Designer proteins in biotechnology

International Titisee Conference on Protein Design at the Crossroads of Biotechnology, Chemistry and Evolution

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Introduction

The structural and functional complexity of proteins as heteropolymeric polypeptide chains is based on diverse interactions between a variety of their hydrophobic, polar and ionizable side chains. These interactions occur in unliganded proteins as well as on association with ligands. This complexity makes it difficult to predict accurately the structure and function of a protein from its primary sequence. Given the vast amount of sequence information produced from the genome projects, as well as the discovery that certain diseases are

due to aberrant protein structures such as amyloid fibrils, there is an increasing need for a better understanding of protein structure and function at the molecular level.

In recent years, progress in predicting protein structure and in the development of new techniques on the basis of evolutionary concepts has led to the design of proteins with improved properties and new functions. The 86th International Titisee Conference highlighted these emerging possibilities. About 60 scientists, working in both basic and applied protein chemistry, discussed new developments from the use of structure-based rational design and *in vitro* evolution strategies. This new research has been developed in many areas, including knowledge-based structure prediction, protein stability, protein–protein and protein–ligand interactions, enzyme catalysis and protein design. In this report, some of the work that was presented is discussed. The selection reflects the author's interests rather than the quality of the talks, which were all excellent.

Protein stability

The three-dimensional structure of a protein is stabilized by hydrogen bonding, van der Waal's interactions, and other polar and non-polar interactions. Although each individual interaction is weak, the sum of several of them can overcome the entropic loss that results from the formation of the structure. Thus, the conformational stability that results from the difference between large enthalpic and entropic values corresponds to the energy of only a few hydrogen bonds.

Whereas this low stability is sufficient for proteins from psychrophilic (cold-adapted) and mesophilic (moderate-temperature-adapted) organisms, proteins from thermophiles and hyperthermophiles (heat-adapted organisms) have evolved much higher conformational stabilities. M.J. Danson (Bath, UK) described an extensive comparison of dimeric citrate synthases derived from organisms that grow optimally at temperatures in the range 10–100 °C. The crystal structures of these proteins are highly homologous. On the basis of these structures, molecular adaptations that increase thermostability could be identified both within and between the subunits. In particular, Danson discussed the formation of inter-subunit ionic networks as an important determinant of stability. Interestingly, the thermostability of citrate synthase was found to be necessary for, but not to guarantee, thermoactivity. That is, reversible subunit dissociation of the

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active dimer precedes irreversible unfolding of the inactive monomers, leading to an optimum temperature of activity that is significantly lower than expected from its thermostability. Consistent with these results, R. Ladenstein (Stockholm, Sweden) reported that glutamate dehydrogenase can be stabilized by introducing ionic networks. His group had shown this by 'transferring' the ionic network observed in the glutamate dehydrogenase of the hyperthermophile *Pyrococcus furiosus* to the less stable enzyme of *Thermotoga maritima*. Whereas single substitutions of either the positively or the negatively charged amino acids of the network destabilized the protein, combining positive and negative mutations led to increased thermostability, in terms of both the half-life of the protein and its melting temperature. However, Ladenstein also noted in his talk that the formation of ionic networks is just one example of a molecular adaptation that leads to thermostability. Statistical and comparative studies of protein structures indicate that an increase in compactness, a decrease in apolar accessible surface area or a decrease in repulsively charged interactions may occur in thermostable proteins. Furthermore, measurements of the temperature dependence of unfolding have shown that the free energy of activation can be a distinct barrier in the case of hyperthermostable proteins, which suggests that the folded state rests in a kinetic trap.

Looking at proteins in another context, J.M. Scholtz (College Station, TX, USA) described the negative effects of electrostatic repulsive forces on protein stability and solubility. The ribonuclease Sa does not have any lysine residues on its surface, and Scholtz showed that introducing a single lysine residue to the surface can increase the stability of the protein, possibly because this disrupts existing electrostatic repulsive interactions. However, further increasing the positive net charge on the surface of ribonuclease Sa, by introducing up to five lysine residues, was also found to decrease the conformational stability of the protein. Even more interesting was the change in the solubility of these variants. The addition of trifluoroethanol has been shown to cause ribonuclease Sa variants to form amyloidogenic fibrils, preferentially at a pH value comparable to the pI of the variant, that is at its lowest solubility. However, in the pH range that is permissible for amyloid formation, the stability of the different variants does not change significantly. Thus, fibril formation does not correlate with protein stability, but rather with low solubility.

Protein stability not only is essential for maintaining protein function *in vitro*, but also can determine the fate of a protein *in vivo*. From a technical point of view, this is important in the production of recombinant proteins. R. Glockshuber (Zürich, Switzerland) has established a new *in vivo* screening system that allows the rapid detection of protein variants with increased thermodynamic stability in the cytoplasm of *Escherichia coli*. The system is based on the simultaneous fusion of the green fluorescent protein (GFP) and the blue fluorescent protein (BFP) to the amino and carboxyl termini, respectively, of the protein (X) to be analysed. Efficient fluorescence resonance energy transfer (FRET) from BFP to GFP in the ternary fusion protein is observed *in vivo* only if protein X is folded. FRET does not occur if BFP and GFP are far apart due to unfolding or intracellular degradation of protein X. This screening system was validated by the identification of novel antibody light-chain variable-domain (V_L) intradomains that have increased thermodynamic stability. The application of this system to various antibody V_L intradomains with different thermodynamic

Table 1 | Overview of combinatorial libraries that couple polypeptides with their encoding genetic sequences (as discussed at the 86th International Titesee Conference)

Library	Proteins displayed	Reference
Messenger RNA display	Decapeptides as ligands for proteins and RNA	Barrick <i>et al.</i> , 2001
Ribosome display	Antibody fragments, new folds	Hanes <i>et al.</i> , 2000 Matsuura <i>et al.</i> , 2002
Bacterial surface display	Cystine-knot proteins	Wentzel <i>et al.</i> , 2001
Phage display	Lipocalins	Beste <i>et al.</i> , 1999; Schlehuber <i>et al.</i> , 2000
	γ-Crystallin	Fiedler & Rudolph, 2002
	Subtilisin	Ness <i>et al.</i> , 2002
	Lipase	Droege <i>et al.</i> , 2003

stabilities revealed a strong correlation between V_L stability and resistance to intracellular proteolysis.

The importance of protein degradation as an intracellular system that allows *E. coli* to cope with stress was highlighted by R.T. Sauer (Cambridge, MA, USA), who described the proteolysis-based regulation of a transcription factor that induces the stress response. This transcription factor is initially inactive due to its binding to a membrane-associated regulatory protein (RseA), and the first step in its activation is the proteolytic cleavage of RseA. The protease that is responsible for this, DegS, is itself membrane anchored. According to structural analyses, DegS is maintained in an inactive state by the binding of its PDZ domain (a domain known to be important for protein–protein interactions) to its protease domain. The protease can be freed either by applying heat stress or by adding peptides that are able to bind to the DegS PDZ domain. Once activated, the protease domain can cleave RseA, which subsequently releases the transcription factor to bring about a full stress response in *E. coli* (Alba *et al.*, 2002). When a recombinant *β-galactosidase* gene was placed under the control of a stress-induced promoter, the protein could be produced according to this mechanism.

Functional analysis and improvement of proteins

The meeting focused mainly on protein design using either knowledge-based or directed evolution techniques, and on assays whereby target proteins can be tested for improved, changed or new activities. Rational protein design has improved in recent years, due to a better understanding and the quantification of the principles that govern protein–protein and protein–ligand interactions. In addition, since the first attempts at *in vitro* protein evolution were made (Hawkins *et al.*, 1992; Stemmer, 1994), several approaches have been established, allowing the generation and selection of protein species that have desired functions from large libraries. These libraries include techniques such as messenger RNA display, ribosomal display, bacterial surface display and phage display (cf. Table 1).

In a simple view of protein function, the active-site residues determine the activity and specificity of an enzyme, whereas the rest of the protein simply allows the specific structure of the active site to be formed. In his analyses of the enzymatic mechanism of dihydrofolate reductase (DHFR), S. Benkovic (University Park, PA, USA) showed that DHFR activity relies on rapid motions in both the active site and the distal parts of the protein, and that the mobile distal regions are highly

conserved in DHFRs from different origins. Even more intriguing was the finding that the motion of distal parts of DHFR are coupled to each other. Thus, a network of long-range interactions coordinates the dynamics of DHFR during its enzymatic cycle.

K.E. Jaeger (Bochum, Germany) gave a report on creating enantioselective enzymes. His group used LimA, a 250-amino-acid lipase that does not select between *R*- and *S*-enantiomers of the substrate, as a starting point for their study. Using a combination of directed-evolution methods, a highly *S*-selective lipase variant containing only six amino-acid substitutions was created (Jaeger *et al.*, 2001). Using other selection conditions, enantioselectivity was reversed, producing a highly *R*-selective variant of the same enzyme. Most of the mutations that were identified were not located in the active site, again highlighting the importance of the distal parts of proteins for enzyme activity and specificity. A more industrial aspect of protein design was discussed by T.V. Borchert (Bagsvaerd, Denmark). For proteins to be used in industrial processes, several biophysical properties often have to be optimized simultaneously; for example, there may be a need for stability and activity at both high temperature and alkaline pH. Borchert reported on the optimization of subtilisins, which are a class of proteases, for five criteria including those mentioned above, using a family shuffling procedure. In this particular DNA shuffling technique, the DNA input consisted of fragments of 26 subtilisin genes from different strains of bacteria. Surprisingly, combining the best parent species, such as variants with the highest stability and the highest activity, often failed to generate an optimally adapted variant. Furthermore, family shuffling starting with natural genes was found to be biased, in that it restricted the number of variants possible in the next generation. As was impressively shown, this limitation can be overcome by using synthetic degenerate oligonucleotides that contain all of the diversity encoded in the parent strains (Ness *et al.*, 2002).

The use of libraries that contain closely related protein variants is not limited to screening for improved enzymatic activity under certain conditions, but also includes the analysis of other protein properties. H. Kolmar (Göttingen, Germany) has used bacterial surface display of the small trypsin inhibitor from *Ecballium elaterium* (EETI) to analyse protein folding. A specific β -turn in the structure of EETI is thought to function as the folding nucleus that directs correct disulphide bond formation in the protein. After complete randomization of the amino-acid sequence of this turn, the resulting EET1 variants were fused to an *E. coli* outer membrane protein, thus allowing their presentation on the bacterial surface (Wentzel *et al.*, 2001). The bacteria were then incubated with a biotinylated form of trypsin, which could bind to structurally intact, and therefore folding-competent, EETI. Using fluorescence-labelled streptavidin, bacteria displaying intact EETI were identified and analysed, and several variants with different sequences in the β -turn were found. Despite the accumulation of amino acids with a general β -turn propensity, no consensus sequence for the β -turn was observed. The structure of the mutated turn did not vary with the different sequences, but the production yield was lower and the folding kinetics were slower in the variants than in the wild-type protein. This example emphasizes the broad range of applications of combinatorial biochemistry that are available to scientific research.

A.G. Beck-Sickinger (Leipzig, Germany) used site-directed mutagenesis and doubly-fluorescence-labelled variants of neuropeptide Y to analyse the interaction of this 36-amino-acid

peptide with its G-protein-associated receptors. The bioactive conformation of neuropeptide Y was elucidated *in vitro* by measuring FRET between the two fluorophores at defined positions in the peptide. In another set of experiments carried out in a cellular system, FRET between fluorophores in receptor fusion proteins proved that G-protein receptors dimerize on binding neuropeptide Y. Finally, Beck-Sickinger described the use of an approach that applies a recently developed GFP-based reporter assay in combination with fluorescence-activated cell sorting to screen for selective agonists of neuropeptide Y signalling. These methods further support the efficiency of fluorescence techniques in the study of protein-protein interactions in biological systems.

A different approach to using sequence libraries and genetic selection was proposed by D. Hilvert (Zürich, Switzerland). Basing their studies on the helical-bundle structure of chorismate mutase, his group established a large set of sequences that encode putative amphiphilic helices consisting of only four hydrophilic and four hydrophobic amino acids. Those sequences that retain the natural amino acids of the active site were then fused, and enzymatically active clones were identified by genetic selection *in vivo*. Using this technique, the minimal requirements for the functional fold of chorismate mutase were identified and the number of sequences compatible with this fold assessed quantitatively. One of the resulting variants showed a rate of catalysis (k_{cat}) only ten times less than that of the original enzyme. These simplified proteins can now be further developed, either by re-introducing amino acids to obtain variants that have higher stability and activity, or by selecting for variants with different activities.

Redesigning the function of a protein

With the techniques available for protein design and directed evolution, the active sites of proteins can be changed such that enzymes with new selectivity or altered specificity are obtained. Restriction enzymes are among the most accurate enzymes known. They recognize short palindromic DNA sequences in a highly redundant manner: only when all contacts to the recognition sequence are formed are the two catalytic centres of the homodimeric enzymes activated and the two strands of the DNA duplex cleaved in a concerted manner. A.M. Pingoud (Giessen, Germany) showed that rational design, as well as directed evolution, make it possible to expand the sequence specificity of *EcoRV* from a six-nucleotide palindrome to one consisting of eight nucleotides. The possibility of creating programmable restriction enzymes—that is, enzymes for which the sequence specificity can be changed by a variable, interchangeable determinant—is now being explored.

In another example of protein redesign, S.L. Mayo (Pasadena, CA, USA) described the conversion of thioredoxin, a catalyst of disulphide reduction, to an esterase. For this reaction, an 'active-site scan' was performed to identify alterations that place catalytically active histidine residues and other mutations that affect substrate binding in favourable positions. The best variants proved to be catalytically active, with k_{cat}/K_M in the order of $200 \text{ M}^{-1} \text{ s}^{-1}$. Although this is comparable to the quality of catalytic antibodies that are designed to perform the same function, the activity is three or four orders of magnitude less than for natural esterases. Mayo suggested that the reason for this discrepancy might be that a static active site was designed, which neglects all of the dynamics that normally occur in an enzyme during the catalytic cycle. This again emphasized that the amino acids that

Fig. 1 | Protein scaffolds that are used to design tailored binding molecules. (A) Crystal structure of the lipocalin-derived anticalin FluA, which was selected against fluorescein as a prescribed hapten (Beste *et al.*, 1999). Fluorescein is bound in the central ligand pocket and shown here with its electron density. Protein residues that form the binding site are shown in green. (Image kindly provided by Arne Skerra.) (B) The maltose binding protein (Protein Database (PDB) entry: 1LLS) was used to engineer a protein able to bind metal ions or small organic molecules in the cleft between the two subdomains (binding site marked by an arrow). (C) Crystal structure of γ -crystallin (PDB entry: 4GCR). One of the β -sheets (marked in red) was randomized to create a new protein surface with specific binding activities. (D) An artificial ankyrin repeat protein was constructed (PDB entry: 1MJ0; Kohl *et al.*, 2003). The variation in the large surface of the loops in these ankyrin repeats should generate customized binding proteins with high affinity for ligands and other proteins.

are distant from the active site and the conformational flexibility of proteins must both be taken into consideration in protein design.

The most common protein fold known is the TIM barrel, represented by the structure of triose phosphate isomerase (TIM), which consists of eight repeats of a $\beta\alpha$ -segment. This fold is present in every class of enzyme, indicating its adaptability to various requirements for substrate binding and turnover. The reason for this flexibility might be that the structural elements that are responsible for conformational stability and those that are involved in catalytic activity are separated in this fold. J. Gerlt (Urbana, IL, USA) and R. Sterner (Cologne, Germany) used site-directed mutagenesis and directed evolution to replace one enzymatic activity of a TIM barrel with another. The striking result was that, in the cases analysed, a single mutation was sufficient to alter the reaction catalysed by the enzymes. These results clearly show the suitability of $\beta\alpha$ -protein topology for the creation of customized enzymes. Interestingly, naturally occurring enzymes often already have a low-level activity in addition to the one that is most biologically relevant, and this is almost certainly the basis of the natural evolution of enzymes. This process is now mimicked by directed-evolution techniques *in vitro*.

Not only the catalytic activity of an enzyme, but also its capacity for binding to other molecules, are amenable to protein redesign. A. Skerra (München, Germany) developed lipocalin

proteins that have altered binding specificities for small molecules. Lipocalins consist of an eight-stranded β -barrel that forms a hydrophobic pocket, inside which their natural ligands, such as retinoic acid or bilirubin, can bind. The opening of this hydrophobic pocket is surrounded by four loops, which were randomized to create new binding specificities (Fig. 1A). Crystallographic analyses of certain variants revealed high levels of plasticity in these loops, suggesting that they could provide different structural backgrounds for the binding of different ligands. One variant that was generated can bind to fluorescein specifically, with a dissociation constant in the low nanomolar range (Beste *et al.*, 1999). A similar binding efficiency for digoxigenin was observed with another variant (Schlehuber *et al.*, 2000). On binding of the ligand, small conformational changes of the protein were detected. This shows the difficulty of producing efficient binding proteins using a rational design approach but, conversely, proves the advantage of directed evolution in certain cases.

In a similar approach, H.W. Hellinga (Durham, NC, USA) used periplasmic binding proteins from *E. coli*, such as the maltose- (Fig. 1B) or ribose-binding proteins, to generate proteins that bind to and 'sense' the presence of metal ions and small organic molecules. Using computational combinatorial design, he

re-designed the natural binding sites of the parent proteins. A special feature of these periplasmic binding proteins is a characteristic bending of the hinge between the two subdomains on ligand binding. Coupling this movement to a change in an electrochemical or fluorescence signal made it possible to monitor the binding of a ligand, thus making it feasible to construct biosensors for zinc ions and for several low-molecular-mass ligands (Benson *et al.*, 2001; de Lorimier *et al.*, 2002). The affinities of these biosensors were in the nanomolar and lower micromolar range.

De novo design of proteins and ligands

Novel binding activities can also be generated from proteins and peptides that do not have any related binding ability. In this case, rational design may be difficult. However, a combinatorial approach coupled with directed evolution is perfectly suited to the creation of a new activity from a protein or peptide scaffold. R. Roberts (Pasadena, CA, USA) took advantage of such a technique, using mRNA display to screen for peptides that bind to the penicillin-binding protein PBP2A. This protein is responsible for the resistance of certain *Staphylococcus* strains to penicillin, and a competitive inhibitor would re-establish the antibiotic effect of penicillin against these bacteria. A library of decapeptides was synthesized by *in vitro* translation and from this, several peptides that bind to PBP2A with high affinity were isolated. In similar experiments, peptides that bind specifically to an RNA hairpin structure were also identified (Barrick *et al.*, 2001).

H. Lilie (Halle, Germany) described the use of γ -crystallin as a scaffold for designing artificial binding proteins. γ -Crystallin is a structural protein of the eye lens that has no catalytic or binding activities (Fig. 1C). To generate an artificial binding activity, eight solvent-accessible residues of a three-stranded β -sheet were randomized. The resulting library was used to select protein species that bind either small molecules, such as oestradiol, or proteins, such as growth hormones and immunoglobulins. In all cases, γ -crystallin variants that bind their respective ligands with affinities in the nanomolar or lower micromolar range were isolated. In future studies, the γ -crystallin library could be used to isolate tumour-specific binding molecules that might be used as fusion partners of therapeutic molecules or vector systems, in order to deliver them to their targets.

The design of a whole protein with a pre-defined function was reported by W. Haehnel (Freiburg, Germany). He described a successful *de novo* design approach for the generation of a four-helix-bundle protein. Previously, such designs have led to protein structures with characteristics that are reminiscent of molten globules, indicating that helix formation was achieved but the interaction between the secondary structural elements was different from that in native proteins. Using combinatorial peptide synthesis and computational rotamer libraries, which are databases that contain all possible side-chain conformations of the amino acids, Haehnel calculated the optimal packing density of the hydrophobic core of the protein. The peptides were bound to a specific template that imposed an ordered interaction of the four helices, and thus led to the formation of a cooperatively stabilized four-helix bundle. With histidine and cysteine residues at defined positions, these proteins were able to bind copper and to ligate haem groups that display redox activity, respectively.

Taking this type of approach a step further, A. Plückthun (Zürich, Switzerland) reported on the first attempts to create new functional

proteins without any structural precedent, based on the linking of cassettes encoding secondary structures. Eventually, such a design may help to address some fundamental questions about why known genomes encode so few distinct protein folds. Recently, Plückthun's group has developed a method for selecting proteins that have desired characteristics using ribosome display. Using this technique, synthetic libraries of either ankyrin repeats (Kohl *et al.*, 2003; Fig. 1D) or leucine-rich repeats were screened for specific binding to targets such as the kanamycin-resistance protein. In this new approach for isolating unique protein folds, the selection pressure used in the ribosome display is based on folding properties such as compactness and non-aggregation (Matsuura *et al.*, 2002). Applying these selection criteria led to the enrichment of species with properties that are characteristic of folded proteins. This approach challenges the molecular principles that govern protein structure and folding.

Conclusions

The 86th International Titisee Conference on Protein Design at the Crossroads of Biotechnology, Chemistry and Evolution highlighted new methods and trends in analysing protein function and in designing proteins with specifically customized qualities. The improvement of computational approaches to analysis of protein structure, protein-protein interactions and protein-ligand interactions, based on the findings that were discussed at the meeting, will be at the heart of a new generation of proteins with novel designed functions. The intriguing field of directed evolution is now firmly established in protein chemistry, and is giving rise to exciting applications, making possible the construction of proteins that are adapted specifically for use as research tools and for biotechnological exploitability. However, it also became clear at the meeting that a great deal of work still needs to be done, both in basic research and industrial development, to make designer proteins a common tool. In this respect, it is significant that the 86th Titisee Conference provided an overview of the current status of protein design and allowed an exchange of ideas and strategies between representatives of the basic research and biotechnology communities.

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