Anticalins versus antibodies: made-to-order binding proteins for small molecules

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Engineering proteins to bind small molecules presents a challenge as daunting as drug discovery, for both hinge upon our understanding of receptor–ligand molecular recognition. However, powerful techniques from combinatorial molecular biology can be used to rapidly select artificial receptors. While traditionally researchers have relied upon antibody technologies as a source of new binding proteins, the lipocalin scaffold has recently emerged as an adaptable receptor for small molecule binding. 'Anticalins', engineered lipocalin variants, offer some advantages over traditional antibody technology and illuminate features of molecular recognition between receptors and small molecule ligands.

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Introduction

The challenge of discovering small molecule ligands for protein receptors is well appreciated by, among others, the pharmaceutical industry, which spent the majority of its $24 billion pharmaceutical research and development budget in 1999 on small molecule drug discovery [1]. The crux of the drug discovery challenge is the currently inadequate understanding of the forces governing molecular recognition. Molecular recognition is also central to essentially all processes in biology. Thus, the study of molecular recognition is a problem of great practical and theoretical importance.

An interesting approach to explore molecular recognition inverts the drug discovery problem, start with a known small molecule and search for a 'receptor' capable of specifically binding the small molecule. While this approach is certainly not a new one, a milestone has recently been achieved in establishing a new class of proteins as user-friendly small molecule binding agents. Using in vitro ligand binding selection from a protein library, researchers in the Skerra laboratory have engineered specific tight binding proteins to several small molecules (Figure 1) [2,3]. These workers have departed from the traditional antibody scaffold as a source of novel binding proteins, using a member of the lipocalin family as a template to create novel proteins dubbed 'anticalins' [2,3].

Though many techniques for producing libraries of proteins are available, the studies reviewed here make use of randomly mutated proteins displayed on the surface of filamentous bacteriophage, viruses capable of infecting only bacteria (reviewed in [4]). Diversity can be targeted to particular regions of a displayed protein through standard molecular biology techniques [5], or can be distributed throughout a displayed protein [6]. The phage display technique allows for in vitro binding selection, a simple and rapid process, followed by multiple rounds of growth in an *Escherichia coli* host to amplify the most fit binding variants and further selection. This process, often described as in vitro evolution, can be readily adapted to many different small molecules for the evolution of novel binding activities of proteins. Using a combination phage-and-plasmid (phagemid) system, monovalent phage display of proteins allows for stringent binding selections that discriminate between high affinity and low affinity binding interactions [5].

In addition to the antibody and anticalin scaffolds, other protein scaffolds have been modified to create libraries of binding proteins (reviewed in [7]). The cytochrome b_{562} scaffold has been adapted to bind *N*-methyl-*p*-nitrobenzylamine conjugated to bovine serum albumin (BSA) [8]. Generally, these binding protein libraries have successfully bound new protein ligands, but not small molecule ligands (reviewed in [7]). For example, the cytochrome b_{562} scaffold fails to bind unconjugated *N*-methyl-*p*-nitrobenzylamine. The engineered lipocalin libraries are distinct from previously described artificial receptor libraries, because the resulting anticalins bind a variety of structurally

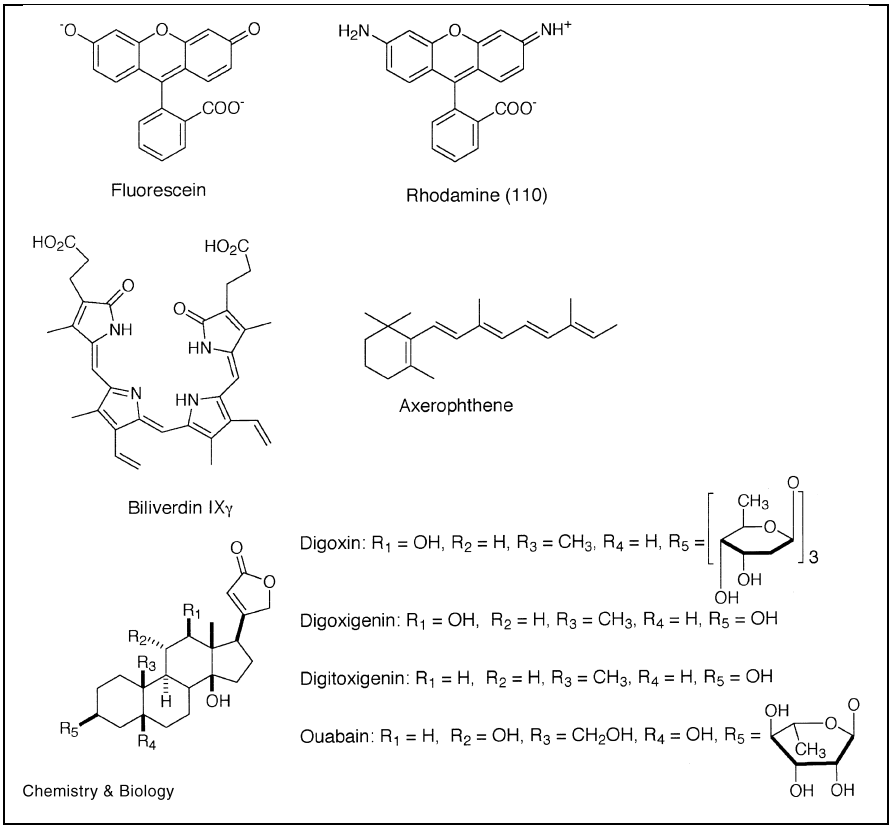

Figure 1. Small molecule ligands for antibodies, lipocalins and/or anticalins. Shown are fluorescein, rhodamine (110), biliverdin IX_γ, axerophthene, digoxin, digoxigenin, digitoxigenin, and ouabain structures.

unrelated small molecules that are not covalently linked to proteins.

Lipocalins from butterflies and pigs

Among the many homologous protein families inherited through evolution from insects to mammals, lipocalins are perhaps uniquely suited for diverse tasks in small molecule binding (reviewed in [9]). The lipocalins are a family of 'professional binding proteins' that specialize in binding hydrophobic small molecules, such as biliverdin IX_γ, a butterfly chromophore; retinal, an important pigment for visualization; or pheromones, for which binding proteins are known in pigs. The structures of several lipocalins bound to their respective ligands have been reported (reviewed in [10] and also [9]).

Various members of the family feature deep to relatively shallow binding sites that are formed by the central core of a β-barrel and protruding loops (see Figure 2). This is clearly seen in the structure of a complex between the butterfly-derived bilin binding protein (BBP) and the natural chromophore biliverdin IX_γ (Figure 2) [11]. From the crystal structure, BBP is a tetrameric lipocalin, with one ligand bound per monomer. The ligand binding site is quite deep (Figure 2) and largely nonpolar, although two residues, Glu(38) and Tyr(94) of BBP, form H-bonds with biliverdin and two ligand-bound water molecules [11]. Furthermore, the side chain of Tyr(94) forms a cap blocking free diffusion of biliverdin from the binding site, yet leaving the carboxylate oxygens of biliverdin accessible to water. By analogy with the ligand binding loops of antibodies, the BBP loop structure presents a logical starting point for the generation of diversity using site-directed mutagenesis. Many different amino acid sequences may be accommodated in such surface loops without global disruption of the scaffold structure. Accordingly, Skerra et al. began their studies by mutating 16 loop residues within a monomeric variant of BBP (see Table 1).

Anticalins: lipocalins with engineered ligand specificity

The phage library of BBP variants was created by fusion of the BBP variant to a phagemid-encoded protein, g3p, which is expressed in the *E. coli* host and incorporated into the virion coat of M13 bacteriophage. Fluorescein was chosen as an initial small molecule ligand, an appropriate choice, since binding affinity is readily measured by spectrophotometric assays, and anti-fluorescein antibodies, useful for structural and affinity comparison, have been previously described. As in the case of antibody-phage libraries [12], ligand-specific binding BBP variants were obtained by screening the phage-displayed anticalin library

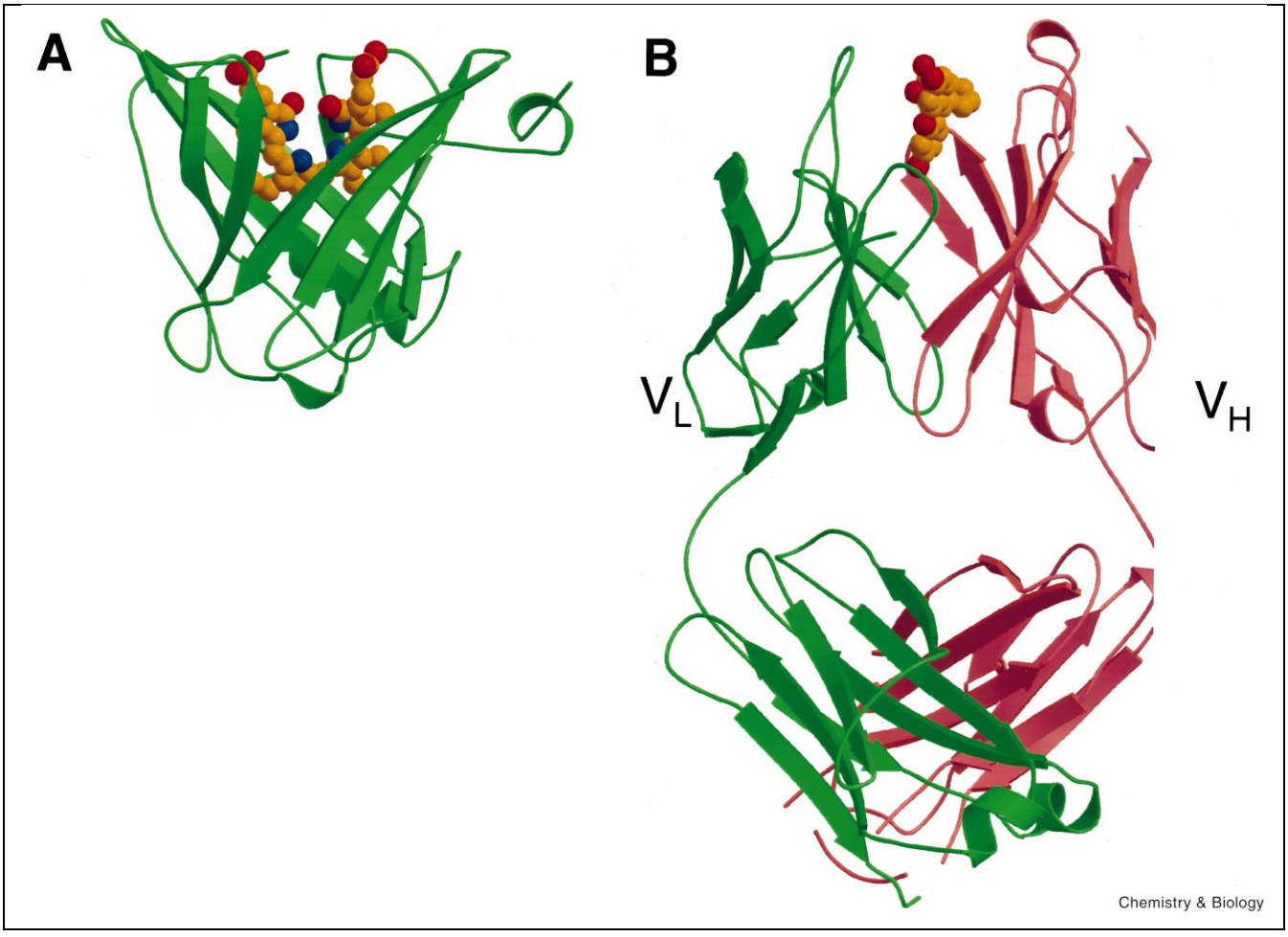

Figure 2. Ribbon diagrams of two artificial receptor scaffolds. **(A)** The lipocalin domain (Protein Database entry 1BBP) of BBP bound to the butterfly chromophore biliverdin IX_γ (depicted with cpk model). One subunit of the lipocalin tetramer is shown, as Beste et al. [2] used a monomeric BBP mutant. **(B)** Fluorescein (cpk) bound to the light (V_L) and heavy (V_H) chains of the anti-fluorescein Fab 4-4-20 (1FLR). The figure was prepared using Molscript-II [27] and RASTER-3D [28].

for binding to BSA-conjugated or RNaseA-conjugated fluorescein. One such anticalin variant, FluA (see Table 1), was identified and found to have 35 nM affinity (K_d) for fluorescein. Remarkably, the ligand specificity was high, with $K_d > 100$ μM for the fluorescein analog rhodamine (see Figure 1).

The BBP structure also provided the basis for a structural model of the fluorescein–FluA anticalin binding interaction. Having noted that basic side chain substitutions occurred in FluA at several randomized sites, Beste et al. [2] demonstrated the importance of two of these side chains. Substitution of Arg(58) with Lys or His(127) with Phe caused losses in fluorescein binding affinity of 59-fold and 13-fold, respectively. Since these residues are important for ligand binding, placement of the modeled fluorescein in the binding site described for BBP was made by analogy with the His (V_L 27d) and Arg (V_H 34) residues of the anti-fluorescein antibody 4-4-20 described below. While this is a tentative model, this placement of the fluorescein molecule within the loop-defined ligand binding site seems plausible.

More recently, Schlehuber et al. [3] reported on steroid binding anticalins. After phage binding selections and colony screening a 16-site randomized BBP-phage library for binding to a digoxigenin-conjugated RNaseA, a selectant was identified with moderate affinity ($K_d \approx 300$ nM) to digoxigenin. This variant, called DigA, was affinity-matured using additional phage display libraries which were randomized at four of the originally mutated positions (residues 34–37), as well as two additional positions (Table 1) involved in an ion pair within BBP. Again, binding selections and colony screening were used to identify optimized

variants. Ultimately, a variant called DigA16 was obtained, which bound the ligand with 30 nM affinity, but had no detectable binding to the related compound ouabain (see Figure 1). Another analog of the target ligand, digitoxigenin, happened to bind even more tightly to this anticalin, with a K_d of 2 nM.

For the DigA16 anticalin, the authors suggest that the steroid is well buried in the anticalin β-barrel. Such a variation on the lipocalin:small molecule binding theme has been observed in the binding of a retinol analog in the structure of the axerophthene:lipocalin (bovine retinol binding protein) complex [13,30]. This axerophthene structure reveals a lipocalin binding site that extends well into the interior of the β-barrel, though the exposed loops are also involved in binding. The degree to which axerophthene is buried by the protein is especially striking (Figure 3D). Thus, the β-barrel region of lipocalins provides additional possibilities for ligand interactions.

The recent successes of anticalin engineering suggest that many different small molecules could be recognized with high specificity by selected members of a lipocalin library, diversified at only 16 residues. The precise interactions involved in anticalin recognition await structural studies on ligand-bound anticalin complexes.

Antibodies with small molecule binding specificity

For comparison with the new anticalin scaffold, the extensive record of antibodies is instructive, as antibodies have proven useful as binding proteins for small molecules. Anti-digitoxigenin, anti-nitrophenol, anti-biotin and anti-fluorescein antibodies are workhorses in biochemistry, as specific binders to covalently modified proteins. Often, these antibodies can bind both the protein-conjugated and the unconjugated small molecule; for example, the structure of an anti-fluorescein antibody bound to free, unconjugated fluorescein has been solved [14]. Antibodies capable of binding small molecules that are meant to mimic the transition states of reactions have been used to explore the role of molecular recognition in reaction catalysis (reviewed in [15]). In addition, the large number of three-dimensional structures of antibodies bound to small molecule ligands illustrates different strategies for one scaffold binding to a variety of molecules.

Fluorescein (Figure 1), long used as a fluorescent molecular probe, was an early target for the generation of antibodies using traditional hybridoma as well as antibody-phage technology [12]. Anti-fluorescein antibodies with binding affinities (K_d) in the range of 100 pM to 10 μM have been reported (reviewed in [16]). The high resolution X-ray structure [14] of one fluorescein binding antibody, known as 4-4-20 Fab, provides a glimpse of how effectively a monoclonal antibody can bind a small molecule (see Figure 3A). Antibodies bind to their antigens through various combinations of contacts made from among six hypervariable complementarity determining regions (CDRs). Five of the six hypervariable CDRs are used by 4-4-20 Fab to construct a binding site for fluorescein. The binding site is a deep cleft, burying 92% of the surface-accessible area of fluorescein. The cleft is formed between

Table 1
Designing anticalin-phage libraries. Each sequence begins with the residue numbered to the left of the sequence.

BBP	1	NVYHDGAC–PEVKPVDNFDWSNYHGKWWEVAKYPNSVEKYGKCGWAEYTPE
BBPm	1	DVYHDGAC–PEVKPVDNFDWSQYHGKWWEVAKYPNSVEKYGKCGWAEYTPE
FluA	1	DVYHDGAC–PEVKPVDNFDWSQYHGKWWEVAKYPSPNGKYGKCGWAEYTPE
DigA16	1	DVYHDGAC-PEVKPVDNFDWSQYHGKWW**Q**VA**A**YPDHITKYGKCGWAEYTPE
RBP	1	ERDCRVSSFRVKENFDKARFAGTWYAMAKKDPEGLFLQDNIVAEFSVD
BBP	51	-GK-SVKVSNYHVIHGKEYFIE-GTAYPVGD-SKIGKIYHKL-TY
BBPm	51	-GK-SVKVSNYHVIHGKEYFIE-GTAYPVGD-SKIGKIYHSL-TY
FluA	51	-GK-SVKVSRYDVIHGKEYFME-GTAYPVGD-SKIGKIYHSR-TV
DigA16	51	-GK-SVKVSRYSVIHGKEYFSE-GTAYPVGD-SKIGKIYHSY-TI
RBP	49	ENGHMSATAKGRVRLLNNWDVCADMVGTFTDTEDPAKFKMKYWGVASF
BBP	91	GGVTKENVFNVLSTDNKNYIIGYYCK-YDEDKKGHQDFVWVLSR-SKVLTGE
BBPm	91	GGVTKENVFNVLSTDNKNYIIGYYCK-YDEDKKGHQDFVWVLSR-SMVLTGE
FluA	91	GGYTR**K**TVFNVLSTDNKNYIIGYSCR-YDEDKKGHWDHVWVLSR-SMVLTGE
DigA16	91	GGVTQEGVFNVLSTDNKNYIIGYFCS-YDEDKKGHMDLVWVLSR-SMVLTGE
RBP	98	LQKGNDDHW-IIDTDYETFAVQYSCRLLNLDGTCADSYSFVFARDPSGFSPE
BBP	141	AKTAVENYLIGSPVVDSQKLV-YSDFSEAACKVNN
BBPm	141	AKTAVENYLIGSPVVDSQKLV-YSDFSEAACKVNN
FluA	141	AKTAVENYLIGSPVVDSQKLV-YSDFSEAACKVNN
DigA16	141	AKTAVENYLIGSPVVDSQKLV-YSDFSEAACKVNN
RBP	149	VQKIVRQRQEELCLARQYRLIPHNGYCDGKSERNIL

Shown are the amino acid sequences of lipocalins BBP [29], BBP mutant (BBPm) used as the anticalin scaffold [2], and bovine RBP [13]; and anticalins FluA [2] for binding fluorescein, and DigA16 [3] for binding digoxin. The 16 sites (and corresponding sites in BBP and RBP) used for generation of the initial phage anticalin libraries are underlined. Other mutations occurring in anticalins selected for ligand binding are shown in bold.

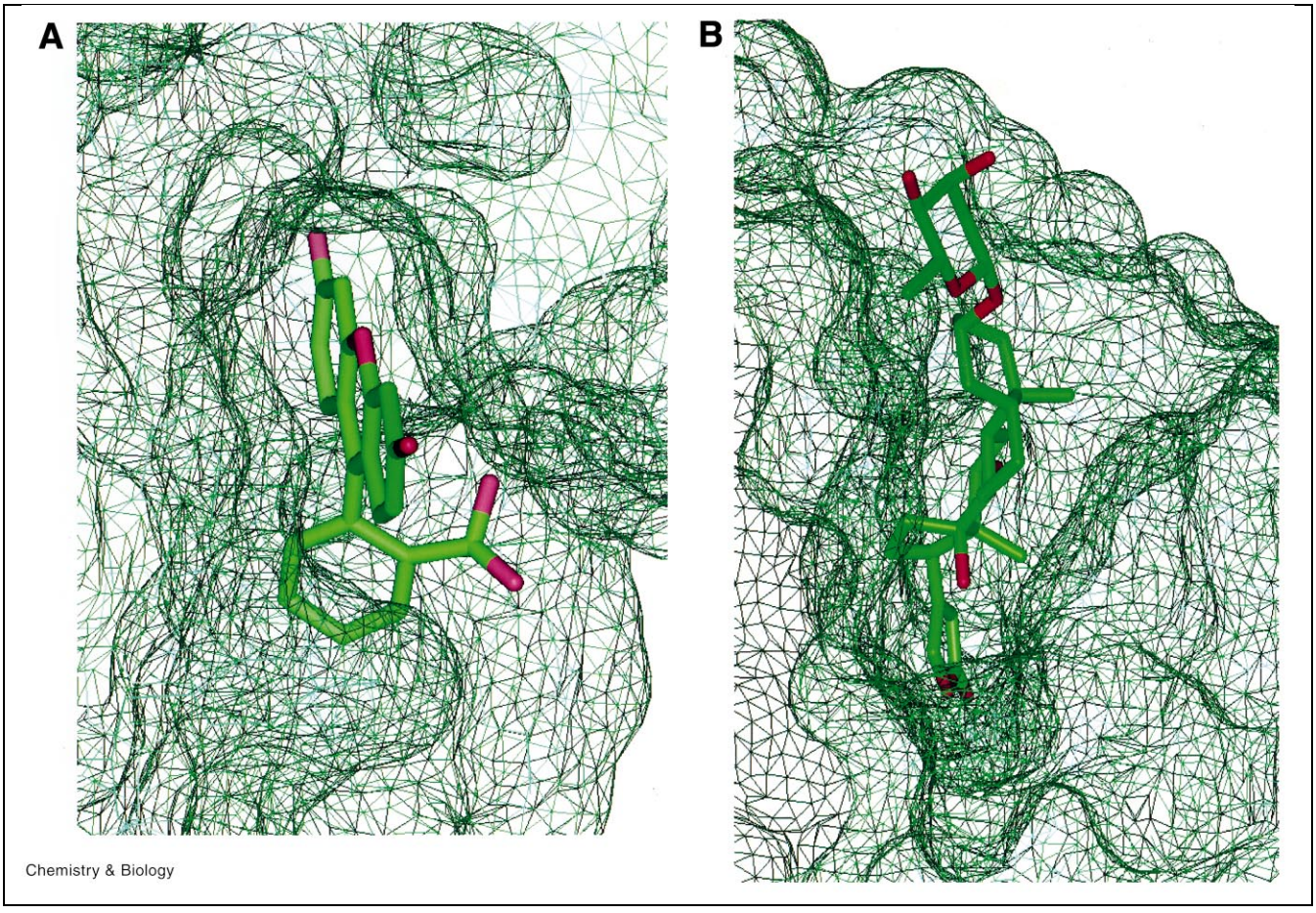

Figure 3. Binding sites for small molecules in antibodies and lipocalins. Ligand structures are shown as connected bonds with heteroatom coloring (red for oxygen, violet for nitrogen) and the surrounding protein as a solvent-accessible surface (InsightII, MSI San Diego, CA, USA). **(A)** The fluorescein:4-4-20 Fab complex (Protein Data Bank ID, 1FLR) illustrates an antibody with a deep binding cleft for fluorescein. **(B)** The digoxin:26-10 Fab complex (1IGJ) demonstrates an antibody binding site that only partially buries the digoxin ligand. **(C)** The biliverdin IX_γ:lipocalin (BBP from the butterfly *Pieris brassicae*) complex (1BBP) shows a ligand bound by the outer loops of a lipocalin. A Tyr side chain forms part of a 'trap door' across the ligand. **(D)** The axerophthene:lipocalin (bovine retinol binding protein) complex (1FEN) exemplifies a lipocalin binding site that extends into the interior of the β-barrel.

CDRs of the two IgG polypeptide chains [17] and lined mostly by hydrophobic residues: a His, Tyr and Trp from the light chain (V_L variable region), as well as a Trp and three Tyr residues from the heavy chain (V_H). In addition, probable H-bonds between antibody and hapten are implied by the structure: a His residue (V_L 27d, residue numbering from [18]) with fluorescein oxygen O1, Arg (V_H 34) as well as Ser (V_H 91) with fluorescein O3, and Tyr (V_L 32) with fluorescein O4; additional H-bonds appear to form with bound water molecules [14]. However, the functional contributions of these H-bonds to the free energy of fluorescein binding is less clear. For example, mutational substitution (reviewed in [14]) of Arg (V_L 34) or His (V_L 27d) by another basic residue, Lys, led to 10-fold or 400-fold reductions in binding affinity, respectively; but removal of the basic functionality via Ala substitutions led to only a 40-fold reduction in affinity (Arg → Ala) or a slight *improvement* in affinity (His → Ala). In contrast, substitutions of hydrophobic residues in the cleft, Trp (V_L 96) or Trp (V_H 33) by Tyr caused decreases in binding affinity of about 60- to 100-fold each. Thus, the shape and hydrophobic character of the cleft appear most crucial to high affinity ligand binding, though H-bonding interactions may enhance specificity. Indeed, the affinities of 4-4-20 for fluorescein and rhodamine are 60 pM and >10 μM, respectively [19], and this has been attributed [14] to the loss of His and Arg contacts in the aza-substituted rhodamine (see Figure 1).

A second series of small molecules, steroids, have been used for the generation of both antibody- and anticalin-based artificial receptors. The X-ray structures of two

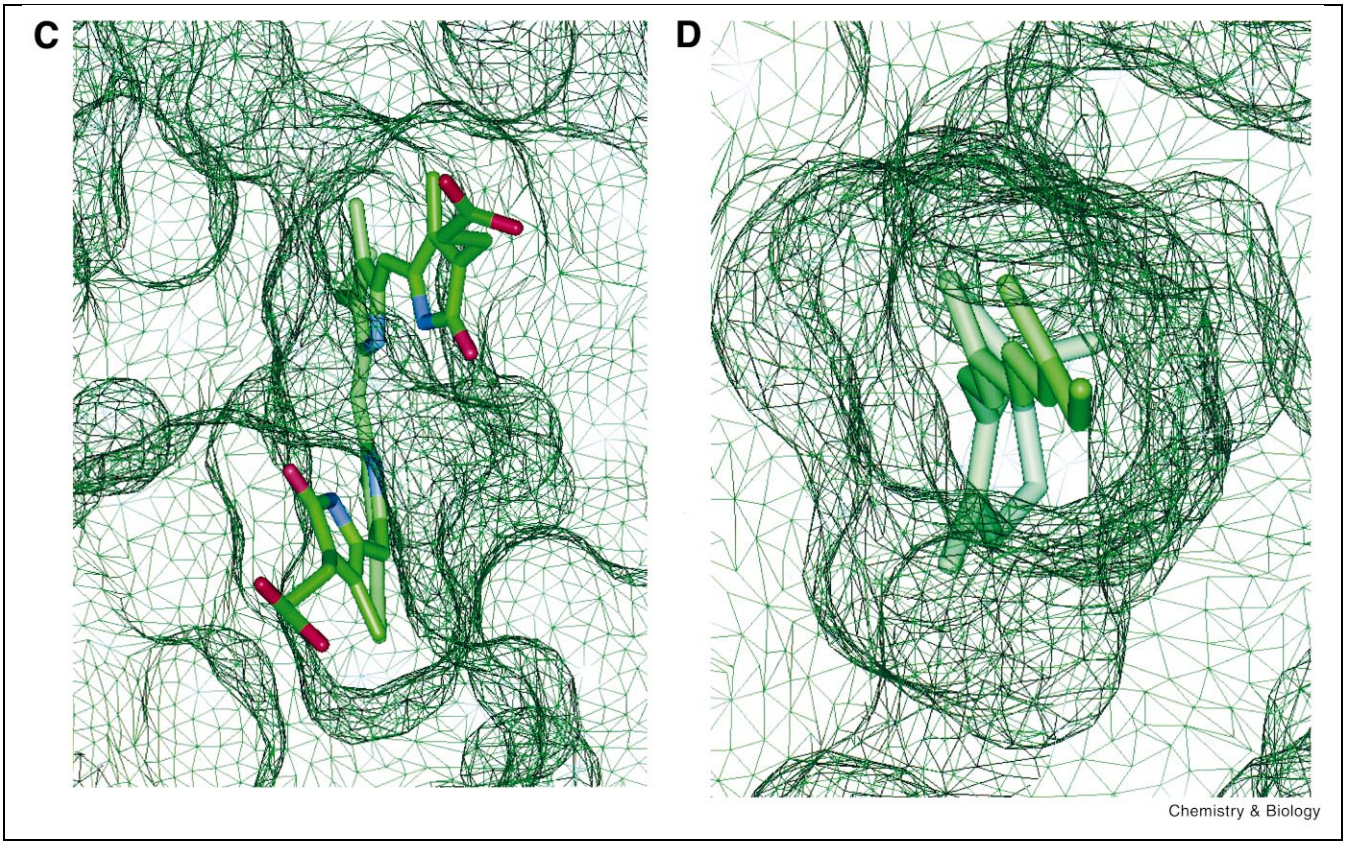

Figure 3 (*continued*).

anti-digoxin antibodies, 26-10 Fab [20] and 40-50 Fab [21] have been reported. Both have broad specificity for binding related molecules such as ouabain (see Figure 1). The structure of 26-10 Fab bound to digoxin reveals a shallow binding site. The ligand carbohydrate groups (see Figure 1), through which conjugation was performed for immunization, protrude from the antigen binding pocket, in which the lactone ring is thoroughly surrounded (see Figure 3B). Interestingly, no H-bonds or salt bridges are seen between antibody and ligand in this structure, leading to characterization of the mechanism of ligand recognition by this antibody as shape complementarity [20]. Similarly, hydrophobic interactions appear to dominate the interaction of the other anti-digoxin antibody, 40-50, whose structure has been reported [21] in complex with ouabain (see Figure 1). Again this binding site is shallow, burying 76% of the solvent-accessible surface area of the ligand. As these authors suggest, hydrophobic interactions appear to be the driving force for ligand binding by this $K_d \approx 0.6$ nM affinity antibody. Although H-bonding does occur in this complex, it has been pointed out that the sites of H-bonding are conserved in the cardiac glycosides; therefore, these interactions cannot yield specificity. In fact, most contact residues in the complex are aromatics, for example, His (V_L 32), His (V_H 35), Phe (V_H 95), Tyr (V_H 100) and Tyr (V_H 100c).

Antibody versus anticalin scaffolds

Despite differences in fold and shape, the antibody and anticalin classes of small molecule binding proteins share much in common. The monomeric anticalin is similar in size to a single chain variable domain (V_L–V_H) of an antibody (see Figure 2) which is often used in phage-displayed libraries (see e.g. [12]). Both form small molecule binding sites or CDRs by forming binding surfaces from flexible loops. A technical limitation for the generation of new binding activities in each case is the need to conjugate small molecules to carrier proteins for in vitro binding selections (anticalins) or immunizations (antibodies).

Anticalins are distinct in that the binding site for small molecules may extend into the interior of a well-ordered β-barrel (see Figure 3). This type of stable secondary structure may have advantages for certain applications (see below), especially when compared to the flexible loops of antibody CDRs.

Antibodies are established tools of biochemistry, with demonstrated high affinities, often with $K_d < 1$ nM, and with remarkable specificity. However, the success at generating anticalins with moderate to high affinity for fluorescein and digitoxigenin, 35 and 2 nM, respectively, with mutations at only 16–17 sites, suggests that anticalins are a robust scaffold for generating small molecule binding proteins quickly and efficiently. Antibodies show sequence diversity at more than 70 positions within the six CDR regions [18]. It is unclear whether, with a small number of side chain substitutions, fluorescein and digoxin binding sites could be engineered into the non-cognate anti-digoxin and anti-fluorescein antibody scaffolds, respectively.

Implications

While molecular recognition has been explored using synthetic non-protein hosts, including combinatorial libraries (reviewed in [22,23]), this review has focused upon adapting protein scaffolds to bind small molecules. Several broad themes emerge from these studies. First, proteins exhibit remarkable malleability in binding to diverse small molecules. This theme mirrors the observation that diverse small molecules can bind to the same binding site; for example, many structurally unrelated small molecules inhibit HIV protease by binding with high affinity to the same protease active site (representative examples reviewed by [24]). Second, the same small molecule can be bound tightly and specifically using very different strategies, by structurally unrelated protein scaffolds. This has also been observed in the diverse protein structures capable of non-covalent binding to the same small molecule (e.g. biotin binding by streptavidin and anti-biotin antibodies). Third, based upon the known structures of lipocalin: small molecule and antibody:small molecule complexes, it appears that the shape complementarity of the binding site, as well as hydrophobic interactions, may often be key to high affinity small molecule binding. Together with a small number of polar interactions, these factors may also contribute to exquisite specificity in ligand recognition. In this sense, the anticalins, having a preformed cavity of fluorescein-like or digitoxigenin-like dimensions, may be easily recruited to bind a diverse range of other small molecules.

Many practical applications may be envisioned for engineered lipocalins. The reported anticalins are proteins derived from a butterfly protein. Thus, these particular anticalins may prove unsuitable for therapeutic applications due to potential immunogenicity. However, human retinol binding protein is a member of the lipocalin family and could presumably be adapted to serve as an artificial receptor in a manner analogous to the experiments described here. Small molecule binding activity could be applied to a number of therapeutic areas, such as the discovery of binders for cholesterol, leukotrienes, toxic shock inducing cell wall fragments, and the like.

As binding proteins for specific small molecules, the anticalins could find a host of potential uses from biosensors to chromatography-based separation technologies. It has been demonstrated that fusion proteins composed of anticalins with reporter enzymes such as alkaline phosphatase can be used for ligand binding assays [3]. In addition, surface plasmon resonance technology has demonstrated the detection of binding events involving molecules of < 200 Da mass [25] using immobilized proteins. Engineered anticalins could provide a ready source of binding proteins for rapidly adapting this technology for routine use in quality control, safety or clinical diagnostics applications. Similarly immobilized proteins could be used for affinity chromatography of target compounds.

Conceivably, the anticalin binding site could be modified to mimic an enzyme active site to evolve proteins with catalytic activity. In fact, members of the lipocalin family can catalyze carotenoid epoxidation in plants [26]. With their β-barrel architecture, it is reasonable to assume that anticalins provide a more rigid ligand binding scaffold than antibodies. Thus, it would be interesting to compare the catalytic activity of anticalins evolved to bind transition state analogs with the analogous catalytic antibodies.

More generally, structural studies of newly engineered anticalins should continue to teach us about the diverse structural features used by proteins to recognize small molecule ligands.

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