

Antibody fragments as tools in crystallography

L. Griffin and A. Lawson

Department of Structural Biology, UCB, Slough,
UK

Accepted for publication 3 May 2011

Correspondence: A. Lawson, Department of
Structural Biology, UCB, 216 Bath Road,
Slough SL1 4EN, UK.

E-mail: alastair.lawson@ucb.com

Summary

While antibody-based therapeutics have become firmly established as front-line drugs, the use of antibodies as research tools in small molecule drug discovery is still in its infancy. In this review we focus on the use of antibody fragments as crystallization chaperones to aid the structural determination of otherwise 'uncrystallizable' or 'undruggable' target proteins. We also highlight a potential application for this technology, in which antibody-mediated structures may be used to inform the design of new chemical entities.

Keywords: antibodies, antibody-mediated crystallization, crystallography, fragments

Introduction

In recent years, small molecule drug discovery has sought increasingly to develop leads from low-molecular-weight fragment hits, identified from screening libraries of only a few thousand compounds [1]. High ligand efficiency, together with effective chemical diversity, are particularly attractive aspects, but the approach places a heavy reliance on structural information to guide the elaboration of initial small molecule fragment hits. High-resolution crystal structures of co-complexes of low-affinity (often mM) initial fragments, binding at sites of biological interest, are needed to rationalize medicinal chemistry towards increased potency and specificity.

Traditional protein targets for small molecules, where this approach has been successful, include enzymes with relatively rigid and deep active sites, in which small molecule fragment hits are protected from exposure to solvent [2]. However, such easily druggable molecules represent only a fraction of the potential proteins which the pharmaceutical industry would like to target, and the structural properties of many proteins involved in protein–protein interactions do not lend themselves readily to this approach. Membrane-bound protein targets and solution phase proteins with a flexible structure, although attractive targets from a biological viewpoint, present major challenges due to their conformational heterogeneity and surface chemistry. In addition, the surfaces at sites of interaction tend to be large and are often apparently featureless. Crystal structures of such proteins are not only difficult to obtain at sufficient resolution to see small molecules bound, but can also be misleading, by providing only a snapshot of the range of potential conformations adoptable by the target.

A number of ways to try to stabilize proteins for crystallography have been developed, including genetic engineering [3], co-complexing with natural ligands [4] and binding of antibody fragments or alternative scaffolds [5]. Recently there has been renewed interest in seeking innovative biological solutions to reducing surface entropy, and some interesting work at the interface of traditional chemistry and biology is starting to show promise.

Protein engineering

Post-purification modification of proteins by, for example, removal of negatively charged sialic acid groups with neuraminidase treatment has been shown to aid crystallization, and enabled the generation of crystals of human chorionic gonadotrophin (hCG) which diffracted to 2.8 Å [6]. The authors go on to comment that recombinant hormones expressed in culture systems, which do not add sialic acid, may provide the best material for crystal growth.

Genetic engineering of proteins, specifically to enhance crystallization, is now common practice to troubleshoot proteins that will not crystallize well. For example, engineering an increase in the hydrophilic surface area through the generation of a fusion protein has been used to enhance crystallization of the lactose permease, an integral membrane protein of *Escherichia coli* [7]. T4 lysozyme has been utilized successfully as a fusion partner by replacing the third cytoplasmic loop in the human β_2 -adrenergic G protein-coupled receptor [8]. An alternative fusion protein approach was used in an attempt to improve the crystal quality of cytochrome *bo*₃ ubiquinol oxidase of *E. coli* [9]. Protein Z, a highly soluble and highly stable form of the fragment B of protein A of *Staphylococcus aureus*, was introduced at the C

terminus of the enzyme, and provided new protein–protein contacts in the crystal lattice. However, the overall resolution was decreased compared to wild-type.

Enzymatic or genetic modification carries with it risk that the conformation adopted by the recombinant target may not be achievable with the native protein, and drug discovery relying on this approach may prove ultimately to be disappointing.

Complexing with natural ligands

Where possible, complexing proteins with their natural ligands may be a useful way to introduce stability. For example, DNA and RNA binding proteins can be co-crystallized with oligonucleotides [4]. However, this approach is somewhat specialized and clearly not universally applicable. The natural ligand may be unknown or not available in sufficient amounts. Crystallization with the natural ligand is not always useful in drug development, as the natural ligand occupies what is likely to be the most potent site for a drug.

Complexing with chaperones

Co-complexing a target protein with an auxiliary protein, which acts as a chaperone, represents a particularly attractive option with wide applicability [10]. Inclusion of the chaperone increases the probability of high-quality crystal formation by minimizing the target conformational heterogeneity through 'locking' or 'clamping' the target in a particular conformation (possibly previously unknown), masking inhibitory surfaces and extending facilitating surfaces. Formation of the crystal lattice based on contacts between chaperone molecules is also likely, in most circumstances, to be an advantage, so that sites of biological interest on target proteins are not occluded by crystal contacts [5]. In addition, a chaperone with a previously characterized structure can facilitate molecular replacement phasing.

Complexing with alternative scaffolds

While antibody fragments such as the Fab, Fv, single-chain Fv and single-domain camelid-derived VHH have proven ability to enhance solubility and stabilize target proteins [5], alternative scaffolds such as affibodies and the helical, but disulphide-free, designed ankyrin repeat proteins with randomized surface residue positions (DARPin) [11–15] have also shown promise.

Affibodies are based on the three-helix scaffold of the Z domain derived from staphylococcal protein A. Recently Eigenbrot *et al.* [16] have demonstrated a ZHER2 affibody binding to the human epidermal growth factor receptor 2 (HER2) at a site distinct from the Fab fragments of pertuzumab and trastuzumab. While it is not clear that the affibody assisted the crystallization process in this case, the

dissociation constant ($K_D = 22 \text{ pM}$) suggests that such constructs have considerable potential in this context.

In contrast, DARPins have proven ability to stabilize proteins for crystallography. Protein engineering approaches applied to the crystallization of the protein kinase polo-like kinase-1 (Plk-1) had been unsuccessful; however, a structure of wild-type apo Plk-1 was achieved at 2.3 Å resolution with the use of a DARPin [17]. In this example, the DARPin masked a Plk-1 surface patch rich in arginines, lysines and glutamates, residues which are unfavourable for forming crystal contacts [18]. In a recent paper [19], a DARPin was used to assist the crystallography of the CC2-LZ domain of nuclear factor kappa B (NF-κB) essential modulator (NEMO), a critical component of the NF-κB signalling pathway, to provide valuable insight into function and regulation. The DARPin enabled a structure of the protein of interest to be obtained at 2.95 Å resolution. In the absence of the DARPin, only a 4 Å structure of a shorter construct was obtained [20], and mutation of a critical lysine residue (K285N) was required to obtain an equivalent structure of apo CC2-LZ [21]. DARPins have also been used to obtain structures for aminoglycoside phosphotransferase [22] and caspase-2 [23], although in the latter case the diffraction quality of the DARPin complex was only 3.24 Å compared to the 1.65 Å structure obtained from the peptide inhibitor complex [24].

DARPins and antibody fragments may be considered complementary in assisting the crystallography of proteins, as largely independent epitopes are defined by the two scaffolds. The detergent-solubilized Na⁺-citrate symporter CitS of *Klebsiella pneumoniae* was co-crystallized successfully with both DARPins and antibody Fab fragments [25]. In this case the location of the binding site at the tip of the antibody fragment, compared with the shallow binding groove formed by the DARPin, conferred a crystallographic advantage. The N-terminal binding site of the Fab enabled protrusion from the target protein's surface, allowing crystals to pack with additional space between the Fab:protein units and minimizing unwanted target protein crystal contacts.

In a parallel study, high-resolution structures of the base-plate BppU-BppL complex of Lactococcal phage TP901-1 were obtained with both DARPins and a camelid VHH antibody fragment [26]. The stoichiometries reflected the respective structures of the chaperones, with three VHHs bound to the trimer, and one DARPin bound at the top of the head domain. As expected, the convex binding site of the VHH sought concave architecture on the target, while the concave DARPin defined a convex epitope. The protruding paratope of the VHH penetrated into a crevice-shaped epitope located between two protomers although, interestingly, the buried surface areas were similar at around 680 Å² in both cases. The affinities of the DARPins and the VHH were also similar (K_D around 1 nM), with some 20 residues mediating hydrogen bonds and Van der Waals contacts in

both cases. VHHs and DARPinS use complementary interaction modes with their targets, determined to a large degree by their intrinsic structures, inherent rigidity and ability to provide multiple crystal contacts.

Complexing with antibody fragments

While DARPinS have become established as tools in crystallization, antibody fragments such as Fab, Fv, scFv and VHH offer both versatility and broad applicability, and function-modifying antibodies, particularly those which bind at allosteric sites, could be of further value in this regard. An additional attribute of antibody fragments is the ability to match the size of the chaperone (50–15 kDa) to the specific target, an important factor to consider as the quality of model-based phasing is dependent upon the molecular mass of the chaperone relative to the total complex [27]. The β -sheet-rich structure of antibodies, with intrinsic capacity for self-assembly through intermolecular anti-parallel interactions, provides a significant advantage over DARPinS, aiding nucleation and promoting dimerization of co-complexes [10]. Antibody fragment-mediated crystallization has been shown to be particularly advantageous for proteins with transmembrane helices and short solvent-exposed loops, such as transporters and ion channels. In these cases the antibody can aid crystallization through increasing the hydrophilic surface area available for formation of an improved crystal lattice. Antibody-based chaperones have also shown their value in trapping proteins in specific conformations, which can occur in solution but which are less common, complementing their use in protein refolding [28]. For example, a Fab fragment was used to crystallize KcsA, locking the proton-activated, voltage-modulated K⁺ ion channel in the physiologically relevant, closed, conformation [29].

An additional advantage, which can be derived from antibody-mediated crystallization, is the utility of the chaperone to provide model-based phasing information. Thus the preferred option for many laboratories when faced with a recalcitrant protein, which defies engineering-based attempts at structure determination, is co-crystallization with an antibody fragment.

The use of Fab fragments as chaperones can be traced back to the work of Laver's group in Australia in the mid-1980s. They showed that whale N9 neuraminidase formed well-ordered crystals only when complexed with Fab fragments from monoclonal antibodies [30]. Rossmann's group used a specific antibody Fab fragment to enable the crystallization of the highly hydrophobic human immunodeficiency virus capsid protein p24 [31]. The enhanced solubility of the complex, provided by the Fab, overcame the susceptibility of p24 to aggregate, and led to crystals which diffracted to at least 2.7 Å. Even at this early stage, Prongay *et al.* [31] had the foresight and vision to propose that the structure of p24-Fab may provide knowledge of the packing arrange-

ment of the capsid structure, and show whether a hydrophobic pocket capable of accommodating modifying anti-viral agents is present. The authors go on to propose that the antibody-enabled structure could be used to find new compounds that bind into the putative functional WIN pocket. Perhaps, some 20 years on, the time is right to revisit this concept in drug discovery.

Crystallization of another human immunodeficiency virus (HIV) target, type 1 reverse transcriptase (HIV-1rt), was aided by an antibody Fab fragment. Attempts to engineer crystallizability through alteration of specific surface amino acids were unsuccessful. However, moderately diffracting crystals were achieved with a HIV-1rt–Fab complex, and in an interesting example of combining the benefits of antibody Fab fragment-mediated crystallization with natural substrate, crystals with enhanced diffraction (3.5 Å) were obtained by the addition of a defined sequence of double-stranded DNA [32].

Advances in antibody engineering around this time led to the generation of minimal antigen-binding fragments such as variable fragments (Fvs) and single-chain (sc) Fvs. In addition to being of smaller size, these Fv fragments conferred the advantage of eliminating flexibility around the elbow region of the Fab [33]. The binding modes of Fab and scFv fragments were compared crystallographically by Kortt *et al.* [34], and shown to assume very similar conformations, thus making Fvs and scFvs ideal alternatives to Fab fragments. As mentioned previously, the relatively large size of Fab fragments can provide a crystal packing advantage as they generally allow greater space in the lattice, although it has been demonstrated equally that Fvs allow spacious and rigid crystal packing, with Fv dimers sufficiently detached to avoid interference of the detergent micelle in a yeast cytochrome *bc₁* structure [35].

The crystallization of membrane proteins presents special challenges due to their contrasting hydrophilic and hydrophobic surfaces, and in 1995 Michel's team was the first to use an engineered Fv fragment to stabilize an intrinsic membrane protein. The hydrophilic regions of membrane proteins are relatively small, thus Ostermeier *et al.* [36] postulated that the probability of obtaining well-ordered crystals could be enhanced if the size of the extramembraneous polar region were increased through the use of antibody fragments. This approach successfully yielded diffraction quality crystals, albeit with a high degree of anisotropy, and resulted in the structure of the detergent-solubilized bacterial cytochrome c oxidase (COX) from *Paracoccus denitrificans* at 2.8 Å resolution. It is important to note that the antibody recognized native enzyme by binding to a conformation-specific epitope while preserving a natural state of the target. All the important crystal lattice contacts were established through the Fvs, which were exclusively involved in polar interactions; no direct interactions between COX molecules were observed. Prior to this work, only a limited number of atomic structures for membrane

proteins had been solved, and no ordered crystals of COX had been obtained. Subsequent investigations generated slightly better diffracting crystals, and the structure was refined further [37].

As a preview to the later, and more sophisticated, Fv-mediated structure, successful structure-determination of a membrane protein was also obtained following co-crystallization with a Fab fragment. A higher-resolution ($< 2 \text{ \AA}$) structure of the KcsA K^+ channel was achieved than had been possible from crystals of the channel alone ($3.2 \text{ \AA}$) [38]. All crystal contacts were observed between Fabs, leaving ample space for the decyl-maltoside detergent micelle. Additional information on ion binding was available from the antibody-mediated structure, due to the altered crystal packing resulting in the presence of a wide passage outside the channel pore. This structure formed the first example in which the presence of the Fab fragment was exploited, through molecular replacement, to aid structure solution.

Just as membrane-bound proteins present challenges to crystallography, the heterogeneity and intrinsic disorder of surface saccharides on glycosylated proteins have been addressed with antibody-based chaperones. While crystallization can be achieved through deglycosylation, this non-natural state can result in abrogation of protein activity and study of inactive protein. In a novel solution, two Fv fragments, acting in parallel on the α and β chains, were used in the elucidation of the crystal structure of hCG [39]. In the crystal, the two Fvs constrained the sialylated hCG in a molecular cage. The Fv fragments were shown to provide strong crystal contacts, leaving sufficient space to enable the non-structured surface saccharides to be accommodated in the cell solvent and not interfere with crystal packing interactions.

An interesting example of the application of antibody technology to structure determination of a long-standing problematic protein is that of jack bean urease (JBU). Crystallization of this nickel-metalloenzyme was reported as early as 1926 [40]; however, due to the crystals diffracting weakly and belonging to a complex cubic space group $F4_132$, no structure of the protein had been determined. Complexation of JBU with an Fv fragment resulted in crystallization in a different space group, $R32$, with the crystals diffracting to $3.35 \text{ \AA}$ [41]. Analysis of the data revealed, for the first time in 76 years, a preliminary structure of the enzyme, with the Fv fragments providing bridges to complete the crystal packing.

Fab-assisted crystallization has been used for other proteins, such as OspA (outer surface protein A) from the Lyme disease spirochete *Borrelia burgdorferi*, which are too soluble to form crystals. The Fab was used to mask a surface patch of highly charged and flexible side chains from arginine, glutamic acid and lysine residues [42]. Fab fragments, binding to each of the four voltage sensors of the KvAP channel from *Aeropyrum pernix*, overcame the inherent flex-

ibility of the channel and enabled the formation of crystals which diffracted to $3.2 \text{ \AA}$ [43]. More recently, a Fab fragment has been used to aid the crystallization of parathyroid hormone-related protein (PTHrP), with diffraction to $2 \text{ \AA}$ resolution reported [44]. Fabs have also been used successfully for the first time as chaperones to overcome the inherent structural flexibility and instability of G protein-coupled receptors for hormones and neurotransmitters, with the structure of the human β_2 adrenoceptor determined at $3.4 \text{ \AA}$ resolution from a complex of an inverse agonist and a Fab fragment, which bound to the third intracellular loop [45].

While Fabs have proven application in enabling crystallization, their utility may be enhanced further with the inclusion in co-complexes of immunoglobulin-binding proteins, such as *Peptostreptococcus magnus* protein L, which binds to the Fab κ light chain [46]. Such 'super chaperones' provide additional spacing and alternative ways of forming the crystal lattice, further increasing the probability of success.

A promising approach, aimed at generalizing antibody-mediated crystallization, is to engineer a tag binding epitope into a known loop region of a protein, thus facilitating the generic application of Fab fragments from well-known, and easily sourced, antibodies. In an early application of this concept, the detergent-solubilized K^+ channel protein KvPae was engineered with a FLAG-binding epitope in a loop region and complexed with an anti-FLAG Fab fragment [47]. Recently an engineered derivative of the scFv fragment of the easily crystallizable antibody 3D5, with specificity for the hexapeptide sequence EYMPME, has been used to co-complex recombinant proteins into which the tag had been inserted at specific sites [48]. The scFv is currently undergoing further engineering to render the CDRs less favourable for crystal contacts. Should this approach prove to be successful with a wide range of targets, it will eliminate the need to generate or select new antibody-binding sites for each new protein, and thus has considerable attraction.

The antibody-mediated crystallography discussed to date has utilized fragments from conventional antibodies; however, in 1993 a novel antibody format was discovered, which would add an additional reagent to the field. The identification by Hamers-Casterman *et al.* [49] of a unique antibody type in the camelid species, termed heavy chain antibodies, redefined the minimal antigen-binding fragment. Although similar in size to the heavy chain variable region domain of Fv fragments from conventional antibodies, the variable regions of heavy chain-only antibodies, termed VHH fragments, display multiple advantages over traditional Fv fragments, including improved stability and reduced aggregation. The first crystallization of a VHH domain, bound to an antigen, identified additional unique features of these antibody fragments, with the CDR-H3 region of the VHH shown to protrude in a convex manner into the catalytic clefts of enzymes [50,51].

The first example of a VHH fragment aiding crystallization of a difficult protein was provided by Loris *et al.* [52] in

the application to the addiction antidote protein – MazE. Crystallization of addiction antidotes is challenging due to low thermodynamic stability and short shelf-life, and for these reasons a camel VHH antibody was introduced to act as a crystallization aid for MazE. Successful crystallization was achieved in the presence of a VHH fragment, with the total amount of structured protein increased significantly from only 45% to 73%. Consideration of the crystal packing showed a repeating arrangement of a MazE dimer sandwiched between two VHH fragments, with all the crystal lattice stabilizing interactions derived from the VHH fragments. These results suggest that the advantageous crystal packing of VHH-mediated crystallization mimics that observed for Fv- and Fab-mediated crystallization. Of further interest is the report that a subsequent structure of the hexameric complex of the antidote MazE, with the toxin MazF (MazE₂MazF₄) displayed the same N-terminal structural arrangement [53], indicating that the presence of the VHH antibody did not disturb the protein structure significantly.

Further examples of VHH-aided crystallization include the EpsI : EpsJ proteins of the bacterial type 2 secretion systems. Solution of the proteins in the absence of VHH had been achieved previously [54]; however, inclusion of a specific VHH fragment resulted in improved crystal packing with alternating VHH/ EpsI : EpsJ layers [55]. Other examples include solution of the N-terminal domain structure of the secretin GspD from enterotoxigenic *E. coli* (ETEC), where crystal growth was promoted through heterotetramer formation [56], and the structure of α -synuclein, where study of aggregation was enabled by the VHH acting as a structurally silent reporter [57].

Having proved the ability of VHHs to facilitate crystallization of difficult proteins, leading groups are now pushing the technological boundaries further in assessing different conformational states of proteins. Domanska *et al.* [58] utilized a VHH domain to trap and characterize intermediates of β 2 microglobulin (β 2 m) to assess fibrillogenesis. In the absence of the VHH, the Δ N6 β 2 m intermediate aggregated within minutes; however, the VHH was shown to act as an efficient crystallization chaperone, capable of trapping the intrinsically unstable Δ N6 β 2 m variant and enabling crystal formation and structure determination. Similarly, Rasmussen *et al.* [59] reported the first crystal structure of a G protein-coupled receptor (GPCR) in an active state. Crystal structures of GPCRs have been limited due to the innate flexibility and inherent instability of the receptors in the absence of ligand; furthermore, the structures documented to date relate to GPCRs in an inactive state. A VHH to the human β 2 adrenergic receptor exhibited G protein-like behaviour, enabling an agonist-bound, active state crystal structure to be obtained, which has provided valuable insight into the process of activation.

As discussed previously, in addition to aiding crystallization, Fab fragments have been used to aid structure solution

through employment of molecular replacement techniques. Similar exploitation of VHH crystallization chaperones has been applied recently to other crystallographic methods of solving the phase problem, such as single-anomalous dispersion (SAD) techniques. Tereshko *et al.* [27] demonstrated that the use of SeMet-labelled VHH as a crystallization chaperone enabled phase determination of the entire complex using SAD, without the need for SeMet labelling of the target protein. The ease with which SeMet can be incorporated into VHH fragments, and the phasing benefits achieved for proteins where molecular replacement is not possible, provides a further distinct advantage of VHH-mediated crystallography.

Of all the crystallization chaperones described to date, the camelid heavy chain antibody-derived VHH fragments offer considerable promise. Their rigidity, thermal stability, solubility, attractive biophysical properties and small footprint speak to their versatility and general applicability, as witnessed by the recent formation of Xaperones.com, a Belgium-based company which specializes in offering VHH-mediated crystallization. Engineering of VHH fragments, specifically for use in crystallography, is already established [27], and is set to become even more sophisticated, as these chaperones are applied to the elucidation of structures of ever more challenging proteins.

Discussion

The literature reviewed above shows how antibody fragments can offer valuable scaffolding for proteins in the early stages of drug discovery, holding targets in conformations which may be amenable to the binding of very weak (mM) small molecule fragment hits. From the data provided by these antibody-assisted structures, medicinal chemists may direct the elaboration of small molecule fragment hits to enable significant increases in potency to be achieved in a short time-frame. The antibodies may then be omitted from the crystal system once the small molecules are potent enough to interact directly with the protein alone. Without the critical antibody-mediated initial stabilization step, small molecule fragments with the potential to become valuable drugs would not be tractable and, as a consequence, lost to medicine.

Conclusion

Antibodies have come a long way in 30 years, from initial doubts about their therapeutic applicability to established blockbuster drug status. Impressive as this progress has undoubtedly been, antibody fragments may yet become of even greater value to the pharmaceutical industry, by enabling the discovery of new generations of small molecule drugs which target successfully previously 'undruggable' proteins. In this context antibody fragments, which stabilize both integral transmembrane and flexible target proteins to

reveal previously unknown conformations, surface features and 'druggable' architecture, may facilitate future structure-based drug discovery in an exciting new way.

Disclosure

Drs Griffin and Lawson are employees of UCB, based at the Slough Research Site in the UK.

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