Engineered proteins as specific binding reagents
H Kaspar Binz and Andreas Plückthun

Over the past 30 years, monoclonal antibodies have become the standard binding proteins and currently find applications in research, diagnostics and therapy. Yet, monoclonal antibodies now face strong competition from synthetic antibody libraries in combination with powerful library selection technologies. More recently, an increased understanding of other natural binding proteins together with advances in protein engineering, selection and evolution technologies has also triggered the exploration of numerous other protein architectures for the generation of designed binding molecules. Valuable protein-binding scaffolds have been obtained and represent promising alternatives to antibodies for biotechnological and, potentially, clinical applications.

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Current Opinion in Biotechnology 2005, 16:459–469

This review comes from a themed issue on Protein technologies and commercial enzymes
Edited by Bernhard Hauer and Brian K Kay

Available online 6th July 2005
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DOI 10.1016/j.copbio.2005.06.005

Introduction
Recombinant and engineered binding proteins have become powerful tools for therapy, in vivo and in vitro diagnostics, drug target analysis and laboratory research. Each of these applications has specific requirements for the binding molecule. These requirements translate to molecular criteria, such as the necessary target affinity and specificity, required protein size, thermodynamic and chemical protein stability, stability in serum, the presence or absence of disulfide bonds, protein domain composition, the presence or absence of post-translational modifications, concerns about immunogenicity, protein expression levels, solubility, and the presence of effector functions or moieties for labeling. Additionally, criteria such as manufacturing cost, shelf-life and intellectual property restrictions can determine whether potential binding molecules will become widely used.

For many applications, antibodies have traditionally been used. Almost all scientific, diagnostic and therapeutic applications require high specificity and a defined molecular composition, thus usually precluding the use of polyclonal antibodies. Monoclonal antibodies, by contrast, are expensive to manufacture, and for all uses except a fraction of therapeutic applications their Fe region is not really required. Antibody fragments in the form of single-chain Fv, Fab and multivalent fragments [1], which can be obtained from synthetic libraries [2] or recombinant libraries from B cells [3], have become important alternatives. They can be generated with greater control of specificity and can be manufactured relatively inexpensively in bacteria.

However, for several applications, such antibody fragments might not be ideal. For example, the stability of recombinant antibodies and antibody fragments relies on disulfide bonds and, despite significant progress [4], intracellular expression in the reducing milieu is only possible for a subset of antibodies [5]. High stability is also pivotal in other applications: for affinity chromatography, stability of the immobilized affinity ligand to very harsh cleaning conditions is essential, as are very low manufacturing costs. Finally, even for therapeutic uses, novel concepts may require fusion proteins and conjugates that would be much easier to manufacture with scaffolds other than antibodies: some antibody fusions are prone to aggregation, while a single cysteine residue that is convenient for conjugation is not as easily handled in a protein with disulfide bonds (such as an antibody fragment) as it would be in a protein without any other cysteine.

Advances in protein engineering and the availability of powerful library selection technologies have allowed the exploration of numerous alternative protein scaffolds for the generation of designed binding molecules throughout the past decade. In essence, the technologies first developed for antibody libraries to recreate the function of the immune system were extended to other protein scaffolds. In the 1990s, affinity maturation or changes of specificity of protease inhibitors using rational engineering and phage display provided the first examples of the use of scaffolds other than antibodies for selecting specific binders (see below). With increasing knowledge about protein–protein interactions, better understanding of protein engineering and the further development of selection technologies, several protein-binding scaffolds have now been explored and found suitable for binding virtually any protein target of choice. In these scaffolds, parts of the surface (typically loops, more rarely the exposed surface of α helices or β sheets) or a ligand-binding cleft of a protein framework are randomized to yield a protein library, which can then be selected towards new functions. In the beginning,
well-studied proteins that are naturally involved in protein–protein interactions were primarily chosen as scaffolds for library generation (Figure 1). The wealth of genomic sequences emerging towards the end of the last century, however, triggered the use of several other protein architectures that were revealed to be frequently used in nature for diverse protein–protein interactions.

We give here an overview of the protein scaffolds that have been used as protein-binding alternatives to antibodies (Table 1). For the sake of clarity, we classify the different protein scaffolds in different groups (Figure 1). It should be noted that this classification is not absolute: scaffolds of one group often share features with scaffolds of other groups. Owing to length restrictions, we will focus on the most recent advances and publications in the field; older studies are mentioned for completeness only if they are not referenced by the more recent studies. For earlier references, the reader is directed to earlier reviews [6–8].

**β-Sandwich and β-barrel proteins**

In antibody variable domains, binding diversity is provided by variation of length and sequence in three loops that connect the strands of the immunoglobulin domain possessing a β-sandwich topology. Many attempts have been made to recreate this concept by using other proteins with β-sandwich or β-barrel topology as the recipients of the diversified loops. Tendamistat [9], fibronectin [10,11†], cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) [12], T-cell receptors [13,14‡] and neo- carzinostatin [15] are examples of β-sandwich scaffolds. CTLA-4, tendamistat, T-cell receptors and neo- carzinostatin all contain disulfide bonds, and hence their use is essentially restricted to applications where antibodies are typically used (Table 1).

In the case of T-cell receptors, the aim was to generate specific binders for peptide–MHC complexes with improved affinity and/or stability, rather than to use this scaffold as a source for general protein binding ligands. Libraries of T-cell receptor mutants have successfully been used in combination with yeast surface display and fluorescence-activated cell sorting [13]. In several cases, the T-cell receptors were displayed as single-chain variants and yielded nanomolar affinity binders. For a long time, the display of T-cell receptors on bacteriophage was...
considered impossible, or at least very inefficient. However, Li et al. [14**] recently published a study, where T-cell receptors could efficiently be displayed on bacteriophage and peptide–MHC binders could be selected with affinities in the picomolar range. The display-enabling trick was to use a stabilizing nonnative interchain disulfide bond in the constant domains.

Li et al. [9] used the α-amylase inhibitor tendamistat in selections against different integrins. First, a loop library was inserted in a loop connecting two β strands of tendamistat and selected against α,β3 integrin. The resulting sequences, mostly containing the known integrin-binding RGD motif, provided the basis for the construction of two further libraries that contained the RGD motif and seven flanking randomized positions. These two libraries were used in selections against different integrins. The selected sequences gave insight into the preferences of the different integrins for particular domains. Among the β-sandwich scaffolds, neocarzinostatin is the most recently explored scaffold. It consists of 113 amino acids and has a bound chromophore. A lysozyme-binding neocarzinostatin variant has been engineered, proving the potential of this scaffold to adopt new binding

### Table 1

Scaffolds used for the generation of protein binders.

<table>
<thead>
<tr>
<th>Scaffold Namea</th>
<th>Fold</th>
<th>Domain size (amino acids)</th>
<th>Cysteines/S–S bondsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA-4</td>
<td>β Sandwich</td>
<td>136</td>
<td>Yes/yes (2)</td>
</tr>
<tr>
<td>Tendamistat</td>
<td>β Sandwich</td>
<td>74</td>
<td>Yes/yes (2)</td>
</tr>
<tr>
<td>10FN3</td>
<td>β Sandwich</td>
<td>94</td>
<td>No/no</td>
</tr>
<tr>
<td>Neocarzinostatin</td>
<td>β Sandwich</td>
<td>113</td>
<td>Yes/yes (2)</td>
</tr>
<tr>
<td>CBM4-2</td>
<td>β Sandwich</td>
<td>168</td>
<td>No/no</td>
</tr>
<tr>
<td>T-cell receptor</td>
<td>β Sandwich</td>
<td>~250</td>
<td>Yes/yes (2)</td>
</tr>
<tr>
<td>Lipocalins</td>
<td>β Barrel</td>
<td>160–180</td>
<td>Yes/yes (0–3)</td>
</tr>
<tr>
<td>Protein A domain</td>
<td>α3</td>
<td>58</td>
<td>No/no</td>
</tr>
<tr>
<td>Im9</td>
<td>α4</td>
<td>86</td>
<td>No/no</td>
</tr>
<tr>
<td>Designed AR proteins</td>
<td>α/β2 Repeated</td>
<td>Variable (67 + n-33)c</td>
<td>No/no</td>
</tr>
<tr>
<td>Designed TPR proteins</td>
<td>α3 Repeated</td>
<td>Variable (18 + n-34)c</td>
<td>No/no</td>
</tr>
<tr>
<td>Zinc finger</td>
<td>α/β (Zn+)</td>
<td>26</td>
<td>Yes/no</td>
</tr>
<tr>
<td>pVIII</td>
<td>Mainly α</td>
<td>50</td>
<td>No/no</td>
</tr>
<tr>
<td>GGNN</td>
<td>α</td>
<td>33</td>
<td>No/no</td>
</tr>
<tr>
<td>WW domain</td>
<td>β3</td>
<td>52 (WW motif 38)</td>
<td>No/no</td>
</tr>
<tr>
<td>SH3 domains</td>
<td>Mainly β</td>
<td>~60</td>
<td>Varying</td>
</tr>
<tr>
<td>SH2 domains</td>
<td>α/β</td>
<td>~100</td>
<td>Varying</td>
</tr>
<tr>
<td>PDZ domains</td>
<td>α/β</td>
<td>~100</td>
<td>Varying</td>
</tr>
<tr>
<td>TEM-1 β-lactamase</td>
<td>α/β</td>
<td>265</td>
<td>Yes/yes (1)</td>
</tr>
<tr>
<td>Green fluorescent protein</td>
<td>β Barrel</td>
<td>238</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>α/β</td>
<td>108</td>
<td>Yes/yes (1)</td>
</tr>
<tr>
<td>Staphylococcal nuclease</td>
<td>α/β</td>
<td>149</td>
<td>No/no</td>
</tr>
<tr>
<td>PHD finger</td>
<td>β/Loops</td>
<td>50–100</td>
<td>Yes/no</td>
</tr>
<tr>
<td>C2</td>
<td>α/β</td>
<td>64</td>
<td>No/no</td>
</tr>
<tr>
<td>BPTI</td>
<td>α/β</td>
<td>58</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>APPi</td>
<td>α/β</td>
<td>58</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>hPSTI</td>
<td>α/β/Loops</td>
<td>56</td>
<td>Yes/yes (2)</td>
</tr>
<tr>
<td>Ecotin</td>
<td>β Sandwich</td>
<td>142</td>
<td>Yes/yes (1)</td>
</tr>
<tr>
<td>LACI-D1</td>
<td>α/β</td>
<td>58</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>LDTI</td>
<td>α/β</td>
<td>46</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>MTI II</td>
<td>α/β4</td>
<td>63</td>
<td>Yes/yes (4)c</td>
</tr>
<tr>
<td>Scorpion toxins</td>
<td>α/β3</td>
<td>25–40</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>Insect defensin A</td>
<td>α/β6</td>
<td>29</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>EETI II</td>
<td>Loops</td>
<td>28</td>
<td>Yes/yes (3)</td>
</tr>
<tr>
<td>CBD</td>
<td>β3</td>
<td>36</td>
<td>Yes/yes (2)</td>
</tr>
</tbody>
</table>

a Abbreviations: APPI, Alzheimer’s amyloid β-protein precursor inhibitor; AR, ankyrin repeat; BPTI, bovine pancreatic trypsin inhibitor; CBD, cellulose-binding domain; CBM4-2, carbohydrate-binding module 4 of family 2 of xylanase from Rhodothermus marinus; C2, chymotrypsin inhibitor 2; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; EETI II, Ebculium elaterium trypsin inhibitor II; 10FN3, tenth fibronectin type 3 domain; HPSTI, human pancreatic secretory trypsin inhibitor; Im9, immunity protein 9; LACI-D1, human lipoprotein-associated coagulation inhibitor domain 1; LDTI, leech-derived trypsin inhibitor; MTI II, mustard trypsin inhibitor 2; PDZ, domain present in the three proteins, post-synaptic density protein PSD-95, Drosophila Discs-Large septate junction protein, and epithelial tight-junction protein ZO-1; PHD finger, plant homeodomain finger protein; pVIII, protein VIII of filamentous bacteriophage; SH2, src homology domain 2; SH3, src homology domain 3; TPR, tetratricopeptide repeat.

b The presence of cysteine residues and disulfide bonds is indicated as well as the number of disulfide bonds.

c The variable n denotes the number of consensus repeat modules in the molecule. The total length is that of the capping modules plus the consensus modules.

d Predicted by homology.
specificities [15]. In this variant, a CDR3 (complementarity determining region 3) loop of a VHH camel antibody domain was grafted at the place of an equivalent loop in neocarzinostatin. At 15 °C the engineered variant had an affinity to lysozyme of 0.5 μM, compared with 20 nM for the loop-donor VHH domain. The neocarzinostatin variant was well expressed with 30–35 mg protein per liter shake-flask culture, but a decrease in stability to 3 kcal/mol compared with 8.8 kcal/mol for neocarzinostatin was also observed. The phage-display selection of testosterone-binding variants from a library of neocarzinostatin, randomized in the chromophore-binding region, showed that this scaffold is also suited for the binding of small molecules [16].

In contrast to the other β-sandwich proteins, fibronectin (Figure 1) does not rely on disulfide bonds and hence might extend the range of uses of antibodies. The tenth domain of type 3 fibronectin (also named 10FN3, FNfn10, trinec, monobody or adnectin) [10,11] is one of the best-characterized scaffolds of this type. This 94 amino acid protein is well expressed in soluble form in bacteria and is thermodynamically stable. Fibronectins with a novel binding specificity to ubiquitin could be generated with an affinity in the micromolar range from a library with two randomized loops using five rounds of phage display [17]. With a similar library, binders to the Src SH3 domain with micromolar affinities were recently selected [11*]. Clones with the typical SH3 domain 1 binding motif PXXP (in single-letter amino acid code, where X is any amino acid) were selected, as well as a sequence containing no PXXP motif. These fibronectins could be used in both western blotting and ‘immuno’-precipitation experiments. In another approach with a slightly different and much more diverse library, binders in the nanomolar range were reported after nine selection rounds of messenger RNA display against tumor necrosis factor α (TNFα) [10]. From these nanomolar binders, picomolar binders could be evolved with further affinity maturation steps [10]. Fibronectin was also successfully used in a yeast two-hybrid approach, indicating that the framework could be interesting for intracellular applications [18].

Lipocalins comprise 160–180 amino acids and form conical β-barrel proteins with a ligand-binding pocket surrounded by four loops. Small hydrophobic compounds are the natural ligands of lipocalins, and different lipocalin variants with new compound specificities (also termed ‘anticalins’) could be isolated after randomizing residues in this binding pocket [19*]. The analogy of their loops to antibody CDRs is an indication that lipocalins might also be used as a source for protein binders. By randomizing these loops and selecting hemoglobin-binding lipocalin variants with micromolar affinities, Vogt and Skerra [20] recently showed that protein binding is indeed possible. More recently, preliminary data on a nanomolar affinity CTLA-4-binding lipocalin variant have been reported [19*], giving first indications that specific, high-affinity protein-binding anticalins can be generated. Lipocalins are usually disulfide-bonded scaffolds and could therefore be an alternative in those applications where recombinant disulfide-containing antibody fragments can also be used. The fatty-acid-binding protein (FABP), also a member of the lipocalin family, has been used as the carrier of an N-terminal peptide library by Lamla and Erdmann [21]. A library consisting of 15 random amino acids was used in ribosome display selections against streptavidin. In seven phage display selection cycles, a peptide–FABP fusion was isolated that had an affinity to streptavidin of 4 nM.

The carbohydrate-binding module CBM4-2 of a bacterial xylanase also has a β-sandwich-like architecture and has been used as a binding protein with novel specificity [22]. Similarly to fibronectin, CBM4-2 does not contain disulfide bonds, is thermodynamically stable and can be expressed at high levels in Escherichia coli. However, unlike the other β-sandwich and β-barrel proteins discussed, where loops were randomized in analogy to antibody loops, CBM4-2 was randomized in the carbohydrate-binding β-sheet surface. A phage display library with a diversity of 1.6 × 10^6 was used to select binders against different carbohydrates as well as against glycosylated human immunoglobulin G4 [22].

**Protein Z and α-helical scaffolds**

One of the first scaffolds investigated that did not belong to the β-barrel or β-sandwich family was protein Z (also named affibody; Figure 1), an engineered domain B of staphylococcal protein A (SpA) [23]. This 58 amino acid three α-helical bundle protein is rather stable (ΔG = 6.6 kcal/mol) and well-expressed in soluble form in E. coli. For protein Z, it was not the loops connecting the secondary structure elements that were randomized, but rather 13 residues on the surface of two α helices. These residues are naturally involved in binding the Fc part of antibodies. In recent years, libraries of protein Z variants have been used to generate binders against at least eight different targets by phage display. Usually, specific binders with micromolar affinities were rapidly obtained. Some of these binders could be evolved to nanomolar binders by a second randomization, followed by further phage display selection rounds. An affibody selected against human CD28 was shown to block the interaction between CD28 and CD80, hence being a therapeutic candidate [24]. Similarly, Wikman et al. [25*] selected protein Z variants that bound to the breast cancer target Her2 with nanomolar affinity, which were also active on Her2-expressing cells. The variant with the highest affinity does not bind to the same site as trastuzumab (Herceptin), which is clinically used in the therapy of breast cancer; nevertheless, these molecules could represent interesting candidates for the development of therapeutic and diagnostic agents.
Particularly interesting are the crystal and nuclear magnetic resonance (NMR) structures of the complex between an affibody and its target, the original protein Z (Protein Data Bank [PDB] IDs 1LP1 and 1H0T; Figure 2) [26**,27**]. The studies reveal the details of the selected interaction and show that most of the randomized surface of the ‘anti-idiotypic’ affibody was involved in the interaction, with a $K_D$ of 6 μM. NMR studies further revealed that this particular affibody seems to be a molten globule that folds only upon binding to its target, protein Z [26**,28,29], thus possibly limiting the observed overall affinity by an entropy loss upon folding.

The bacterial nuclease inhibitors Im7 and Im9, naturally made by colicin-producing strains to protect themselves, are also α-helical proteins that could be used as alternative binding or inhibiting proteins. Indeed, by combining error-prone polymerase chain reaction (PCR) with an in vitro compartmentalization selection procedure, Bernath et al. [30] evolved Im9, the inhibitor of colicin E9, into a colicin E7 nuclease inhibitor that showed some features of Im7, the natural inhibitor of colicin E7 that is homologous to Im9. Besides the generation of nuclease inhibitors with new specificities, these well-characterized immunity protein scaffolds could also be considered for the generation of binding molecules with new binding specificities.

Repeat proteins

With the increasing availability of genomic sequencing data, it became obvious that nature has evolved repeat proteins as another important class of binding molecules, next to antibodies [31]. Ankyrin repeat (AR), armadillo repeat (ARM), leucine-rich repeat (LRR) and tetratricopeptide repeat (TPR) proteins are the most prominent members of this protein class (Figure 3). Repeat proteins are composed of homologous structural units (repeats) that stack to form elongated domains [31]. The binding interaction is usually mediated by several adjacent repeats, leading to large target interaction surfaces (Figure 3).

AR protein libraries have been used for the generation of binding molecules [32**]. In this case, the chosen

![Figure 2](https://www.sciencedirect.com/current-opinion-in-biotechnology/current-opinion-in-biotechnology.2005.16.459-469)
Natural repeat proteins binding their target proteins or peptides. This illustration shows the variety of interactions repeat proteins can perform and, thus, the potential of repeat proteins as alternatives to antibodies. The repeat proteins are depicted as a black ribbon, while the target proteins or peptides are shown in a grey surface representation. Four repeat proteins were used to prepare this figure using MolMol [80]: the ankyrin repeat (AR) protein mouse guanosine-adenosine rich repeat binding protein β1 (GABP) binding to the α subunit (PDB ID, 1AWC); the porcine leucine-rich repeat (LRR) protein ribonuclease inhibitor binding bovine ribonuclease A (PDB ID, 1DFJ); the tetratricopeptide repeat (TPR) protein region of human Pex5 binding the peroxisomal targeting signal peptide PTS1 (PDB ID, 1FCH); and the mouse armadillo repeat (ARM) protein importin-α binding the nuclear localization peptide of the Xenopus laevis N1N2 phosphoprotein (PDB ID, 1P1N).

Peptide-binding scaffolds

Many protein chip applications require peptide-binding reagents. Besides antibodies, several natural peptide-binding scaffolds have successfully been used for the generation of binders to peptides. Yet, most of these recognize only very short motifs and typically show only micro- to nanomolar affinities. These domains are all involved in cellular signaling and include SH3 [39–41], SH2 [42], PDZ [43,44,45] and WW [46] domains (Table 1). These proteins usually recognize peptides in a specific context: SH3 domains bind peptides that have a polyproline II helix conformation and usually contain a proline-rich motif; PDZ domains (Figure 1) typically bind C-terminal peptides and thus recognize the terminal COO− group; and SH2 domains are usually involved in binding of phosphorylated peptides. The example of PDZ variants, which can be used in western blotting, ‘immuno’-precipitation and affinity chromatography [45**], shows the power of these peptide-binding proteins. Nevertheless, the applicability of these scaffolds is restricted to specific peptides close to the sequence they naturally recognize, leaving room for the development of domains that can be generically used for high-affinity peptide binding. Two such scaffolds could be TPR or ARM proteins (Figure 3).
Scaffolds presenting constrained peptides

The proteins mentioned so far were designed (with a few exceptions) to bind the target with more than one loop and sometimes with a surface provided by the domain fold. With a few exceptions [26, 27, 32, 36, 47, 48], no crystal structures of selected complexes exist and it is therefore possible that in some cases the selected loops bind the target merely as constrained peptides. In this section, we will summarize the cases where this is the intended mode of binding. We will not discuss the many constrained peptide libraries that were used directly in phage or ribosome display or cases in which the peptide is typically restricted in conformation by a disulfide bond.

In several approaches peptides were inserted in constrained loop regions of other proteins. Fibronectin, green fluorescent protein (GFP) and lipocalins have been used to display constrained peptides, either as a loop insertion or as C-terminal fusions. Thioredoxin A (TrxA; Figure 1) [49] and staphylococcal nuclease [50] are two early examples of the display of constrained peptides. The main aim of these approaches is to protect the peptides from proteolytic degradation and/or to constrain their conformation [51]. In this manner, the integrity and thus the functional diversity of the peptide library is maintained, and the conformational restriction might allow the achievement of higher affinities. In a comparative study, Klevenz et al. [52] inserted two different peptides in TrxA, staphylococcal nuclease and GFP. While one peptide interacted with its target independently of the scaffold, the other peptide was only able to interact within the TrxA scaffold context, as revealed by yeast two-hybrid and glutathione S transferase (GST) precipitation experiments.

Recently, the cysteine-rich plant homeodomain (PHD) finger domain of the transcriptional cofactor Mi2β (second domain; Mi2β-P2) was investigated as a scaffold for the generation of novel binding molecules [53]. This PHD finger domain is stabilized by two zinc ions, which are complexed by seven cysteines and one histidine residue. Sequence alignments of different PHD domains and NMR analyses revealed that two loops (loops 1 and 3) are highly flexible both in terms of sequence and structural plasticity, suggesting that these loops could bear altered sequences. This loop-alteration tolerance was confirmed by mutagenesis and sequence insertion. A Mi2β-P2 variant with a PVDLS sequence inserted in loop 3 was made, creating a folded domain with affinity for the transcriptional corepressor CtBP2. This construct could efficiently be used in GST ‘immuno’-precipitation experiments and in yeast two-hybrid experiments, the intracellular applicability of this scaffold was demonstrated.

Small scaffolds

Another way to circumvent the loss of entropy upon binding an unfolded flexible peptide to a target is to present the peptide in a conformationally frozen form. The introduction of a disulfide bond is often used to restrict the conformational flexibility of peptides. Another possibility is to use peptides that adopt a rigid conformation on their own. As in earlier approaches, where small domains such as zinc-finger domains [54], coiled-coil peptides or single helices [55] and pVIII of filamentous bacteriophage (Figure 1; Table 1) [56] were used to present conformationally uniform peptide libraries, Sia and Kim [57] used the GCN4 leucine-zipper for the rational construction of human immunodeficiency virus 1 (HIV-1) inhibitors with nanomolar affinity. They grafted 19 amino acids from a helical peptide derived from gp41 of HIV-1 onto GCN4, leading to a 34 amino acid peptide that can inhibit the HIV-1 envelope-mediated membrane fusion with IC50 (inhibition constant) values in the nanomolar range.

Scaffolds with intrinsic detection means

Protein chip applications, enzyme-linked immunosorbent assays (ELISAs) or localization studies require the binding of the target polypeptide to be easily detected. Traditionally, radioactive or fluorescently labeled detection agents, detectable fusion proteins, or strategies involving secondary detection reagents are used for this purpose. An alternative approach is to use binding proteins with intrinsic detection means, such as an enzymatic activity or fluorescence (Figure 1). GFP [58-61] and β-lactamase [62] are the most thoroughly tested examples. The β-barrel protein GFP was used both for library insertions in loops connecting the β strands [60, 61] and as an N-terminal fusion protein for random peptide libraries [58, 59]. In the latter, more recent approaches, individual library members could successfully be screened for either cellular localization (3.1% to 4.8% of library members showed some localization tendencies) or mediation of cell cycle arrest.

β-Lactamase (Figure 1) variants with new binding specificities have been isolated from libraries where one or two loops were randomized [62]. Altogether, seven different libraries were constructed and tested. Using phage display, binders could be isolated against monoclonal antibodies, streptavidin or ferritin. After affinity maturation, ferritin binders with low nanomolar affinities were isolated [62]. For some binders, the target interaction did indeed modulate the enzymatic activity. β-Lactamase therefore appears to be a sensitive detection probe.

Protease inhibitors

Owing to their importance in blood clotting and many other pharmaceutically relevant processes, protease inhibitors were among the first scaffolds to be chosen for protein engineering (Table 1). So far, protease inhibitors have always been adapted to novel protease targets and affinity and specificity can usually be improved. Also, the high affinity translated to extremely high inhibition
also used in loop grafting studies with loops of de
and selection, the charybdotoxin and other scaffolds were
binding specificities via surface residue randomization
and selection, the charybdotoxin and other scaffolds were
also used in loop grafting studies with loops of defined
sequence. In an extension of preceding work, a scylla-
toxin variant carrying a CD4 loop in its β-hairpin was
optimized such that the affinity and inhibitory effect of
the chimera equaled the potency of CD4, and an inhibi-
tory effect of one designed variant on HIV-1 infection
was shown in cell culture [72*]. A similar study has been
performed with charybdotoxin [71]. As these CD4
mimetic proteins induce a conformational change in
the HIV-1 protein gp120, leading to exposure of cryptic
antigen parts, they were suggested as vaccine candidates,
similar to what has been suggested for different α-con-
toxin variants [74,79]. Different applications have also
been reported for EETI-II. In addition to being used as a
Sendai virus epitope carrier, EETI-II was used as a
scaffold for the presentation of constrained peptides for
selections against the parental target, bovine trypsin [78].
In the case of the insect defensin A [77], a phage library
with a diversity of 3 × 10^8 members presenting con-
strained randomized peptides (seven amino acids) in
defensin A, was prepared. This library was used in selec-
tions against TNFα, two TNF receptors and a mono-
clonal antibody, and phage enrichments could be
observed for all four targets.

Conclusions
Well over 30 different protein scaffolds have been inves-
tigated as alternatives to antibodies. These proteins are of
different topologies and folds and different structural
elements mediate the target interactions, offering a large
set of options. Proteins selected from libraries of such
scaffolds can be used in manifold applications such as
affinity chromatography, western blotting, tissue staining,
and diagnostic applications. Some can also be used as
intracellular inhibitors in target discovery and validation,
as well as potentially in therapy. Unpublished work on,
for example, γ-crystallins and ubiquitin (http://www.
siclproteins.de), transferrin (http://www.biorexis.com),
C-type lectin-like domains (http://www.borean.dk) and
low-density lipoprotein receptor domain A (http://www.
avidia.com) shows that the field of alternatives to anti-
bodies is still dynamically developing. Particularly for
peptide binding, generically applicable scaffolds are still
sought. ARM and TPR proteins could represent solutions
to this problem. To reach a state of maturity comparable
to recombinant antibodies, where a wealth of data on the
structure of antibody–antigen complexes, biophysical
properties and both natural and biosynthetic affinity
maturation strategies have helped to shape both libraries
and selection technologies, similar studies will have to be
carried out with alternative binding molecules. X-ray
crystallography, NMR experiments or biophysical ana-
lyses have only been performed for a very limited number
of synthetic binding molecules; however, with the first
examples of atomic coordinates of binding molecules in
complex with their protein targets, detailed insight of the
mode of interaction of three scaffolds was gained
[26**,27**,32**,36**,47,48]. This might stimulate future
Tanaka et al. [63] selected high-affinity LDTI-based
thrombin binders and inhibitors in two rounds of phage
display. Inhibition was restricted to thrombin and trypsin,
while factor Xa, plasma kallikrein and neutrophil elastase
were not inhibited. This study was further extended [64],
and highly specific inhibitors to plasmin and neutrophil
elastase were selected. To improve plant defense against
aphids (soft-bodied insects), MTI II was subjected to
phage display selection against trypsin and chymotrypsin
[63,66]. Picomolar (trypsin) and nanomolar (chymotryp-
sin) inhibitors were obtained in four selection rounds.
The authors suggest that such MTI II variants could be
incorporated in transgenic crops to increase resistance
against sucking insect pests.

The periplasmic E. coli protease inhibitor ecotin (Figure 1)
was selected to bind urokinase-type plasmino-
gen activator (uPA) in several different approaches. The
knowledge gained from all these experiments was used to
select picomolar uPA inhibitors by combining phage
display and rational design [68]. The use of ecotin was
recently investigated in even more detail [67*] and inhibi-
tors against several proteases (plasma kallikrein, mem-
brane-type serine protease 1 [MT-SP1] and factor XIIa)
were selected from combinatorial ecotin libraries with up
to 20 randomized amino acids, using six to seven rounds of
phage display. The most potent inhibitor had an affinity
of 11 pM to plasma kallikrein. Competition with soluble
proteases of undesired specificity was used in phage
display selections to increase the inhibitor specificity.

Small disulfide-bonded scaffolds
Small disulfide-bonded proteins usually exhibit a high
thermodynamic stability and are known to bind a broad
range of targets such as proteins, sugars and lipids. In this
respect, the scorpion toxins charybdotoxin [69–71], scylla-
toxin [72*,73] and α-conotoxin [74] (Figure 1; Table 1),
the cellulose-binding domain of cellulases [75,76], the
insect defensin A [77] (secreted by certain larvae to attack
bacterial membranes), and the Echallium elaterium trypsin
inhibitor II [78] have been used as scaffolds for generating
new binding molecules. While the cellulose-binding
domain and charybdotoxin were used to generate novel
binding specificities via surface residue randomization
and selection, the charybdotoxin and other scaffolds were
also used in loop grafting studies with loops of defined
design and experimental strategies to obtain such novel binding proteins.

Acknowledgements

The authors would like to thank Per-Ake Nygren, Patrik Forrer, Michael Stumpp and Patrick Amstutz for helpful comments.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


An informative review giving perspectives on licocals as drug candidates.


The interaction of an anti-idiotypic affibody with protein Z was studied both at the level of the free affibody and at the level of the structure of the complex (see also [27*]). The affibody appears as a molten globule that adopts the three-helical bundle fold upon binding to the target.


Crystallographic study of the same complex as described in [26*].


Using designed ankyrin repeat protein libraries of varying repeat numbers [33], the authors selected nanomolar binders against maltose-binding protein and two mitogen-activated protein kinases using ribosome display. The crystal structure of the complex between a selected binder and maltose-binding protein was determined confirming the scaffold design.


A combined selection/screening approach using designed ankyrin repeat protein libraries allowed the identification of inhibitors to a bacterial aminoglycoside phosphotransferase. Inhibition could be shown both in vitro and in vivo, where the best inhibitors led to a phenotype comparable to the gene knockout.


One of the aminoglycoside phosphotransferase inhibitors described earlier [33] was analyzed in more detail, revealing an allosteric enzyme inhibition mechanism, and co-crystallized with the target. The crystal structure emphasized the allosteric inhibition mechanism again and showed that the inhibitor trapped the enzyme in a catalytically inactive state.


40. Hiipakka M, Huotari P, Manninen A, Renkema GH, Saksela K: Protein libraries allowed the identification of inhibitors to a bacterial aminoglycoside phosphotransferase. Inhibition could be shown both in vitro and in vivo, where the best inhibitors led to a phenotype comparable to the gene knockout.


45. Reina J, Lacroix E, Hobson SD, Fernandez-Ballester G, Rybin V: Using a rational design approach PDZ variants with new peptide-binding specificities were generated with micromolar affinities. The variants could be used for western blotting, affinity purification and in ‘immuno’-precipitation experiments.


In analogy to previous studies, the protease inhibitor ecotin was used for the selection of specific, picomolar affinity inhibitors of plasma kallikrein.


Generation of a CD4 mimetic, chimeric scorpion toxin that binds to viral particles and diverse HIV-1 envelopes with CD4-like affinity.


