High-affinity binding to staphylococcal protein A by an engineered dimeric Affibody molecule

Malin Lindborg1, Anatoly Dubnovitsky2,5, Kenneth Olesen3, Tomas Björkman4, Lars Abrahmsén1,6, Joachim Feldwisch1 and Torleif Hård2,7

1Affibody AB, Gunnar Asplunds Allé 24, SE-171 63 Solna, Sweden, 2Department of Molecular Biology, Swedish University of Agricultural Sciences (SLU), Box 590, SE-751 24 Uppsala, Sweden, 3Department of Biomedicine, University of Gothenburg, Box 440, SE-405 30 Gothenburg, Sweden, 4GE Healthcare Bio-Sciences AB, Björkgatan 30, SE-751 84 Uppsala, Sweden, 5Present address: Department of Clinical Neuroscience, Center for Molecular Medicine L8:04, Karolinska Institutet, SE-17176 Stockholm, Sweden and 6Present address: Algeta ASA, PO Box 54 Kjelsås, N-0144 Oslo, Norway

Off to whom correspondence should be addressed.
E-mail: Torleif.Hard@slu.se

Received May 7, 2013; revised July 3, 2013; accepted July 7, 2013

Edited by Arne Skerra

Affibody molecules are engineered binding proteins, in which the three-helix bundle motif of the Z domain derived from protein A is used as a scaffold for sequence variation. We used phage display to select Affibody binders to staphylococcal protein A itself. The best binder, called ZpA963, binds with similar affinity and kinetics to the five homologous E, D, A, B and C domains of protein A, and to a five-domain protein A construct with an average dissociation constant, \( K_D \), of \( \sim 20 \) nM. The structure of ZpA963 in complex with the Z domain shows that it interacts with a surface on Z that is identical in the five protein A domains, which explains the multi-domain affinity. This property allows for high-affinity binding by dimeric Affibody molecules that simultaneously engage two protein A domains in a complex. We studied two ZpA963 dimers in which the subunits were linked by a C-terminal disulfide in a symmetric dimer or head-to-tail in a fusion protein, respectively. The dimers both bind protein A with high affinity, very slow off-rates and with saturation-dependent kinetics that can be understood in terms of dimer binding to multiple sites. The head-to-tail (ZpA963)_{H} dimer binds with an off-rate of \( k_{off} \leq 5 \times 10^{-6} \text{s}^{-1} \) and an estimated \( K_D \leq 16 \) pM. The results illustrate how dimers of selected monomer binding proteins can provide an efficient route for engineering of high-affinity binders to targets that contain multiple homologous domains or repeated structural units.

Keywords: molecular recognition/phage display/protein engineering/protein–protein interactions/protein structure

Introduction

Affibody molecules are small binding proteins selected from combinatorial libraries in which the 58-residue Z domain is used as a scaffold for sequence variation (Nord et al., 1997; Löffblom et al., 2010). The Z domain is homologous to the five individually folded E, D, A, B and C domains of protein A and it was derived by introducing a chemically stabilizing mutation in the B domain (Nilsson et al., 1987). Affibody binders have been selected for a large number of protein targets (see Löffblom et al., 2010 for a review), including the HER2 receptor extracellular domain (Orlova et al., 2006; Eigenbrot et al., 2010) and the amyloid-β peptide (Grönwall et al., 2007; Hoyer et al., 2008).

An array of protein A-based products has been developed for applications involving antibodies that bind protein A through their Fc domain. These include protein A linked to dyes, biotin, gold particles, enzymes and magnetic, latex or agarose beads. A readily produced and cheap non-Fc binder might be developed for use in quality control and purification of protein A-linked reagents. A small robust protein A binder might also be used for affinity purification of protein-A fusion proteins. A broad potential application would be as a tag on re-combinant proteins, expanding the application of the wide range of protein A reagents developed for Fc (or IgG) interaction outside of the immunoglobulin field.

Another motivation to select and study protein A binders is that five homologous sites are available for binding. This opens for the construction of a high-affinity protein A binding molecule in which a dimer or higher order motif engages two or more protein A domains in a complex. Hence, we wanted to study if and how high-affinity binders of a multi-domain protein can be engineered by linking selected monomer binders.

Here, we describe the selection and characterization of a protein A binding Affibody molecule (ZpA963) that binds to the five domains with similar nanomolar affinities. We determine the structure of ZpA963 in complex with the Z domain to understand the basis for this multiple specificity. We also demonstrate that picomolar protein A binding can be achieved when two ZpA963 subunits are linked into dimers.

Materials and methods

Phage display selections

A combinatorial phage display library was prepared essentially as described earlier (Nord et al., 1995; Nord et al., 1997; Grönwall et al., 2007). The pool that was used for the present selection contained 3.4 × 10^9 variants of the Z domain, with random amino acid residues at positions 9, 10, 11, 13, 14, 17, 18, 24, 25, 27, 28, 32 and 35 (Grönwall et al., 2007). The 34.3 kDa protein A target with subunits E, D, A, B and C and a C-terminal cysteine was obtained in lyophilized form (GE Healthcare), dissolved in phosphate-buffered saline (PBS) buffer (2.68 mM KCl, 137 mM NaCl, 1.47 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.0) and stored frozen. Biotinylation of protein A was carried out in PBS buffer by mixing protein A, an excess of the sulfhydryl-reactive EZ-link PEO-maleimide peptide (Groenwall et al., 2007; Hoyer et al., 2008).
activated biotin (Thermo Scientific) at room temperature for 2.5 h. Unbound biotin was removed by gel permeation chromatography on a NAP-5 column (GE Healthcare). Biotinylated protein A was stored at 4°C.

Phage selections were carried out in four cycles following a protocol used previously for a biotinylated target (Lindborg et al., 2011). Briefly, tubes and streptavidin (SA) beads (Dynal) were pre-blocked in PBS-T (PBS buffer with 0.1% Tween-20) supplemented with 0.1% gelatin. The phage library stock was precipitated using polyethylene glycol with NaCl and dissolved in PBS-T at pH 7.0 with 0.1% gelatin. All incubations were performed at room temperature. Non-specific binders were first removed by pre-incubation with SA beads. The remaining phages in the supernatant were mixed with biotinylated protein A and incubated with rotation for 2.0 h (cycle 1) or 1.5 h (cycles 2–4). The protein A target concentrations were 50, 10, 2 and 0.4 nM in cycles 1, 2, 3 and 4, respectively. SA beads were added to the mixtures and the incubation was continued for 10 min. The beads were washed with PBS-T and the number of washing steps was increased for each cycle (Grönwall et al., 2007). The remaining bound phages were eluted with 500 μl low pH elution buffer (0.1 M glycine–HCl, pH 2.2) for 10 min and neutralized with 450 μl PBS and 50 μl 1 M Tris-HCl, pH 8.0. The eluted phages were amplified for the next cycle of selection (Hansson et al., 1999).

DNA sequence analysis

DNA sequencing was performed with ABI PRISM® dGTP, BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit according to the manufacturer’s recommendations, using biotinylated oligonucleotides. The sequence reactions were purified by binding to magnetic SA-coated beads in a Magnatrix 8000 robot and analyzed on ABI PRISM® 3100 Genetic Analyzer. The sequences were verified using the software Sequencher (Gene Codes Corp.).

Enzyme-linked immunosorbent assay for analysis of protein A binding

Affibody molecules (Z domain variants) selected randomly from phage clones remaining after four rounds of selection were expressed and screened for protein A binding activity using an enzyme-linked immunosorbent assay (ELISA). The expression was carried out directly from the pAffiI phagemid vector, which yields the Z domain variants as albumin-binding domain (ABD) fusion proteins that become secreted in the periplasm, as described previously (Lindborg et al., 2011). Briefly, single colonies of selected clones were used to inoculate tryptic soy broth (TSB) + yeast extract (YE) medium (30 g/l TSB and 5 g/l YE) supplemented with 100 μg/ml ampicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 20 h at 37°C. The cell pellets were re-suspended in PBS-T and frozen at −80°C for outer cell membrane disruption. The frozen samples were thawed in a water bath and clarified by centrifugation, giving supernatants (periplasmic fractions) containing fusion proteins of prospective protein A binding Z variants and ABD. The ELISA was performed as follows: 100 μl periplasmic fraction was transferred to MaxiSorp 96-well plates (Nunc), coated with human serum albumin (Sigma; 6 μg/ml in PBS) and blocked with 2% skim milk in PBS-T, for 1.5 h incubation. The plates were washed five times with PBS-T in a SkanWasher 300 (Skatron) prior to addition of 100 μl of 1 μg/ml biotinylated protein A per well and incubation for 1.5 h. After washing the wells five times, SA-horseradish peroxidase (Dako) diluted 1:5000 in PBS was added to the wells and incubated for 1 h. TMB substrates A and B were mixed 1:1 and added to the washed wells and incubated for 30 min according to the manufacturer’s instructions (ImmunoPure TMB Substrate Kit; Thermo Scientific). Stop solution (2 M H2SO4) was added and the absorbance at 450 nm was measured in a SmartSpec 3000 spectrophotometer (BioRad).

Cloning, expression and purification of Affibody molecules

Three Affibody molecules, denoted ZpA963, ZpA670 and ZpA964, were selected for further experiments and cloned into expression vectors containing a T7 promoter (Studier et al., 1990), a multiple cloning site flanked by sequence coding for an N-terminal His6-tag and a C-terminal cysteine, and a kanamycin resistance gene. The protein obtained from this vector is GSSHHHHHHHLQ[Z variant]WVDC, where the Z variant denotes the selected Affibody molecule sequence. To produce an additional monomeric ZpA963, the C-terminal cysteine residue was mutated to an alanine using the QuikChange kit (Stratagene). To produce head-to-tail dimeric ZpA963, the monomeric construct harboring a C-terminal cysteine was cut and a second ZpA963 gene was introduced, resulting in a plasmid for expressing GSSHHHHHHHLQ[Z variant]2VDC. Escherichia coli BL21(DE3) cells (Novagen) were transformed with the expression plasmids and cultivated at 37°C in TSB + YE medium supplemented with 50 mg/l kanamycin. At an optical density (OD600nm) of 1, expression of the Affibody genes was induced through the addition of IPTG to a final concentration of 0.5 mM, and the cultivation was incubated for another 5 h before harvest. Pelleted bacteria was dissolved in immobilized metal ion affinity chromatography (IMAC) buffer A (500 mM NaCl, 20 mM Tris-HCl, 10 mM imidazole, pH 8.0) and disrupted by sonication.

Monomeric Cys-flanked Affibody molecules were purified by IMAC using Talon metal affinity resin (Clontech). After IMAC purification, buffer exchange from IMAC buffer B (500 mM NaCl, 20 mM Tris-HCl and 500 mM imidazole pH 8.0) to PBS was performed using gel permeation chromatography on PD-10 columns (GE Healthcare).

Different variants of ZpA963 were purified from E.coli BL21 Star (DE3) cells lysates using IMAC chromatography on 5 ml HiTrap Chelating HP columns (GE Healthcare) followed by size-exclusion chromatography on a HiLoad 16/600 Superdex 75 prep grade column (GE Healthcare). Purified proteins were concentrated using 5 kDa cut-off Vivavspin columns (GE Healthcare), flash-frozen in liquid nitrogen and kept at −20°C.

Surface plasmon resonance analysis

One set of surface plasmon resonance (SPR) experiments involved analysis of each of the five protein A domains binding to Affibody molecules immobilized on the carboxylated dextran surface of a Biacore CM5 chip. Three molecules were analyzed using the four flow cells (His6-ZpA963-Cys, His6-ZpA670-Cys, His6-ZpA964-Cys and a blank control) on a Biacore 2000 instrument (GE Healthcare). The immobilization was performed by thiol coupling via C-terminal cysteine, according to the manufacturer’s recommendations. One surface on the chip was activated and deactivated for use as a reference cell. The protein A domains (E, D, A, B and C)
produced as ABD-[protein A domain]-Cys (Jansson et al., 1998) were diluted in HBS-EP buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid, 0.005% Tween-20 and pH 7.4) to a final concentration of 10 μM and injected at a flow rate of 5 μl/min for 5 min at 25°C. After 5 min of dissociation, the surfaces were regenerated with one (for ZpA670 and ZpA964) or three (for ZpA963) injections of 0.05% sodiumdodecyl sulfate (SDS). BIAevaluation 3.2 software (GE Healthcare) was used for evaluation of Biacore data.

A second set of SPR experiments involved analysis of ZpA963 variants to the five-domain protein A fragment immobilized on CM5 chips on a BIACORE X100 instrument (GE Healthcare). Binding of the ZpA963 monomer (without a C-terminal cystein) was analyzed at 25°C in HBS-EP. Binding of the cysteine-linked (ZpA963)2Cys and head-to-tail (ZpA963)2ht dimers were analyzed and compared in the same HEPES buffer, with 300 mM NaCl and 0.01% Tween-20 (the free cysteine at the C-terminus of (ZpA963)2ht was blocked by N-ethylmaleimide). These experiments were conducted at flow rates of 10 μl/min and chip regeneration was performed with 0.1% SDS. Binding and dissociation curves were analyzed using the BIAevaluation 4.1 software (GE Healthcare).

**Isothermal titration calorimetry**

The Z domain was expressed and purified as described previously (Lendel et al., 2002; Wahlberg et al., 2003). Protein concentrations were determined spectrophotometrically using extinction coefficients obtained from the ExPASy server (www.expasy.org). Isothermal titration calorimetry (ITC) was performed at 25°C on a VP-ITC calorimeter (MicroCal) with a cell volume of 1.42 ml. Proteins were dialyzed twice for 4 h against 1 l of 50 mM potassium phosphate and 75 mM NaCl at pH 7.5. All solutions were degassed prior to the ITC experiments. ZpA963 at a concentration of 10 μM was used as titrant in the cell and Z domain at 100 μM as titrant in the syringe. Typically, an initial 3 μl injection was followed by 24 subsequent 12 μl injections. The heat of post-saturation injections was averaged and subtracted from each injection to correct for heats of dilution and viscous mixing. The initial injection was not considered in the analysis. Data were processed using MicroCal Origin 7.0 software provided with the calorimeter. The observed binding isotherms were subjected to a non-linear least-square fit to a generic binding model in which binding affinities and stoichiometry were treated as unknown parameters.

**Nuclear magnetic resonance spectroscopy and structure determination of the ZpA963-Z complex**

Isotope labeled proteins were obtained from cultures in M9 medium with 3 g/l 13C glucose, 2 g/l 15N3H4Cl and 2 g/l 13C,15N-enriched Celtone (Spectra Stable Isotopes). Nuclear magnetic resonance (NMR) samples of the ZpA963 complex contained either 0.5–1 mM homogeneously 13C,15N-labeled Z and unlabelled ZpA963 in 25% molar excess, or 13C,15N-labeled ZpA963 and excess unlabeled Z, in 75 mM NaCl, 20 mM sodium phosphate, 0.1% azide and 7.5% D2O at pH 5.6 (uncorrected reading). The relative excess of the unlabeled protein ensured that all 13C,15N-labeled protein existed in the bound state. NMR experiments were performed at 25°C using Varian Inova 800 and 900 MHz spectrometers (Agilent Technologies) of which the latter was equipped with a cryogenic probe. The data were processed using NMRpipe (Delaglio et al., 1995) and analyzed using CcpNmr Analysis version 2.1 release 3 (Vranken et al., 2005).

The C-terminal cysteine residue in the ZpA963 construct would potentially interfere with structure determination due to dimerization of the Affibody molecule. It was therefore mutated to an alanine using the QuikChange kit (Stratagene). NMR spectra of the Z complex with ZpA963 with C-terminal alanine or cysteine were identical, except for the resonances of those residues, indicating identical structures.

Backbone and side chain resonance assignments were obtained from standard triple-resonance experiments (Varian BioPack software) including 3D HNCO, 3D HNCA, 3D HN(CO)CA, 3D CACBNH, 3D CACB(CO)NH, 3D 15N-TOCSY, 3D HCCH-COSY and 3D HCCH-TOCSY. Aromatic side chains were assigned using 2D (HB)CB(CGDCE)HE experiments. Proton resonance assignments could be completed to 99%.

NMR spectra were analyzed using the CCPN data model (Vranken et al., 2005) to exchange data with CcpNmr Analysis. All of the eight NOESY spectra mentioned above were used and calibrated within ARIA without spin diffusion corrections with a 6.0 Å distance cutoff. The NOEs were automatically assigned based on the (manually assigned) chemical shifts with tolerances of 0.02 and 0.04 ppm for directly and indirectly detected proton dimensions, respectively, and 0.5 ppm for heteronuclear dimensions. Manually assigned NOEs were used, but not trusted. The structural rules, bound corrections and network anchoring options were not used. Structure calculations were performed by simulated annealing in eight iterations starting from linearized peptides. During the iterations, parameters for violation tolerance and violation threshold were gradually changed from 1000 to 0.1 Å and 0.5 to 0.3, respectively, and the ambiguity cut-off parameter for partial assignments was decreased from 1.0 to 0.8. The default ARIA simulated annealing protocols and parameters were used throughout. The dihedral angle upper and lower bounds predicted by DANGLE were applied in structure determination, but a few dihedral angle constraints that conflicted with the NOEs after the final iteration were removed. The calculations were then repeated in two iterations without otherwise altering the constraint set (i.e. no more new assignments) to generate 50 structures of which 20 were kept based on low conformational and constraint violation energies. Water refinement was not applied. Structural quality was evaluated using built-in ARIA 2.3 routines and Procheck (Laskowski et al., 1993). Constraint statistics and structural statistics have been summarized in Table I.

Molecular graphics figures were created using PyMOL (Schrödinger). The NMR chemical shifts, experimental constraints and the final ensemble of 20 structures have been
were identified as strong protein A binders in an ELISA assay. Three strong binders, denoted ZpA963, ZpA670 and ZpA964 (Fig. 1), were selected for further characterization.

Affinity and binding kinetics of the three chosen binders to each of the five domains of protein A were analyzed using SPR. ZpA963 and ZpA670 both display similar affinity for separate E, D, A, B and C domains (Fig. 2A and B). However, slower off-rates can be observed with ZpA963, indicating that it is a stronger binder than ZpA670. ZpA964 shows comparably strong binding to the B and C domains and weaker affinity for the A, D and E domains (Fig. 2C). We chose ZpA963 for further examination based on similar affinity and slow off-rate kinetics for binding to each of the five protein A domains. Equilibrium binding of ZpA963 to the Z domain was analyzed using ITC. The data are consistent with one-site binding stoichiometry and an equilibrium dissociation constant of $K_D = 48 \pm 8 \text{ nM}$ (Fig. 2D).

**Structure of the Z:ZpA963 complex**

The structure of the complex between the Z domain and the ZpA963 Affibody molecule in solution was determined using NMR (Table I and Fig. 3A). The original three-helix bundle scaffold of the Z domain is retained in ZpA963 and the structures of the Z domain in the free and ZpA963-bound states are similar (not shown). The topology of the complex is such that the anti-parallel $\alpha$-helices 1 and 2 of ZpA963 interact with the corresponding $\alpha$-helices 1 and 2 of the Z domain at an angle of 55° (Fig. 3B). The interaction interface is predominantly non-polar (66%) and a total of 1626 Å² interaction surface area becomes inaccessible to water. These measures, the binding surface on the Z domain and the nature of the interactions with a central hydrophobic core and polar interactions at the edges, including four hydrogen bonds (Fig. 3C), are similar to those of the previously studied Z:ZSPA-1 complex (Eklund et al., 2002; Wahlberg et al., 2003). However, the orientations of the two ZpA963 and ZSPA-1 Affibody molecules in their complexes with Z are very different as further discussed below.

The binding surface of ZpA963 involves interactions by 10 of the 13 residues that were randomized in making the phage library (Figs 1 and 3C). The interaction also involves two additional non-randomized residues Phe5 at the N-terminus and Ile31 at the core of the binding surface. Ile31 may be classified as a so-called hotspot residue common to all protein A domains (Jendeberg et al., 1995; Lendel et al., 2006). This property is retained in Affibody molecules as Ile31 is completely buried at the center of the binding surface in the present Affibody molecule structure, as well as all of the four other Affibody molecules for which the structure has been determined in complex with the corresponding target (Wahlberg et al., 2003; Lendel et al., 2006; Hoyer et al., 2008; Eigenbrot et al., 2010). Correspondingly, Ile31 is also at the center of the Z domain interaction surface in the present Z:ZpA963 complex as well as in the previous Z:ZSPA-1 complex.

**Structural basis for recognition of the protein A domains**

The surface of the Z domain that is conserved in the five protein A domains is illustrated in Fig. 3C. With one exception the amino acids of $\alpha$-helices 1 and 2, to which ZpA963 binds, are identical in the five protein A domains. This is presumably the basic reason why the binding of ZpA963 to different domains (Jendeberg et al., 1995; Lendel et al., 2006; Hoyer et al., 2008; Eigenbrot et al., 2010). Correspondingly, Ile31 is also at the center of the Z domain interaction surface in the present Z:ZpA963 complex as well as in the previous Z:ZSPA-1 complex.

**Results**

**Binding protein selection and characterization**

A 34.4 kDa protein A fragment containing the E, D, A, B and C domains was biotinylated at its C-terminal cysteine residue and used as target in phage selections. Affibody binders were selected from a phage display library containing $3.4 \times 10^9$ Affibody variants. Four rounds of selection and phage amplification were performed while decreasing the target concentration by factors of five from 50 nM in the first round to 0.4 nM in the final round. Phage clones remaining after four rounds of selection were expressed and screened for protein A binding. Fourteen clones representing eight different Affibody sequences
domains is so similar. The exception is the His18 in the Z domain, whose side chain forms a hydrogen bond with Gln10 in ZpA963. His18 is present in the Z, B, and C domains, but is replaced by Asn in the A, D, and E domains. However, an alternative hydrogen bond may form between an Asn18 in protein A (Z) and Gln10 in ZpA963 (Fig. 4A and B), implicating similar interactions between ZpA963 and all of the five protein A domains.

Comparison of the sequences of the other two Affibody molecules (ZpA670 and ZpA964) with that of ZpA963 suggests that their binding modes to the Z domain are similar. For instance, selected large non-polar side chains (Trp14, Phe17 and Leu35), which together with (wild type) Ile31 constitute most of the Z:ZpA963 interaction surface, are all present in ZpA670 and ZpA964 (although Leu35 is Met35 in the latter). Similarly, Gly24 was selected in all of the three binders,

Protein A binding Affibody molecule

Fig. 1. (Upper panel) Sequence alignment of the E, D, A, B and C domains from staphylococcal protein A and the (non-native) Z domain. The B domain sequence is used as a reference. The helical secondary structure has been indicated by gray boxes. (Lower panel) The Z domain and the three selected Affibody sequences with the Z domain sequence as reference. Black boxes enclose sites that were randomized in the phage display library. Underlined residues in ZpA963 are in contact with the Z domain in the complex. The bold font indicates that the interface residues in ZpA963 (red) are identical in ZpA670 and/or ZpA964.

Fig. 2. Binding of Affibody variants to separate protein A domains. (A–C) SPR binding kinetics of individual protein D, E, A, B and C to immobilized Affibody molecules ZpA963, ZpA670 and ZpA964, as indicated. The protein A domains were injected at a concentration of 10 μM. Dissociation kinetics was monitored while injecting buffer. (D) ITC measurement of ZpA963 binding to the Z domain at 25°C in phosphate buffer at pH 7.5. The solid line represents a fit of the data with a binding stoichiometry \( n = 0.82 \pm 0.02 \) and dissociation constant \( K_D = 48 \pm 8 \) nM.
presumably because there is no room for any side chain at this position at the interaction interface (Fig. 3C).

Given similar binding modes for all of the three selected binders, one can attempt to understand the preferential binding of ZpA964 to the B and C domains over the A, D and E domains (Fig. 2C). Comparison of the sequences of the five protein A domains shows that the only place where the B and C domains (as a group) differ from the A, D and E domains (as another group) occurs at positions 18 and 19 (Fig. 1). Here, B and C contain His18 and Leu19 while A, D and E contain Asn18 and Met19. A hydrogen bond between Asp10 in ZpA964 to His18 in the B and C domains (corresponding to the hydrogen bond made by Gln10 in ZpA963, discussed above) seems possible (Fig. 4C). However, the Asn18 side chain in the A, D and E domains could be too short to reach the Asp10 in ZpA964 to function as a hydrogen bond donor (Fig. 4D), which might explain the weaker binding of ZpA964 to A, D and E.

**Dimeric Affibody constructs bind protein A with high affinity**

The property of ZpA963 to bind all five protein A domains offers the potential to achieve high-affinity binding by dimeric Affibody constructs that simultaneously bind two protein A domains. Two alternative dimeric constructs were made; the first by linking ZpA963 tail-to-tail through a disulfide between the C-terminal cysteine residues, and the second by making a fusion protein where two ZpA963 units are fused head-to-tail. These dimers are referred to as (ZpA963)$_2$Cys and (ZpA963)$_2$htt, respectively.

Binding of ZpA963 monomer and dimers to an immobilized five-domain protein A fragment was analyzed and compared using SPR. The SPR saturation levels for monomeric ZpA963 binding can be analyzed using a one-site binding model with a dissociation constant $K_D = 24 \text{nM}$ (Fig. 5A; $K_D = 20 \text{nM}$ in a separate control experiment). This affinity should be considered as an apparent $K_D$ for pseudo-one-site binding since the five protein A domains are not identical. It is nevertheless comparable with the $K_D = 48 \text{nM}$ that was obtained for ZpA963 binding to the Z domain using ITC (Fig. 2D).

Both dimeric ZpA963 constructs bind protein A with affinities that are considerably higher than that of the monomer (Fig. 5B and C). However, the binding is complex as manifested in saturation-dependent dissociation kinetics: dissociation at high Affibody molecule concentrations involves a
Fig. 4. Suggested basis for different bindings of ZpA963 and ZpA964 Affibody molecules to different protein A domains. (A) Detailed view of the (observed) hydrogen bond between Gln10 in ZpA963 and His18 in the Z domain, that most likely is present also in complexes with the B and C domains of protein A. (B) Modeling of the corresponding hydrogen bond distances for ZpA963 binding to the A, D and E domains with an Asn residue at position 18. (C and D) Modeling of the corresponding hydrogen bond distances for the ZpA964 Affibody molecule with an Asp residue at position 10. The different hydrogen bond distances can account for different binding affinities as discussed in the text.

Fig. 5. SPR binding kinetics of ZpA963 and two ZpA963 dimers to an immobilized five-domain protein A construct. (A) Steady-state SPR at saturation of ZpA963 monomer. The fit corresponds to pseudo-one-site binding with $K_D = 24$ nM. The inset shows the kinetics data. (B) Experimental binding kinetics (gray lines) and theoretical fit (black lines) for the (ZpA963)$_2$Cys symmetric dimer (from top: 16, 8, 4 and 3 nM; lower concentrations are indicated in the figure). Best-fit kinetics at low saturation levels are $k_{on} = 12 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$ and $k_{off} = 8 \times 10^{-5} \text{ s}^{-1}$. (C) Experimental binding kinetics (gray lines) and theoretical fit (black lines) for the (ZpA963)$_2$htt head-to-tail dimer (from top: 80, 40 and 20 nM; lower concentrations are indicated). Best-fit kinetics at low saturation levels are $k_{on} = 3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ and $k_{off} \leq 5 \times 10^{-6} \text{ s}^{-1}$ (no measurable dissociation within 2000 s). Different maximum saturation levels in (A–C) are due to variations in the protein A density on the CM5 chips used for the experiments. (D) Schematic illustration of symmetric Affibody dimer binding to the five protein A domains sites at low and high concentrations that is used to explain the saturation-dependent kinetics observed in (B) and (C).
rapid and a very slow phase, whereas only the slow phase is observable at low concentrations. This is most pronounced with the tail-to-tail dimer. Possibly, excess protein A (domains) at low Affibody dimer concentrations allows both domains of each type of dimer to find a protein A domain to engage in binding, resulting in strong binding with slow off-rate. However, competition sets in as the saturation increases and the fully saturated protein A will bind five Affibody dimers with weaker affinity as only one of the two subunits in each dimer is involved. (This interpretation of the kinetic data is illustrated schematically in Fig. 5D.) The dissociation kinetics will then initially be more rapid, but the remaining binders will eventually redistribute into two-site binding with slow off-rate as the saturation level drops.

We therefore estimated the affinity of dimeric ZpA963 binding by analyzing binding and dissociation kinetics at low levels of saturation. Best-fit on-rates are \( k_{\text{on}} = 12 \times 10^5 \) and \( k_{\text{on}} = 3 \times 10^5 \text{M}^{-1} \text{s}^{-1} \) for (ZpA963)\(_2\)Cys and (ZpA963)\(_2\)htt, respectively. The off-rate for (ZpA963)\(_2\)Cys is \( k_{\text{off}} = 8 \times 10^{-5} \text{s}^{-1} \) corresponding to an apparent \( K_D \) value of \( \approx 66 \) pM. The off-rate for (ZpA963)\(_2\)htt is more than an order of magnitude slower and difficult to measure precisely. However, an estimate of the upper limit to \( k_{\text{off}} \leq 5 \times 10^{-5} \text{s}^{-1} \) corresponds to an apparent \( K_D \leq 16 \) pM. Protein A binding by the (ZpA963)\(_2\)htt head-to-tail dimer is therefore >1000 times stronger than that of the monomer, when protein A is in excess.

Discussion

The primary objective of this work was to select a good protein A binder. The selected ZpA963 Affibody molecule binds to each of the five E, D, A, B and C domains of protein A with similar kinetics and it binds a five-domain protein A fragment with an (average) \( K_D = 24 \) nM. The structure of ZpA963 in the complex with the Z domain (which is very similar to domain B) clarifies the basis for multi-domain binding. We previously studied another protein A binding Affibody molecule (ZSPA-1), selected from another library (Eklund et al., 2002; Wahlberg et al., 2003; Lendel et al., 2004). ZSPA-1 also recognizes the five domains of protein A and the Z domain, but the affinities are moderate, in the order of \( K_D = 2–6 \) μM. (The binding affinity of ZSPA-1 was eventually improved by disulfide engineering to \( K_D = 130 \) nM for binding to the Z domain (Wahlberg and Härd, 2006)).

The ZpA963 Affibody molecule recognizes the same surface on helices 1 and 2 of the protein A domains as ZSPA-1. This is probably not a coincidence, because the Affibody molecules are derived from a protein A domain and have essentially the whole surface except the binding surface in common with the E, D, A, B and C domains. Any Affibody molecules present in the library that bind these common surfaces would be expected to have a propensity for self-association. The selection procedure, therefore, presumably promotes binding to a surface that is not shared, which is the surface on helices 1 and 2 that is varied in the phage display library.

The ZSPA-1 Affibody molecule (Eklund et al., 2002; Wahlberg et al., 2003) also binds with helices 1 and 2 to helices 1 and 2 of the Z domain, but the overall orientation ZSPA-1 is rotated by ca. 160° compared with that of ZpA963 (Fig. 6A–C). Still, the interaction surface on Z is essentially the same in the two complexes and the nature of the interactions is similar, as mentioned (Fig. 6D and E). For instance, both complexes involve the same non-polar interaction surface of the Z domain. This surface can be described as a central flat non-polar surface and a non-polar cavity. The flat non-polar surface is composed of Phe13, Leu17 and Ile31. In both complexes this surface interacts with the Ile31 of the Affibody molecule (and in the ZSPA-1 complex also with Leu34). The non-polar cavity on Z is lined with Phe13, Ile31, Leu34 and aliphatic carbons from Lys35. In the complexes, this cavity holds Phe17 from ZpA963 or Trp35 from ZSPA-1.

Furthermore, the non-polar interactions are surrounded by polar interactions and multiple hydrogen bonds. Also, these involve essentially the same set of side chains on the Z domain. For instance, Glu24 on Z accepts a hydrogen bond from Ser32 on ZpA963 and Lys7 on ZSPA-1, and Tyr14 on Z donates hydrogen bonds to the backbone carbonyl oxygens (Gln10 on ZpA963 and Asp24 on ZSPA-1, respectively). There are also differences in how the Z domain surface adopts to the Affibody molecule surfaces (Fig. 6D and E). For instance, the side chains of Tyr14 and His18 of the Z domain, which reorient to become stacked in the complex with ZSPA-1 (Wahlberg et al., 2003), have different conformations in the ZpA963 complex. Also, the N-terminus of the Z domain interacts with the ZpA963 complex via Phe5, but is not interacting with the Affibody molecule in the ZSPA-1 complex. These differences and similarities illustrate how quite different binders can be selected to the same protein surface.

A second objective of this study was to explore the use of multi-domain binding in protein engineering. We find that a head-to-tail dimer of ZpA963 binds a five-domain protein A fragment with \( K_D \leq 16 \) pM. The maximum effect on affinity that is theoretically achievable with two covalently linked ligands binding to two equivalent sites on a target protein is a 2-fold increase of the binding free energy. The \( K_D \leq 50 \) nM for a monomer ZpA963 binding to the Z domain corresponds to a binding free energy \( \Delta G_{\text{bind}} \leq -9.8 \text{ kcal mol}^{-1} \) at room temperature, giving a maximum achievable binding free energy of \( \Delta G_{\text{bind}} \leq -14.5 \text{ kcal mol}^{-1} \) (\( K_D \sim 2.5 \) nM) for dimeric binding. With the (ZpA963)\(_2\)htt head-to-tail dimer we obtain \( \Delta G_{\text{bind}} \leq -19.6 \text{ kcal mol}^{-1} \) (\( K_D \sim 1.5 \)) for dimeric binding. This entropy loss can only be overcome with a completely rigid linker that fixes the two subunits in the correct orientation for binding. A second source of binding free energy loss is any rearrangement of the protein A domains that is required to accommodate two covalently linked ligands compared with two ligands that are not linked.

The kinetics for binding of dimeric Affibody constructs to protein A is complex. We argue that the complexity arises from different binding modes at high and low levels of saturation as depicted schematically in Fig. 5D. However, other mechanisms might also be at work. It is for instance not clear to what extent the protein A domains need to reorganize to receive the Affibody dimers as two-site ligands, as mentioned above, and such conformational reorganization should be reflected also in the binding kinetics. It is possible that the dimeric constructs are both suboptimal for binding two protein A domains in a favorable conformation, in which the binding
motif matches the (unknown) preferred domain architecture of protein A. Conformational rearrangements and steric restrictions then presumably also explain different binding affinities of the (ZpA963)2 htt and (ZpA963)2 Cys dimers. In this case, a slightly longer linker and/or head-to-tail arrangement of monomers in (ZpA963)2 htt appears to be more favorable for accessing two sites on protein A than coupling of the C-termini at helix 3 with a shorter linker into the (ZpA963)2 Cys symmetric dimer.

Finally, we note that affinity improvement by using multimeric Affibody constructs to bind repeated or homologous domains of a target could be a useful tool in protein engineering. Many potential target proteins, such as multi-domain enzymes or cell surface proteins, contain repeated homologous domains. Repetitive surface units are also present and available for binding in fibrous protein structures, in amyloid and in the protofibrils that in many cases are precursors to amyloid formation. Our results suggest that linking of monomeric binding modules in these cases can provide a straightforward way to obtain higher affinity binding molecules.

Acknowledgements

We thank Mrs Jenny Björs at Affibody AB for technical assistance and Dr Bertil Macao at the University of Gothenburg for assistance with protein production and mutagenesis. This work was supported by grants to T.H. from the Swedish Research Council (VR 621-2011-5812) and by GE Healthcare Bio-Sciences AB.

References

M. Lindborg et al.


