containing a 5′ ribosyl cytosine attached to puromycin) into the translation product. Translation of templates containing puromycin at the 3′ end produces a diffuse, low-mobility band, as assayed on urea polyacrylamide gel electrophoresis, that the authors assign as '*in vitro* virus virion', the putative mRNA–protein fusion; however, recent investigation of the effects of linker length on fusion formation [12••] casts doubt on these results (see discussion below).

Optimization of fusion synthesis

In order to simplify selections using the fusion system, the yield of fusion synthesis needed to be improved. Isolated observations indicated that under certain conditions as much as 50% of the *in vitro* synthesized peptide was converted to fusion. It is now clear through a detailed set of optimization experiments that this level of fusion formation can be routinely achieved, resulting in the majority of the peptide and a significant percentage (≥40%) of the template being converted to mRNA–protein fusion product [12••]. Three main features seem to be important: post-translational addition of Mg^{2+} and/or K^+; post-translational incubation (15–60 min) at room temperature if K^+ and Mg^{2+} are added, or incubation at low temperature (16–48 h) if they are not; and the use of a flexible linker or a standard linker of the correct length [12••]. The optimum linker length for fusion synthesis seems to be between 25 and 30 nucleotides. Linkers shorter than 19 nucleotides and longer than 45 nucleotides show little or no fusion formation. These results argue that the long linker used by Yanagawa and coworkers (132 nucleotides) would be unlikely to result in fusion formation [11].

In vitro selection using a highly complex peptide library

In order to provide a test of the fusion system, a library containing 2×10^{13} different mRNA–peptide fusions was generated and screened for binding to an anti-Myc antibody (P Burgstaller *et al.*, unpublished data). Fusions containing 27 consecutive random codons were screened over six rounds. After five rounds with an average enrichment of more than 200-fold per round, the pool could be immunoprecipiated as efficiently as the wild type Myc epitope alone. Sequence analysis of 116 clones indicated that 113 were unique and contained a consensus very similar to the Myc sequence, while three either contained the exact Myc epitope EQKLISEEDL, or two point mutations that changed isoleucine to valine. Functional analysis of 12 clones confirmed that all bound the anti-Myc antibody with affinities similar to that of the wild type sequence.

Synthesis of mRNA–protein fusions

In addition to the peptides already mentioned, a growing number of proteins have been successfully expressed as mRNA–protein fusions. Among these are lambda protein phosphatase [12••], β-lactamase, the B1 domain of protein G (S Li and RW Roberts, unpublished data), the Z domain of protein A, the green fluorescent protein, and single-chain antibodies (scFvs) (S Hale, L Sun, R Wagner, personal communication). It now appears that any protein that can be made *in vitro* using the reticulocyte lysate translation system can be made as a mRNA–protein fusion, opening protein systems ranging from 1–100 kD to exploration.

Ribosome display: the rudiments of selection

Almost 40 years ago, it was realized that a small fraction of the proteins made within a living cell can be found associated with the ribosome [13]. In an elegant set of experiments it was shown that ribosomes isolated from isopropylthio-beta-D-1-thiogalactopyranoside (IPTG)-induced *Escherichia coli* retained a small but reproducible amount of ribosome-associated β-galactosidase. Through the 1970s and early 1980s, a number of laboratories used these mRNA–ribosome–protein complexes to isolate specific mRNAs (see [14] and references therein). After initial arguments that ribosome display could be used as a vehicle for protein selection [15], Kawasaki [P1] filed a patent on the process, and the proposed selection cycle (Figure 2b) was implemented as a means to generate and screen large peptide libraries [16,17]. Both the *in vitro* translation techniques used to generate ribosome display libraries and the selection techniques involved have recently been reviewed elsewhere [17].

Generation and screening of single-chain antibody libraries

Recently, ribosome display has been extended from application in peptide libraries to explore protein selection *in vitro* using scFvs [18••,19•,20••]. In a heroic set of experiments, Hanes and Plückthun [18••] determined conditions that allowed expression and selection of functional antibody sequences in a bacterial translation extract. Synthesis and screening of libraries in these extracts presents significant technical challenges because of the instability of the mRNA templates. Through optimization of translation time and ribonuclease inhibitors, 0.2% of the input template was recovered as mRNA–ribosome–protein complexes (in principle, allowing libraries containing $\geq 10^{11}$ proteins to be generated). Using this system, it was possible to enrich an anti-hemagglutinin scFv by approximately two orders of magnitude per round of selection. He and Taussig [19•] have reported an even more striking enrichment of 10^4–10^5 per round for scFv fragments expressed in reticulocyte lysate. In both systems, a known antibody was enriched through repeated rounds of selection and amplification.

The Plückthun laboratory followed its initial demonstration by searching for antibodies that target sequences in the yeast transcription factor GCN4 [20••]. They began by constructing an scFv library from three mice, each immunized with a different preparation of the target peptide. The library was screened in two ways, with puzzling implications for future

experimental design. In the first, sterilized milk was used as a blocking reagent to decrease nonspecific complex formation, while in the second it was omitted. Not surprisingly, the majority of clones (75%) isolated from the first selection specifically bound the target sequence, while the second screen yielded only 3/24 functional clones (12%). However, it is the second pool that resulted in the isolation of the best clone, termed g5, that contained a mutation resulting in a dissociation constant value (K_d) of $\sim 4 \times 10^{-11}$ M.

The advantages of *in vitro* selection

The effects of larger libraries

Increasing the library size provides two key advantages: it improves the likelihood that very rare sequences can be isolated; and it increases the diversity of sequences isolated in a given selection. Simply put, functional molecules are likely to be rarer than nonfunctional sequences. This simplistic statement has been put on quantitative footing when considering the general case of protein–ligand interactions. Lancet *et al.* [21] have developed a relationship between library size and the K_d of the best binder in the library that one could expect to find for a randomly chosen ligand. Their analysis predicts the relatively weak affinity ($K_d \sim 10^{-5}$ M) present in the olfactory system and the high affinity binding ($K_d \sim 10^{-9}$ M) seen in immunoglobulin G interactions on this basis. While the absolute binding constant with a given complexity will surely depend on the selection, the general trends should remain intact. In particular, the model predicts that increasing library size 10,000-fold (from 10^8 to 10^{12} sequences) should increase the best binding affinity found by more than 300-fold. The increase in library size will also provide a commensurate increase in the diversity of sequences found with a given K_d. This variety is likely to be an invaluable aid in our understanding of protein recognition and catalysis.

Removal of unwanted selection pressures

Not all sequences express well in bacteria (and phage) or in yeast. Folding, transport, membrane insertion and complexation provide significant *in vivo* selection pressures against certain proteins and scaffolds. These pressures are likely to be significantly reduced *in vitro*, where efficient translation is the only requirement. It is well documented that partially unfolded proteins are rapidly degraded in bacteria [22]. Indeed, careful controls are needed in phage display experiments to ensure that the library has not been degraded during expression and processing to the cell surface [23]. One example of this *in vivo* selection pressure is the poor expression of murine antibodies in bacteria and phage [24]. Netzer and Hartl [25] have argued that eukaryotic proteins may express poorly in bacteria because of differences in the protein synthesis and folding machinery; however, the origins of selection pressure against murine antibodies are not clear, as humanization often results in improved expression [24].

In vitro evolution and recombination

One major limitation of yeast and bacterial selection systems is that they do not readily allow application of powerful *in vitro* mutagenesis [26–28] and recombination techniques once the initial library is constructed [29,30•,31•]; however, both mRNA–protein fusions and ribosome display selections cycles can easily be adapted to incorporate both techniques, via error-prone and sexual PCR (i.e. DNA shuffling), respectively, during the amplification step in every cycle of selection. In addition, the ability to work entirely *in vitro* provides exquisite control to the experimenter to choose the conditions for evolution. Bartel and Szostak [32] have shown how a population of several RNA sequence classes present after the first few rounds of a selection, can change so that new members come to dominate the pool as *in vitro* evolution is applied in later rounds. The change in composition of the library is likely to be caused by competition between domains to acquire beneficial mutations. Some domains that are almost optimized at the beginning of the experiment will be unable to improve, while others, which began as suboptimal, thrive in later rounds due to one or more positive mutations. This affinity maturation may be an inherent characteristic of totally *in vitro* protein selection schemes because of the modest level of mutagenesis present in both reverse transcription and Taq polymerase amplification. Indeed, the anti-GCN4 antibodies isolated by the Plückthun lab (including the best binder) probably resulted from a number of point mutations in progenitor sequences that were either neutral or improved function [20••].

Once a number of beneficial point mutations exist within a pool of homologous structures, these may be tested combinatorially using *in vitro* recombination [30•]. This technique has the added benefit that neutral mutations can be distinguished from beneficial ones by back-crossing with an excess of the wild type sequence. The combination of mutagenesis and recombination techniques provides a powerful strategy to improve protein function. By comparison, application of these methods to libraries present in a living host requires either the use of mutator strains or a laborious protocol involving isolation, manipulation and re-transfection of the library back into the host [33].

Conclusions

The ribosome display and mRNA–protein fusion systems allow the synthesis and screening of peptide and protein libraries with 10^{11} to more than 10^{13} members, respectively, by far the largest currently accessible. Technical improvements, particularly in the fusion system, have opened pathways to some extremely exciting future experiments. First, synthesis and screening of protein libraries containing as many as 10^{15} different sequences should be possible through simple scale-up. Second, libraries constructed from cDNA or genomic fragments would allow all the proteins or domains present in an entire genome to be synthesized as fusions or ribosme complexes. Combination of these complexes with array techniques currently used to examine mRNA levels could provide tools to exhaustively determine a protein's interaction partners *in vitro* [34,35]. Improvements in encapsulation and sorting technologies may provide avenues to isolate multiple turnover catalysts directly [36].

Finally, incorporation of unnatural amino acids [37,38] provides a powerful strategy in the development and dissection of protein-based reagents for recognition and catalysis.

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