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Purification Systems Based on Bacterial Surface Proteins

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1. Introduction

Affinity purification is based on the selective and reversible interaction between two binding partners, of which one is bound to a chromatography matrix and the other may be either a native target protein or a recombinant protein fused with an affinity tag (Cuatrecasas et al. 1968). Recombinant DNA-technology allows straightforward construction of gene fusions to provide fusion proteins with two or more functions. The main intention is to facilitate downstream purification; however gene fusions may also improve solubility and proteolytic stability and assist in refolding (Waugh 2005). There are many fusion partners for which commercially available purification systems exist, ranging in size from a few amino acids to whole proteins (Flaschel & Friehs 1993; Terpe 2003). A commonly used purification handle is the poly-histidine (His) tag, enabling purification of the recombinant protein on a column with immobilized metal ions (Hochuli et al. 1988). Other commonly used tags include the FLAG peptide (binding to anti-FLAG monoclonal antibodies), the strep-tag (binding to streptavidin), glutathione S-transferase (binding to glutathione) and maltose binding protein (binding to amylose) (Terpe 2003). Many affinity chromatography strategies also exist for the purification of native proteins, however these are slightly less specific and generally purify classes of proteins, as individual proteins each need a specific ligand. Today, many different ligands are available that can separate specific groups of proteins, for example phosphorylated, glycosylated or ubiquitinylated proteins (Azarkan et al. 2007).

Several bacterial surface proteins that show high affinity against different host proteins as immunoglobulins (Ig:s) and serum albumin, but also other host serum proteins, have been identified, see table 1 for examples. These proteins have different specificities regarding species and immunoglobulin classes and also bind to different parts of the immunoglobulin molecules. Therefore they have proven to be highly suitable for applications within protein purification. Many such proteins are expressed by pathogenic strains of the *Staphylococci* and *Streptococci* genera, and one biological function of these surface proteins is to help the bacteria evade the immune system of the host by covering the bacterium with host proteins (Achari et al. 1992; Sauer-Eriksson et al. 1995; Starovasnik et al. 1996). A significant property of serum albumin is the capability to bind other molecules and act as a transporter in the

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blood. Bacteria able to bind albumin may therefore also benefit by scavenging albumin-bound nutrients (de Chateau et al. 1996). One of the most studied immunoglobulin-binding proteins is the surface-exposed protein A of *Staphylococcus aureus*. Several animal models have demonstrated a decreased virulence for mutants of *S. aureus* that lack Staphylococcal protein A (SPA) on their surface (Foster 2005). Another staphylococcal surface protein, *S. aureus* binder of IgG (Sbi), has also been described (Atkins et al. 2008; Zhang et al. 1998). Several cell surface proteins binding immunoglobulins and other host proteins have also been discovered in *Streptococcus* strains. Streptococcal protein G (SPG), which binds both to immunoglobulins and serum albumin of different species (Kronvall 1973), is the most investigated. Proteins M, H and Arp (short for IgA receptor protein) are expressed by the human-specific pathogen group A streptococci and have different specificities (Akerstrom et al. 1991; Akesson et al. 1990; Fischetti 1989; Lindahl & Akerstrom 1989; Smeesters et al. 2010). Protein L is expressed by the anaerobic bacterial species *Finegoldia magna* (formerly known as *Peptostreptococcus magnus*). It has been shown that this protein binds to the light chains of human IgG molecules (Bjorck 1988). Another protein expressed by *F. magna* is the peptostreptococcal albumin-binding protein (PAB), which displays high sequence similarity with the albumin-binding parts of SPG. However, the species specificity differs somewhat and PAB binds mainly to albumin from primates (Lejon et al. 2004). Protein B, which is expressed by group B streptococci, binds exclusively to human IgA of both subclasses as well as its secretory form (Faulmann et al. 1991).

Among the identified staphylococcal and streptococcal immunoglobulin-binding surface proteins, SPA (Grov et al. 1964; Oeding et al. 1964; Verwey 1940) and SPG (Bjorck & Kronvall 1984) have been subjects for substantial research and have found several applications in the field of biotechnology. SPA exists in different forms in various strains of *S. aureus*, either as a cell wall component, or as a secreted form (Guss et al. 1985; Lofdahl et al. 1983). This indicates that the function of SPA stretches beyond only immune system evasion and SPA has for example been shown to activate TNFR1, a receptor for tumor necrosis factor-α (TNF-α), with pneumonia as a possible outcome (Gomez et al. 2004). SPA includes five homologous immunoglobulin-binding domains that share high sequence identity (Moks et al. 1986). SPG contains, apart from two or three regions binding to IgG, also two or three homologous domains binding serum albumin, depending on the strain (Kronvall et al. 1979). Although they differ somewhat regarding sequence length, there is great homology between the variants (Olsson et al. 1987). The IgG-binding domains of SPG differ from their counterparts in SPA, regarding subclass and species specificity as well as structure (Bjorck & Kronvall 1984; Gouda et al. 1992; Gronenborn et al. 1991; Kronvall et al. 1979). Today, SPA and SPG are widely used in different biotechnological areas, the most widespread being affinity purification of antibodies and proteins fused with the fragment crystallizable (Fc) antibody region. Other applications are for example depletion of IgG or albumin from serum and plasma samples (Fu et al. 2005; Hober et al. 2007). The selective affinity of SPA and SPG for different immunoglobulin types enables efficient isolation of specific antibody subclasses from an immunoglobulin mixture. SPA and SPG bind both to the Fc- and fragment antigen-binding (Fab)-portions of the antibody, the latter enabling purification also of antibody fragments (Akerstrom et al. 1985; Erntell et al. 1988; Jansson et al. 1998). The history behind these proteins, along with their structural and binding properties will be discussed in section 2. In this section we will also cover some applications of SPA and SPG in protein purification and related areas. As both proteins consist of

repeated homologous domains, a natural development has been to investigate the utility of them individually. In section 3 we introduce how these domains have been generated and how they have found applicability in the protein purification field. With the recombinant DNA technology, it has become more feasible to create proteins with new properties and several improvements have been made to the domains of SPA and SPG regarding for example stability and binding specificity using rational design or combinatorial engineering. Modified domain variants have proven to be very useful as ligands in affinity purification of antibodies and as fusion partners for purification of target proteins. The engineered proteins have been used in a wide range of applications, including affinity chromatography and depletion. These efforts are presented in section 4, where we also discuss possible future developments.

Protein	Origin	Binding specificity
Staphylococcal protein A (SPA)	*S. aureus*	IgG, IgM, IgA of different species
S. aureus binder of IgG (Sbi)	*S. aureus*	IgG of different species (weak binding to IgM)
Streptococcal protein G (SPG)	Group C and G streptococci	IgG and albumin of different species
Protein M	Group A streptococci	Human IgG, IgA, albumin among others
Protein H	Group A streptococci	Human IgG
Protein Arp	Group A streptococci	Human IgA (weak binding to IgG)
Protein B	Group B streptococci	Human IgA
Protein L	*F. magna*	Human IgG
Peptostreptococcal albumin-binding protein (PAB)	*F. magna*	Human albumin

Table 1. Overview of some staphylococcal and streptococcal surface proteins that bind different immunoglobulin classes, albumin and other host serum proteins.

2. Protein A and protein G applied in protein purification

SPA and SPG represent the best-characterized bacterial surface proteins. Several structures of their immunoglobulin- and albumin-binding, in the case of protein G, domains have been solved. Species specificities and affinities of the full-length proteins as well as individual domains have been determined. Based on the interesting properties and accumulated knowledge regarding these proteins, they have found many different applications in the field of biotechnology. In this section, we will first present some background information on the proteins, before describing some examples of where the proteins have been utilized in different applications related to protein expression and purification.

2.1 Staphylococcal protein A

The interaction between SPA and IgG has been widely studied and SPA has for a long time been used as a tool in many biotechnological applications (Langone 1982). The molecule was discovered already in 1940, when extraction of cells of the J13 strain of *S. aureus* yielded an antigenic fraction, which was found to consist of proteins (Verwey 1940) and the protein received its name in 1964 (Oeding et al. 1964). It was observed in 1958 that SPA stimulated an immune response in rabbits, wherefore it was believed that SPA participated in an antigen-antibody interaction. However, it was later shown that the observed interaction between SPA and the immunoglobulin did not involve the antigen-binding site, but rather the constant Fc-region and the interaction was therefore denoted a “pseudo-immune”

reaction (Forsgren & Sjoquist 1966). This interaction causes many immunological effects similar to an antigen-antibody interaction, including complement activation and hypersensitivity reactions (Martin et al. 1967; Sjoquist & Stalenheim 1969).

The gene for SPA was sequenced in 1984 (Uhlen et al. 1984) and the corresponding protein was shown to be a surface protein of about 58 kDa consisting of a single polypeptide chain. The protein can be divided into three regions with different functions. The N-terminal part consists of a signal peptide (Ss) followed by five homologous IgG-binding domains (E, D, A, B and C) and the C-terminal region (X and M) anchors the protein to the bacterial cell wall (Abrahmsen et al. 1985; Guss et al. 1984; Lofdahl et al. 1983; Moks et al. 1986; Schneewind et al. 1995; Uhlen et al. 1984), see figure 1.

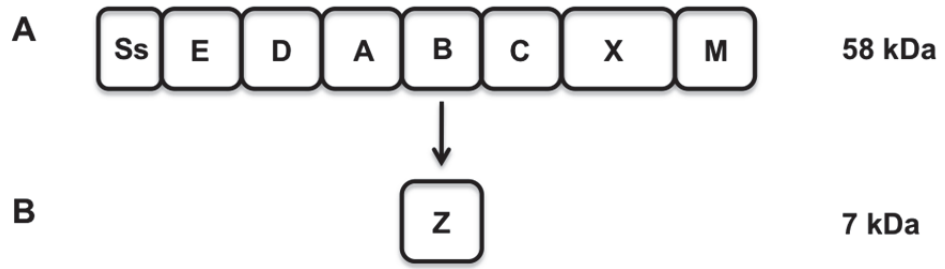

Fig. 1. (A) Organization of the different regions of SPA; An N-terminal signal sequence (Ss), which localizes the protein to the cell surface, five homologous IgG-binding domains (E, D, A, B and C) and two domains for anchoring of the protein to the cell wall (X and M). (B) The IgG-binding Z-domain, which is an engineered version of the B-domain, discussed in section 3.

SPA is produced by many strains of *S. aureus* and most of them typically produce a cell wall-bound variant. Usually about 85% of the protein is anchored to the cell wall whereas 15% exists as a soluble protein in the cytoplasm, however some strains produce the soluble variant exclusively (Movitz 1976). SPA is produced in the form of a precursor protein that contains a 36 amino acid N-terminal signal sequence, which directs the protein to the cell wall before it is cleaved off. There is a high sequence identity between the five IgG-binding domains. A "homology gradient" along the protein sequence has been established as two regions lying next to each other show a higher degree of sequence identity than two domains situated further apart. This indicates that the IgG-binding domains have evolved through step-wise gene duplications. The gene sequence of SPA reveals an unusually large number of changed nucleotides compared to changed amino acids, indicating that an evolutionary pressure has aimed to preserve the primary amino acid sequence (Sjodahl 1977; Uhlen et al. 1984). The sequence similarity between the five domains varies between 65-90% (Starovasnik et al. 1996), see figure 2. The E- and C-domains, situated closest to the N- and C-terminus, respectively, exhibit higher sequence dissimilarity when compared to the other domains. The C-domain seems to have diverged more to the cell wall anchoring part X, however without affecting the IgG-binding affinity (Jansson et al. 1998; Sjodahl 1977). Region X anchors the protein to the bacterial cell wall by binding to peptidoglycan with the N-terminus, thereby exposing the IgG-binding regions to the extracellular space (Schneewind et al. 1995; Sjodahl 1977; Ton-That et al. 1997).

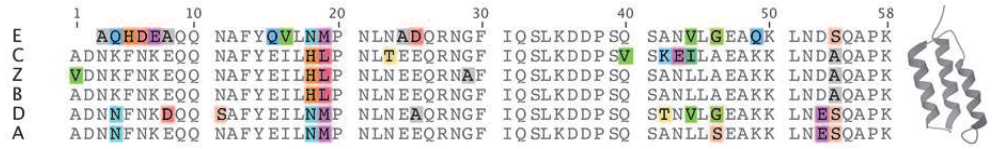

Fig. 2. Sequence alignment of the five immunoglobulin-binding domains of SPA and the synthetic Z-domain. Differences are highlighted (Nilsson, B. et al. 1987). The structure of the domains is also shown. (Reconstructed from PDB structure 1Q2N) (Zheng et al. 2004).

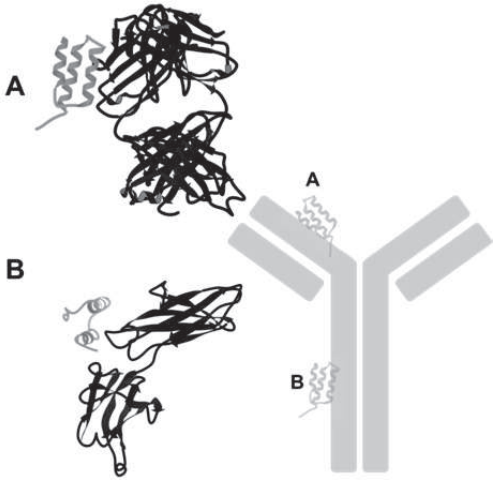

Fig. 3. Overview of binding sites of domains from SPA to (A) Fab and (B) Fc (Reconstructed from PDB structures 1DEE and 1FC2) (Deisenhofer 1981; Derrick et al. 1999; Graille et al. 2000; Lejon et al. 2004; Sauer-Eriksson et al. 1995).

The first crystallographic structure of the B-domain in complex with Fc showed a structure of two helices and the part corresponding to the third helix being irregularly folded (Deisenhofer 1981). However, further studies have been performed on several of the IgG-binding domains and a triple helix conformation has been determined in both the bound and unbound state. No significant difference in structure between the domains has been observed (Deisenhofer 1981; Gouda et al. 1992; Graille et al. 2000; Starovasnik et al. 1996) and only a minor side chain rearrangement of a phenylalanine occurs upon binding, as observed in the E-domain (Starovasnik et al. 1996). Each IgG-binding domain of SPA recognizes two separate binding sites on the immunoglobulin molecule located on the Fc and Fab parts, respectively (figure 3).

Eleven amino acids have been suggested to be important for the Fc interaction. All of them are situated in the first two helices of each IgG-binding domain and they are highly conserved within the five homologous regions (Deisenhofer 1981; Moks et al. 1986; Uhlen et al. 1984). Even though the third helix is not involved in Fc binding, it has been shown that it has structural importance. For example, truncated mutants of domain B where residues corresponding to the third helix are deleted have decreased affinity to Fc due to an overall loss in stability (Gouda et al. 1992). A region at the CH2 and CH3 interface of Fc interacts

with SPA (Graille et al. 2000), but neither CH2 or CH3 can bind to SPA independently (Haake et al. 1982). Region E, which is situated closest to the signal peptide, differs the most from the other domains in this region (Moks et al. 1986). When comparing all five domains, domain B shows the lowest number of substitutions and its sequence can therefore be seen as a consensus sequence for all IgG-binding domains (Uhlen et al. 1984). Several binding studies have shown an approximate affinity to Fc of 5 nM for SPA and between 10-100 nM for the individual domains as determined by Surface Plasmon Resonance (SPR) (Karlsson et al. 1995; Nilsson, J. et al. 1994; Roben et al. 1995). A decreased affinity to Fc has been observed for the E-domain compared to the other four domains, which demonstrate similar affinities (Jansson et al. 1998; Moks et al. 1986). However, even though individual domains demonstrate similar affinities to Fc, a greatly increased apparent affinity is observed when several domains are combined (Ljungberg et al. 1993). Despite the fact that SPA contains five IgG-binding domains, it only has the capacity to bind on average 2.5 IgG molecules simultaneously (Ghose et al. 2007).

Besides IgG, SPA also interacts weakly with IgM and IgA. However, those low affinity interactions involve the Fab part of the antibody rather than Fc (Inganas 1981; Ljungberg et al. 1993). There have been contradictions regarding the ability of individual IgG-binding domains to bind to Fab, and initially only certain domains where proposed to be responsible for the binding (Ljungberg et al. 1993). However, it was later shown that all five domains bind both Fc and Fab individually. The affinity of SPA to Fab has been determined and dissociation equilibrium constants in the range of 2-200 nM, depending on the VH3 genes, have been reported. The affinity of individual domains is lower and reported numbers lay in the range 100-500 nM as determined by SPR (Jansson et al. 1998; Roben et al. 1995). The crystal structure of domain D bound to Fab of human IgM has been solved and the binding site is non-overlapping with the binding site to Fc. The binding site on the SPA domains involves highly conserved residues in the second and third helix and the loop in-between (Graille et al. 2000). The binding site on Fab involves residues situated on four β-strands in the VH region, see figure 3B. The interactions involve mainly polar side chains, as opposed to the binding between SPA and Fc, where the binding site is mainly hydrophobic (Graille et al. 2000). Only one position in each domain of SPA participates in both the interaction with Fc and Fab and its contribution is small in both cases. A single domain of SPA can bind to Fc and Fab simultaneously, as has been shown for domain D in an enzyme-linked immunosorbent assay (ELISA) (Roben et al. 1995) and for domain E in competition assays using affinity chromatography and calorimetry (Starovasnik et al. 1999).

IgG origin	SPA binding	SPG binding
Human	Yes*	Yes
Mouse	Yes**	Yes
Rat	No	Yes
Rabbit	Yes	Yes
Cow	Yes	Yes
Goat	No	Yes

Table 2. Some examples of IgG-binding specificities of SPA and SPG (* IgG1, IgG2, IgG4 and some IgG3, ** IgG2 an IgG3 but not IgG1).

SPA binds to IgG from different species, with varying affinity. In one study, a competition assay was used to analyze the binding to IgG of sera from 80 animals. (^{125}I)SPA was incubated with sera and the fraction of non-bound protein was analyzed by capturing the protein on IgG-coupled beads. The results showed a 10^6-fold variation in affinity between species (Richman et al. 1982). SPA binds to IgG from for example human, mouse and rabbit, but not rat (Reis et al. 1984; Richman et al. 1982), see table 2. The protein is not only species-specific, but also subclass-specific and binds for example to murine IgG2 but not IgG1 and to all human IgG subclasses except IgG3. The interaction between SPA and IgG does however not seem to be entirely subclass-specific. Some allotypes of human IgG3 bind to SPA, whereas some do not and a possible explanation includes an amino acid in a loop of CH3, which is either a histidine or an arginine (Haake et al. 1982; Reis et al. 1984; Scott et al. 1997). The affinity against human IgG1 has been shown to be higher than the binding against IgG2 and IgG4 (Reis et al. 1984) and binding is only observed to Fab parts from the human VH3 family and their homologues in other mammalian species. However, this is a common family from which about 50% of inherited VH genes originate (Graille et al. 2000).

2.2 Streptococcal protein G

SPG was discovered in 1973, when it was found that β-hemolytic streptococci carried IgG-binding proteins on their surfaces (Kronvall 1973). Different groups of streptococcal strains were determined to bind immunoglobulins with different affinities. Group A streptococci infect only humans, while group C and G streptococci also commonly infect animals (Myhre & Kronvall 1977). These observed differences led to the introduction of a classification system, where SPA was classified as a type I Fc-binding protein, SPG from group A streptococci as type II and proteins from human group C, G and L streptococci as type III proteins (Myhre & Kronvall 1977). There is no major difference between the binding characteristics of streptococcal proteins from groups II and III when it comes to human IgG subclasses (Kronvall et al. 1979). Bovine group G streptococci together form the type IV Fc receptor group. They show a limited specificity and bind weakly to human IgG (Myhre & Kronvall 1981). Group C streptococcal strains from the species *S. zooepidemicus* form another group, with properties similar to SPA with regard to human IgG specificity, however this group differs from the type I proteins when it comes to specificity to non-human IgG:s (Myhre & Kronvall 1980). The protein G molecules from groups C and G have the same principal structures and share high sequence similarity (Sjobring et al. 1991). Apart from binding to IgG, SPG can also bind serum albumin (Kronvall et al. 1979). There are many different group C and G streptococcal strains and they have been classified into three different groups based on size and binding patterns. SPG from certain strains have lost their serum albumin-binding capacity and the affinity against IgG differs about ten times between different strains. That protein G from all strains bind IgG, while the affinity against albumin has been lost in some strains, indicates that the evolutionary pressure on keeping the immunoglobulin-binding properties of SPG is greater than keeping the affinity against albumin. Hence, IgG-binding would seem to be essential for the bacteria, while binding to albumin seems less critical (Sjobring et al. 1991). Two strains that have been widely studied are G148, containing three IgG-binding and three serum albumin-binding domains and GX7809, containing two of each (Olsson et al. 1987). Apart from this, there is a very high sequence homology between SPG from the two strains (>99%) and only five mutations

(including two silent) have been observed. This indicates a very recent divergence of the two proteins or a deletion of part of the gene (Olsson et al. 1987). In this chapter, we will focus primarily on SPG from group G streptococci and the Fc-binding proteins type III.

Based on the nucleotide sequences of G148 and GX7809 (Fahnestock et al. 1986; Guss et al. 1986), the protein has been divided into several regions. An N-terminal signal sequence (Ss), followed by a serum albumin-binding region (A1-A3) and an IgG-binding region (C1-C3) separated by a spacer region (S). The protein also includes a region that anchors it to the bacterial cell wall (W) (Akerstrom et al. 1987; Olsson et al. 1987), see figure 4. The C-terminal IgG-binding domains are denoted C1-C3 or B1-B2, depending on the strain. However, B1 and B2 are identical to C1 and C3, respectively (Achari et al. 1992; Fahnestock et al. 1986; Sauer-Eriksson et al. 1995), wherefore from now on in this text the domains will be referred to as C1-C3. The IgG-binding domains are very stable, despite the lack of stabilizing disulfide bonds (Achari et al. 1992). They each constitute 55 amino acids and are separated by two 15 amino acid spacers, D1 and D2 (Guss et al. 1986; Lian et al. 1992). There is only a two amino acid difference between C1 and C2, four additional substitutions exist between C1 and C3 and consequently four amino acids differ between C2 and C3 (Achari et al. 1992; Gronenborn & Clore 1993; Sauer-Eriksson et al. 1995), see figure 5. Despite the high sequence similarity, the affinity against IgG is different for the different domains (Lian et al. 1992). Similarly to SPA, there is a homology gradient between the domains, which indicates that they have arisen through gene duplications (Guss et al. 1986). It seems as if initially, one IgG-binding domain was split into the C1 and C3 parts with a spacer in-between. The C2-domain seems to originate from both of these domains; the N-terminal end from C1 and the C-terminal end from C3. This is further indicated as the two spacers D1 and D2 are identical and probably diverged relatively recently (Guss et al. 1986; Olsson et al. 1987). With this evolutionary explanation, one is inclined to believe that protein G from strain GX7809, which contains only two IgG-binding domains, represents a variant that stayed at the intermediate stage (Olsson et al. 1987). Comparisons made to the IgG-binding domains of SPA reveal no sequence homology even though the proteins compete for the same binding site on IgG and the two different IgG-binding domains are therefore an excellent example of

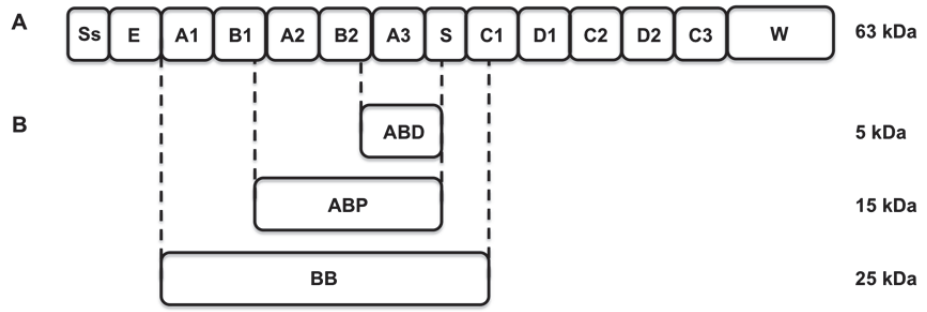

Fig. 4. (A) Organization of the different regions of SPG; An N-terminal signal sequence (Ss), which localizes the protein to the cell surface, a serum albumin-binding region (A1-A3) and an IgG-binding region (C1-C3) separated by a spacer region (S) and a part for anchoring of the protein to the cell wall (W). (B) Derivatives of the serum albumin-binding region that have been used in biotechnological applications.

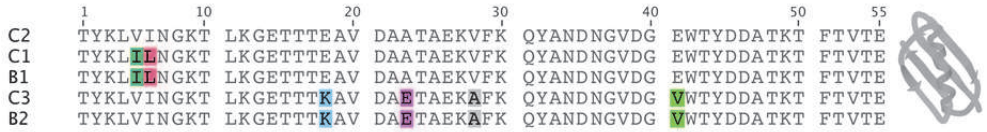

Fig. 5. Sequence alignment of the immunoglobulin-binding domains of SPG. Differences are highlighted (Fahnestock et al. 1986; Guss et al. 1986). The structure of the domains is also shown (Reconstructed from PDB structure 1FCC) (Sauer-Eriksson et al. 1995).

convergent evolution (Frick et al. 1992; Olsson et al. 1987). This is further strengthened by the notion that an eleven amino acid long peptide binding to Fc can inhibit binding of both SPA and SPG (Frick et al. 1992). The structure shared by the IgG-binding domains of SPG is different from that of the domains of SPA. Several studies have revealed it to be a four-stranded β-sheet of two β-hairpins connected by an α-helix and short loop regions (Achari et al. 1992; Gronenborn et al. 1991; Lian et al. 1992; Lian et al. 1991; Sauer-Eriksson et al. 1995).

SPG from strain G148 has three serum albumin-binding domains, whereas SPG from GX7809 has only two, each of about 46 amino acids. The differing number of repeats can be explained similarly as for the IgG-binding domains (Kraulis et al. 1996; Kronvall et al. 1979; Olsson et al. 1987). The binding sites of IgG and serum albumin on SPG are situated on opposite sides of the molecule and IgG cannot inhibit the binding of SPG to serum albumin (Bjorck et al. 1987). The structure of the albumin-binding unit is very similar to the structure of the IgG-binding domains of SPA, although the helices differ somewhat in length (Gouda et al. 1992; Kraulis et al. 1996). This interesting observation suggests a possible evolutionary relationship despite the lack of sequence homology (Kraulis et al. 1996). No structural data exists on the complex between human serum albumin (HSA) and the albumin-binding domains of SPG, however a complex between albumin and a highly sequence similar albumin-binding domain, the second GA (G-related albumin-binding)-module of PAB (sometimes referred to as ALB8-GA) has been determined using both NMR and crystallography. No significant structural change was observed upon binding for either of the two proteins (Cramer et al. 2007; Johansson et al. 1997; Lejon et al. 2004). The GA-module shows almost 60% sequence identity to the albumin-binding domains of SPG and it is even likely that the GA-module originates from these domains, wherefore this structural data is likely to also correspond to the albumin-binding domains of SPG (de Chateau & Bjorck 1994; de Chateau et al. 1996), see figure 6. At least 16 homologous albumin-binding domains from four different bacterial species have been identified (Johansson et al. 2002a). Taken together, this indicates that the fold, stability and mode of interaction of the homologs are very similar (Johansson et al. 1997; Lejon et al. 2004).

The interaction surface between Fc and the SPG-domain (C1-C3) can be divided into three centers; region I is a network of hydrogen bonds and consists of two residues in the center of the α-helix separated by three residues so that they are pointing in the same direction, region II also contains amino acids in the α-helix, very close to the two from region I and also separated by three residues. Region III includes one residue in the C-terminal end of the α-helix, two residues in the N-terminus of the third β-strand and two in the loop connecting

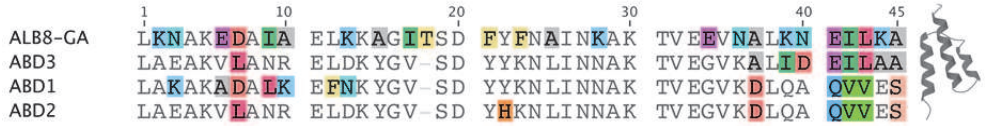

Fig. 6. Sequence alignment of the albumin-binding domains of SPA together with the second GA-module derived from *F. magna* (ALB8-GA). Differences are highlighted and only the 44-45 amino acid motifs that are most highly conserved are displayed. The structure of the domain is also shown (Reconstructed from PDB structure 1GJT) (Johansson et al. 2002a).

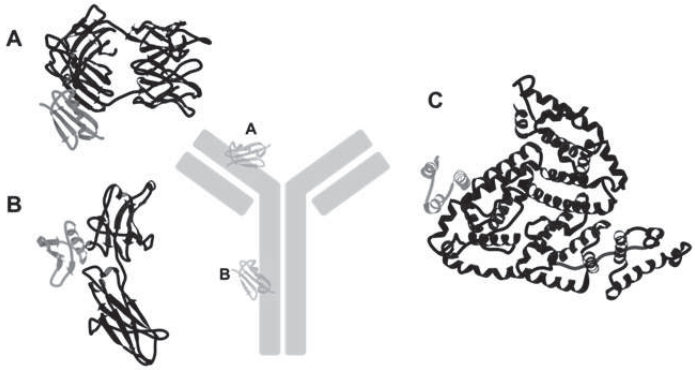

Fig. 7. Overview of binding sites of IgG-binding domain C2 from SPG to (A) Fab and (B) Fc. The binding of the GA-module of PAB to HSA is shown in (C) (Reconstructed from PDB structures 1QKZ, 1FCC and 1TF0) .

them (Sauer-Eriksson et al. 1995). SPG-domains bind to the cleft between CH2 and CH3 (figure 7A), as opposed to the domains of SPA, which bind more on the CH2 side of Fc. However, in the complex, the third strand of the SPG-domain is situated approximately in the same region as the first Fc-binding helix of the SPA-domain. Consequently, SPA and SPG cannot simultaneously bind the same Fc-molecule (Stone et al. 1989). Furthermore, the interactions between SPG and Fc involve many charged and polar residues, forming hydrogen bonds and salt bridges while the binding between SPA and Fc involve mostly hydrophobic interactions (Sauer-Eriksson et al. 1995). The strength of the binding to Fc has been determined using SPR to around 20-100 nM for the C2-domain and low nanomolar values for the whole SPG molecule have been reported (Akerstrom & Bjorck 1986; Gulich et al. 2002; Sagawa et al. 2005). The binding of SPA and SPG to Fc is pH-dependent. SPG binds most efficiently to IgG at pH 4-5 and the binding is weakened with increased pH. SPA on the other hand, binds with highest affinity at pH 8 (Akerstrom & Bjorck 1986). This difference is due to the composition of the interaction interfaces. C3 has higher affinity to Fc than C1 and C2, all differences in binding affinity have been explained based on structural data (Lian et al. 1992; Sauer-Eriksson et al. 1995). The difference in binding can partly be explained by the existence of a carboxylic acid in the binding site of SPG-domain C3, a position in the α-helix, which is an alanine in C1 and C2 (Achari et al. 1992; Sauer-Eriksson et al. 1995). It has also been speculated that the substitution Glu/Val located on the third β-strand in C1/C3 would improve binding to Fc, this has however been argued (Gronenborn & Clore 1993; Sauer-Eriksson et al. 1995).

Similarly to SPA, SPG also has the capability to bind Fab although the binding strength is about ten times weaker than the affinity to Fc (Bjorck & Kronvall 1984; Erntell et al. 1983; Erntell et al. 1988; Sagawa et al. 2005). The affinity constant for domain C2 has been determined to around 150 nM (Sagawa et al. 2005). As for SPA, the binding sites to Fc and Fab are not overlapping. The α-helix does not participate in the Fab-binding, but instead the second β-strand forms an extended β-sheet structure with the last β-strand of the CH1-domain of Fab, see figure 7B, and the interactions are mainly between main chain atoms (Sauer-Eriksson et al. 1995). The third IgG-binding domain of SPG has been analyzed in a crystal complex with Fab and forms twelve hydrogen bonds with Fab residues. Eight of these are between SPG and main-chain atoms on the Fab CH1-domain, contributing to the broad specificity of SPG to different IgG subclasses and species. The remaining four hydrogen bonds are between SPG and side chain atoms on the Fab CH1-domain, and these amino acids are highly conserved among γ heavy chain subclasses (Derrick & Wigley 1994). SPG binds well to IgG whereas, in contrast to SPA, no binding has been observed to IgA or IgM (Achari et al. 1992; Bjorck & Kronvall 1984; Kronvall et al. 1979). However, SPG has broader subclass specificity than SPA and it binds to all four subclasses of human IgG, whereas SPA shows no affinity against certain human allotypes of IgG3. A histidine residue in a loop on CH3, situated in the binding interface of SPA and IgG, may explain this observation. Histidine possibly blocks the interaction and in allotypes of IgG3 binding to SPA, this amino acid has been substituted for an arginine. In SPG, this residue does not participate in the Fc-binding, which explains why SPG binds well to all IgG3 allotypes (Haake et al. 1982; Sauer-Eriksson et al. 1995; Shimizu et al. 1983). SPG has also been shown to bind more strongly than SPA to IgG from several species including human, mouse, rat, cow, rabbit and goat (Akerstrom & Bjorck 1986; Akerstrom et al. 1985; Fahnestock et al. 1986). However, there have been contradictions to this statement and results showing no significant difference in the binding strengths of SPA and SPG have also been published (Guss et al. 1986; Kronvall et al. 1979). The abilities of SPA and SPG to bind a large number of IgGs from different species were determined using a competitive ELISA setup (GE Healthcare, Antibody Purification Handbook 18-1037-46, 2007). From this study none of the proteins could be denoted the overall superior binder; they have different advantages.

Early biochemical analysis of the interaction between SPG and HSA revealed that the binding site involved mainly the second domain of HSA and engages loops 7-8 (Falkenberg et al. 1992). Isolation of a small fragment of HSA corresponding to these residues inhibited the binding between intact HSA and SPG (Falkenberg et al. 1992). This finding also suggested that all albumin-binding domains of SPG share the same epitope on HSA, which is in concordance with the evolutionary hypothesis that gene duplications gave rise to the multiple homologous domains. Each of the three albumin-binding regions in SPG is approximately 5 kDa in size (Johansson et al. 2002a). The third domain has been most extensively investigated. It consists of 46 amino acids folded into a three helical bundle as determined by nuclear magnetic resonance (NMR) (Kraulis et al. 1996). The sequence is devoid of cysteines and the structure does not depend on any stabilizing factors such as bound ligands, metal ions or disulphide bonds. This is true also for the larger constructs containing two and a half (ABP) or three (BB) sequential domains (Stahl & Nygren 1997), see figure 4. NMR-perturbation studies have indicated that the albumin-binding residues are mainly localized to the second helix and the loop preceding it (Johansson et al. 2002b). Those observations are in agreement with a mutational analysis of the binding site to HSA on the

albumin-binding domain from SPG (Linhult et al. 2002). By comparing the binding of different point mutants as well as a few double and one triple mutant, the binding site was deduced to reside mainly in the second helix. The first helix does not take part in the binding and only small parts of the third helix are involved (Linhult et al. 2002). It has also been demonstrated that a variant with a truncated N-terminus has a significantly reduced affinity against HSA (Johansson et al. 2002a). Only five N-terminal amino acids differ somewhat in a crystal structure of the domain compared to the NMR-data. In the crystal structure of the GA module, amino acids are more ordered and extend the first helix by an additional turn. The GA-module mainly binds to the surface of domain II of HSA. The structural data from the complex between the GA-module and HSA shows that the binding interface is of hydrophobic nature with two bordering hydrogen bond networks (Lejon et al. 2004). The binding surface is centered around a tyrosine of the GA-module in a hydrophobic cleft on HSA. This residue, as well as the flexibility of the backbone structure, has been linked to the broader host specificity of the albumin-binding domain derived from SPG compared to the GA-module from *F. magna* (He et al. 2006; Johansson et al. 2002b; Lejon et al. 2004). Bacteria that express the GA-module have only been isolated from humans, whereas streptococci expressing SPG are known to infect all mammalian species (Johansson et al. 2002b). This may also explain the higher affinity of the GA-module against HSA as compared to the domains derived from SPG (Johansson et al. 2002a). The third albumin-binding domain of SPG binds strongly to human, mouse, rabbit and rat serum albumin, among others, with a low nanomolar affinity as determined by SPR. It binds less efficiently to hen and horse serum albumin and only weak or no binding is observed to albumins of bovine origin (Falkenberg et al. 1992; Johansson et al. 2002a; Linhult et al. 2002; Nygren et al. 1990; Raeder et al. 1991). When comparing affinities against IgG and albumin from different species, it is common that strong binding to one of the molecules means weaker binding to the other. The only exception is man, as SPG binds well to both human IgG and human albumin (Nygren et al. 1990).

2.3 Utilization of full-length SPA and SPG in protein purification

SPA and SPG are today used in a number of applications concerning protein purification. The most widespread application is the use of SPA and SPG coupled to chromatography resins for the purification of antibodies and Fc-tagged recombinant proteins (Lindmark et al. 1983; Ohlson et al. 1988). Even though both SPA and SPG bind to antibodies, as mentioned above they bind with different specificities to immunoglobulins of various species and subclasses. This makes the proteins suitable for slightly different applications. Furthermore, SPA has a higher stability than SPG, making it more suitable for large-scale, industrial applications (Boyle 1990; Hober et al. 2007). SPG is usually the protein of choice when isolation of the total IgG fraction of a sample is desired. Due to its broader specificity, SPG would generate a higher yield of antibody from a sample containing for example human antibodies of different subclasses. On the other hand, SPA can be a better choice for isolation of specific subclasses of IgG. SPA has been reported to separate mouse IgG1, IgG2a and IgG2b in pure fractions. Surprisingly, in this study, mouse IgG1 was found to bind to the SPA columns under certain conditions with very high salt concentrations. The binding of SPA to IgG is pH sensitive and different pH were used to separately elute IgG1, IgG2a and IgG2b from the column (Ey et al. 1978). Affinity membranes with SPA or SPG have also been used to purify human and murine IgG (Dancette et al. 1999). An important issue in

chromatography is the possibility to clean the column after purification to be able to reuse it. This is most efficiently done with high concentrations of NaOH, but unfortunately this is a problem in affinity chromatography since it often results in denaturation of proteinacious ligands. However, SPA has been shown to cope well with high NaOH concentrations with only a small decrease in binding capability (Hale et al. 1994). Use of SPA has been evaluated in therapeutic applications as well, for example in the treatment of autoimmune disorders. Patients with an autoimmune disease produce autoantibodies, which can be removed from the blood by plasma exchange. A simpler way to remove the antibodies, without unwanted removal of all other serum components, is immunoadsorption. IgG is selectively removed from serum by immobilized SPA, however whether or not this is the reason for the success of the treatment is controversial. Two products are currently available on the market for immunoadsorption; the ProSorba® column and the ImmunoSorba® column, which are both accepted by the food and drug administration (Matic et al. 2001; Poullin et al. 2005; Silverman et al. 2005).

An early study demonstrated the successful use of SPG in a western blot setup, where an iodine-labeled variant of SPG was used as a secondary detection reagent. A mixture of antigens was separated on a sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antigen-specific antibodies were bound to the membrane before the iodine-labeled SPG was added for detection (Akerstrom et al. 1985). SPA and SPG have also been used in immunocapture, with the aim to capture target proteins from a complex sample. Antigen-specific antibodies are cross-linked to an SPA or SPG matrix, which ensures correct orientation and hence no blocking of antigen binding sites, leading to a higher yield of immunocaptured material (Kaboord & Perr 2008; Podlaski & Stern 2000; Sisson & Castor 1990).

When analyzing serum samples, the problem arises that there is a huge difference in abundance of different proteins. A concentration range spanning ten orders of magnitude from the least to the most abundant proteins, represented by IgG and albumin, has been reported. This makes it hard to analyze the low abundant proteins, which are often of interest in for example biomarker analysis or plasma profiling (Anderson & Anderson 2002). Depletion of IgG using SPG (Faulkner et al. 2011; Fu et al. 2005) prior to further analysis is common to decrease the complexity of a sample. Different types of matrices are commonly used, such as porous particles, monoliths and affinity membranes (Urbas et al. 2009). There are several products on the market using natural, recombinant or even stabilized derivatives of SPA and SPG on affinity media. The media are mostly agarose, sepharose or acrylamide (Grodzki & Berenstein 2010; Hober et al. 2007). A different approach has been evaluated for depletion of serum proteins using antibody fragments, in combination with SPA and SPG. Specific antibody fragments bind serum proteins and the complexes are subsequently captured using a combined SPA and SPG resin (Ettorre et al. 2006).

SPA has been used as a fusion partner to simplify production and purification of recombinant proteins (Nilsson, B. et al. 1985). SPA is a stable protein and generates functional fusion proteins when produced in different bacterial hosts (Abrahmsen et al. 1985; Nilsson, B. et al. 1985). Different variants of SPA have been used; often the X region has been deleted to hinder the protein from being incorporated into the bacterial cell wall (Nilsson, B. & Abrahmsen 1990; Uhlen et al. 1983). Several properties of SPA make it a good fusion partner; the acidic properties of SPA can help stabilize basic proteins at neutral pH

(Nilsson, B. & Abrahmsen 1990). SPA does not contain any cysteines, which could otherwise interfere with the target protein through formation of disulphide bridges (Uhlen et al. 1983). The secretion signal associated with SPA enables secretion of the fusion protein if the membrane anchoring region of SPA is deleted (Abrahmsen et al. 1985). Introduction of a cleavage site between SPA and the target protein enables cleavage of SPA after purification (Nilsson, B. & Abrahmsen 1990). SPA has also been used as a fusion partner to antigens in the production of antibodies by immunization of animals. In this context the protein acts as an adjuvant to increase the immune response towards the antigen (Lowenadler et al. 1986).

3. Domains of bacterial surface proteins

Both SPA and SPG are multi-domain proteins with several domains filling the same function. A protein domain is defined as the smallest structural unit that alone possess characteristics that are associated with the whole protein (Holland et al. 2006). It can fold independently, and should have the same conformation as when included in the whole protein. Furthermore, a protein domain should be able to function on its own. All IgG- and serum albumin-binding domains of SPA and SPG have these properties and can therefore be used individually. Protein domains have several advantages compared to their full-length ancestors, wherefore a natural development has been to utilize single or multiple IgG- or albumin-binding domains from SPA and SPG as replacements for the full-length proteins. One advantage is their small size, which both decreases the protein production cost and simplifies the production procedure, for example due to a more straightforward folding process. Protein domains also have the advantage of easy characterization, for example structural and binding studies are more easily performed with smaller proteins. The isolated binding property contained in a small domain enables efficient use as capture ligands on columns for affinity chromatography. Alternatively, protein domain(s) may be fused to a recombinant target protein to facilitate recovery by affinity purification on easily prepared IgG or albumin media (Nygren et al. 1988). However, commercial matrices are not widely available, perhaps due to the harsh elution conditions required. The smaller domains have many additional advantages, which make them favorable as fusion partners. (I) Domains of SPA or SPG have surface-exposed termini, assuring that the fusion tag will not interfere with the structure of the fusion protein. (II) They do not contain any cysteines that can form disulphides with the fusion protein and interfere with the folding process. (III) The domains are highly soluble and refold easily after treatment with denaturants, which can aid the refolding of the fusion partner. (IV) Fusion proteins can be produced at high levels in *Escherichia coli* and still remain soluble. (V) It is generally easy to insert cleavage sites for proteases between the fusion tag and the target protein, which enables recovery of native protein. Different hosts have successfully been applied for production of fusion proteins with domains of SPA and SPG, including gram-positive and –negative bacteria, yeast, plant, mammalian and insect cells (Stahl et al. 1997). Protein domains from SPA and SPG, and commonly slightly modified versions of these, are also frequently used as affinity ligands for purification of antibodies, antibody fragments and Fc-fused proteins, which is a common strategy to express proteins in mammalian hosts (Hober et al. 2007; Ljungquist et al. 1989; Lo et al. 1998). In the case of SPG, another advantage with using single domains instead of the full-length protein is the fact that SPG is a dual affinity protein, binding to both IgG and albumin. This dual affinity is a drawback for example in antibody purification, as antibodies

are commonly purified from serum in which serum albumin is present at high abundance. Several examples where advantageous properties of domains of SPA and SPG are exploited are given below.

In this section we focus on different approaches that utilize mono- or multi-domain derivatives of the IgG-binding domains from SPA or the albumin-binding domains from SPG. Those domains have been most widely explored in the context of protein expression and purification. The most thoroughly studied and used IgG-binding domain from SPA is the B-domain; from which the synthetic stabilized Z-domain has been designed. Although the Z-domain is by definition a synthetic domain, its widespread use in a large range of applications makes it a natural focus of this section. In the brief part that follows, the emphasis is on different uses of the immunoglobulin-binding domains of SPG, which have found their main application areas as models in studies of protein folding and dynamics rather than within the field of protein purification. Of the albumin-binding domains of SPG, the third domain has been investigated and utilized the most. Therefore, the use of this domain is the main topic for the section covering domains of SPG.

3.1 Domains of protein A

As mentioned above, each IgG-binding domain of SPA independently folds into a three-helix bundle that can bind to the Fc or Fab region of an antibody. All five IgG-binding domains of SPA have high sequence identity (figure 2), although when comparing them one by one, the IgG-binding domain B was found to contain the least substitutions and it may therefore be seen as a consensus sequence of the IgG-binding domains (Uhlen et al. 1984). A pair of modifications has been introduced into the B-domain, with the aim to increase its stability and potential as a fusion partner. The modified variant of the B-domain has been given the name Z (Nilsson, B. et al. 1987).

3.1.1 The Z-domain derived from the B-domain of protein A

The B-domain of SPA is the most thoroughly studied of the five IgG-binding domains and has been subject to rational improvements yielding the synthetic Z-domain. Two amino acids have been changed, mainly to increase the chemical stability of the protein. An Asn28-Gly29 dipeptide has been changed to Asn28-Ala29 to ensure resistance to hydroxylamine (Nilsson, B. et al. 1987). This facilitates efficient removal of Z after purification, by the introduction of a hydroxylamine cleavage site in the joint between Z and the fusion protein. As the asparagine in the dipeptide cleavage site is believed to be involved in Fc binding, the glycine was instead mutated. The Z-domain also lacks methionines, which makes it stable against proteolytic cleavage with cyanogen bromide (Nilsson, B. et al. 1987). To facilitate the cloning procedure, an *AccI* cleavage site was introduced by exchanging Ala1 to Val1, situated outside the first helical region (Nilsson, B. et al. 1987). All of the five native IgG-binding domains of SPA exhibit binding to both Fc and Fab (Jansson et al. 1998). However, the modifications incorporated in the B-domain to produce Z resulted in a loss of affinity to the Fab-part of the antibody, although the Z-domain retains its Fc-binding capacity along with high stability and solubility. Binding studies show that neither Z, ZZ or a pentameric variant of it bind Fab (Ljungberg et al. 1993). The Fab-interaction involves the second and the third helix of the IgG-binding domains of SPA, and position Gly29 has been shown to be important (Graille et al. 2000). In the Z-domain, this position is mutated to an alanine, which

could explain the loss of binding. As many as ten repeat domains of Z in succession have been expressed in bacteria, the long construct was however susceptible to some homologous recombination even in a RecA-negative host (Nilsson, B. et al. 1987). This observation may explain the high frequency of silent mutations found in the native SPA-gene and suggests a selection pressure to avoid homologous recombination of the regions encoding the domains (Nilsson, B. et al. 1987).

As the original B-domain, the Z-domain consists of 58 amino acids. Despite the substitutions in Z compared to the B-domain of SPA, the structures are very similar and the Z-domain has also been determined to be a three-helical bundle (Jendeberg et al. 1996; Tashiro et al. 1997). Helices two and three are situated in an anti-parallel fashion and the first helix is anti-parallel to the second helix but slightly tilted. NMR has been used to evaluate the conformation of Z and the dimer ZZ in solution and circular dichroism (CD) spectroscopy has been used to investigate structural changes upon binding to Fc. Only minor structural changes were observed in both the monomer and the dimer during complex formation. In addition, both the bound and unbound states were shown to contain a structured third helix (Jendeberg et al. 1996), as opposed to the original crystal complex where the third helix is not well resolved (Deisenhofer 1981). In several studies, the dimer ZZ has been used instead of the monomeric Z-domain. ZZ has been shown to bind more strongly to Fc than does the monomeric Z, due to a lower off-rate achieved through the avidity effect (Nilsson, J. et al. 1994). ZZ has been suggested to be a preferred arrangement for many applications, which yields strong Fc binding in combination with efficient secretion and small overall size (Ljungquist et al. 1989; Nilsson, B. et al. 1987).

The interaction between the Z-domain and human IgG1 has been further investigated, for example by the construction of four single amino acid mutants. Amino acids were chosen that were thought to be important in the binding surface, based on structural data from the crystal complex (Deisenhofer 1981). The mutants were evaluated in a competition assay where radioactively labeled Z was used as a tracer. All four mutants were found to have a decreased affinity against IgG1 compared to Z, which led to the conclusion that positions Ile31, Lys35, Leu17 and Asn28 are important for Fc-binding (Cedergren et al. 1993). Those results also confirmed the importance of Asn28 for binding; this position is found in the hydroxylamine site that was altered as part of the development of Z. It was later shown that the kinetics of the interaction of Fc with the B- or Z-domain were indeed identical (Jendeberg et al. 1995; Starovasnik et al. 1996).

The Z-domain has found use in several ways in the field of protein purification, mostly as a fusion tag for efficient production and purification of recombinant target proteins. Usage of the Z-domain as a fusion tag enables production and purification of recombinant proteins with very high yields. The majority of proteins that have been purified fused to the Z-domain have been produced as soluble proteins and several examples exist where ZZ-fusions have facilitated the recovery of proteins secreted into the periplasmic space or to the culture medium (Hansson et al. 1994; Uhlen et al. 1992). For example, human insulin-like growth factor II was produced as a secreted fusion to ZZ and affinity purified on IgG Sepharose (Wadensten et al. 1991). In a similar strategy, a secreted protein built up from a repeat-structure of a malaria antigen (M5) tagged with a dimeric Z-tag could be recovered from the culture medium by expanded bed adsorption and ion exchange chromatography. The initial capture was followed by a polishing step by affinity purification facilitated by the

IgG-binding fusion (Hansson et al. 1994). Z displays fast kinetics, enabling the use of high flow rates and columns with immobilized IgG can be reused many times (Uhlen & Moks 1990). Fusion proteins including the Z-domain are easy to detect by immunoblotting after purification, as Z binds to antibodies normally used in these setups (Stahl et al. 1997). The Z-domain has been used as a solubilizing fusion partner as it folds easily and may therefore aid the *in vitro* folding process of proteins with complex folding patterns. It has also been used as a fusion partner to insulin-like growth factor I (IGF-I), a protein with a complicated folding pattern that involves formation of three disulfide bonds. The fusion tag was shown to confer a higher overall solubility to IGF-I, which was shown to be at least 120 times more soluble when fused to either Z or the dimer ZZ. In addition, Z also decreased the degree of multimerization of IGF-I (Samuelsson et al. 1994; Samuelsson & Uhlen 1996; Samuelsson et al. 1991). The Z-domain has also been used in the production of very insoluble proteins in the form of inclusion bodies. As IgG-sepharose columns are resistant to 0.5 M guanidine hydrochloride, it is possible to perform the purification step in the presence of a chaotropic agent, which keeps the target proteins in a soluble state (Stahl et al. 1997). The D-domain of SPA has also recently been shown to function as a solubility and stability enhancing tag (Heel et al. 2010).

Competitive elution protocols have been developed as a milder alternative to the strategies normally used. This concept has been proven effective for Z-fusion proteins eluted with bivalent ZZ, which has a roughly 10-fold higher apparent affinity as a result of avidity effects (Nilsson, J. et al. 1994). The feasibility of the competitive elution strategy was demonstrated for a Z-fusion to the Klenow fragment of DNA polymerase I expressed in *E. coli* (Nilsson, J. et al. 1994). The competitor in this study was also tagged by a dimeric albumin-binding domain to facilitate effective removal after elution from the IgG column by capture of the competitor on an HSA column, without interfering with the tag still present on the final product. This approach should in principle be applicable to fusions to an albumin-binding domain as well, provided the purification steps are used in the reverse order for a protein tagged with a monovalent albumin-binding tag. It is possible to recombinantly introduce a proteolytic cleavage site in the joint between the Z-domain and the recombinant protein to enable removal of the fusion tag after purification. Several efficient cleavage agents have been identified for removal of Z (Forsberg et al. 1992). In one study ZZ was fused to proinsulin and three different short linkers containing trypsin cleavage sites were introduced between the tag and the target protein (Jonasson et al. 1996).

The Z-domain can also be utilized for purification of antibodies or Fc-fused target proteins, similarly to the full-length SPA or SPG. However, the IgG-binding domains may more easily be engineered to facilitate site-directed immobilization on a solid support. For example thiol-directed immobilization has been employed, where a C-terminal cysteine was recombinantly introduced to enable immobilization of Z, ZZ or pentameric Z. The C-terminal residue had little impact on the binding capacity for Fc, determined by measuring the amount of protein eluted from the column. This strategy is advantageous since the ligands are correctly oriented and no ligands are truncated since an intact C-terminus is required for coupling to the column (Ljungquist et al. 1989). Furthermore, the Z-domain has been used as a means for site-directed immobilization of antibodies on cells or viruses. For example, yeast cells have been engineered to express a dimeric form of the Z-domain on the cell surface (Nakamura, Y. et al. 2001). The engineered cells were applied as renewable

immunosorbents for affinity purification of antibodies from serum. In addition, cells expressing Z were used for detection of antigens, after a primary incubation of the sample with target-specific immunoglobulins. Other examples include the capture of antibodies on phage (Mazor et al. 2010), and display of Z on baculovirus (Ojala et al. 2004) or *E. coli* (Mazor et al. 2008; Mazor et al. 2007).

3.2 Domains of streptococcal protein G

The immunoglobulin-binding domains C1-C3 have been expressed, purified and studied independently (Akerstrom et al. 1985; Akerstrom et al. 1987). Whereas the immunoglobulin-binding domains of SPA have been the subjects of a large number of studies, the domains of SPG conferring the same binding activity have however not been as extensively investigated in the context of bioseparation. They have been utilized for antibody purification, mostly due to the broader subclass specificity compared to SPA. However, the immunoglobulin-binding domains of SPG have been best characterized and utilized as models to deepen the understanding of protein folding and dynamics. Regions responsible for albumin binding have also been isolated (Nygren et al. 1988). Fragments spanning two and a half (BB) or three (ABP, albumin-binding protein) of the albumin-binding motifs of SPG have been expressed and characterized (Larsson et al. 1996; Nygren et al. 1988). A smaller albumin-binding segment that has been widely studied alone comprises the third albumin-binding repeat flanked by a few amino acids from the B2- and S-regions, respectively (Nygren et al. 1990). This molecule is referred to as the albumin-binding domain (ABD) in the text.

3.2.1 The IgG-binding domains derived from SPG

SPG contains three homologous IgG-binding domains, referred to as C1-C3. The immunoglobulin-binding domains each consist of 55 amino acids and fold into a four-stranded β-sheet connected by an α-helix and short loops (Akerstrom et al. 1985; Lian et al. 1992). In analogy to the albumin-binding domains of SPG and the IgG-binding domains of SPA, C1-C3 are unusually stable to harsh thermal or chemical treatment and can be effectively refolded after denaturation (Alexander, P. et al. 1992). Each domain comprises non-overlapping binding-sites for both the Fc- and the Fab-regions of IgG from several subclasses (Erntell et al. 1988; Lian et al. 1992). The subclass specificity of SPG is broader than for SPA since the immunoglobulin-binding domains also bind IgG3 (Bjorck & Kronvall 1984). SPG has been widely used for purification of immunoglobulins or antibody fragments (Akerstrom et al. 1985; Cassulis et al. 1991; Hober et al. 2007).

The immunoglobulin-binding domains of SPG have not been as extensively used as gene fusions or ligands for affinity capture as the domains of SPA, perhaps as a result of the later identification of SPG and lower tolerance to alkaline conditions of the immunoglobulin-binding domains compared to SPA. However, SPG is widely used for purification of antibody fragments and the inherent tolerance for chaotropic agents facilitates rigorous cleaning (Winter et al. 1994). A few diverse examples of fusions to C-domains are exemplified here to illustrate some additional applications. The C1-domain has been used to increase the expression levels and aid in refolding of small recombinant proteins or peptides (Cheng & Patel 2004; Nadaud et al. 2010; Pazehoski et al. 2011). A repeat of the C3-domain has in another study been combined with luciferase to form a fusion protein with ability to

detect antibodies bound to bacteria through a light-emitting reaction (Nakamura, M. et al. 2011). Another fusion strategy produced an adherent protein able to capture antibodies in microwells when a hydrophobic domain of elastin was combined with an immunoglobulin-binding domain from SPG (Tanaka et al. 2006). As mentioned above, the use of the C1-C3 domains of SPG has been more focused around basic biophysical questions. Since the initial structural characterization of the C1-domain (Gronenborn et al. 1991) all three IgG-binding domains of SPG have become popular model systems for studies on protein stability, folding, structure and dynamics (Alexander, P. et al. 1992; Clore & Schwieters 2004; Derrick & Wigley 1994; Franks et al. 2005; Hall & Fushman 2003; Ulmer et al. 2003). The vast number of studies within those fields have been reviewed elsewhere and lie out of the scope of this chapter. The surprising structural similarity between the albumin-binding domains of SPG and the immunoglobulin-binding domains of SPA (Falkenberg et al. 1992) has motivated several studies where the folding patterns and sequence-structure relationships have been experimentally dissected. Interestingly, it was recently demonstrated that a domain with the same immunoglobulin-binding fold as found in C1-C3 could be transformed into a three-helix bundle domain, similar to the albumin-binding domains of SPG, with acquired affinity against albumin through a defined mutational pathway (Alexander, P. A. et al. 2009; He et al. 2005).

3.2.2 The albumin-binding domains derived from SPG

Different regions of the albumin-binding part of SPG have been affinity purified by an effective one-step HSA-chromatography protocol (Nygren et al. 1988). This method has also been applied to a wide range of proteins fused to different albumin-binding fragments of SPG. Those albumin-binding affinity tags have, in analogy to SPA-based tags, been shown to be proteolytically stable, highly soluble and possible to produce in high yields (Larsson et al. 1996; Nilsson, J. et al. 1997b; Nygren et al. 1988; Stahl et al. 1989). Due to the harsh conditions required to elute tightly bound proteins from HSA columns, different approaches using milder routines have been evaluated. The low pH most often applied for elution may be harmful for the fusion partner of interest. For ABP-fusion proteins, other elution strategies including heat (Nilsson, J. et al. 1997a), high pH (Makrides et al. 1996) and lithium diiodosalicylate (Lorca et al. 1992) have been successfully investigated. Furthermore, the different binding affinities measured for albumin from different species has also been proposed as a means to achieve milder elution conditions by for example using albumin from mouse as the affinity ligand instead of the human equivalent (Nygren et al. 1990). The albumin-binding fragments of SPG that have been studied are easily refolded and retain activity after harsh treatment (Oberg 1994, as cited in Kraulis et al. 1996). This property has been utilized to facilitate recovery and refolding of fusion proteins from inclusion bodies (Murby 1994, as cited in Murby et al. 1996; Stahl & Nygren 1997). It can sometimes be an advantage to produce proteins as inclusion bodies since high production yields can be achieved and the insoluble proteins are protected from proteolysis (Murby et al. 1996). An ABP-fusion has been combined with hydrophobicity engineering to express and recover a slightly modified variant of a very insoluble and easily degraded fragment of the human respiratory syncytical virus (RSV) major glycoprotein G (Murby et al. 1995). In another study (Murby 1994, as cited in Murby et al. 1996), efficient recovery was demonstrated in the presence of chaotropic agents (0.5 M guanidine hydrochloride) for precipitation prone

fragments of the fusion glycoprotein F from the same virus expressed as ZZ- or BB-tagged fusions.

Sometimes removal of the fusion tag is necessary to obtain a product of desired quality. Several chemical and enzymatic methods for tag removal have been devised (Arnau et al. 2006; LaVallie et al. 2001). Chemical methods are often scalable and relatively inexpensive, they may however produce side-chain modifications, denaturation of the target protein or be too unspecific to be generally applicable to many larger proteins (Parks et al. 1994). In general, more specific agents, such as proteases, are required to avoid unwanted cleavage in the coupled target protein. Strategies where the protease carries the same tag as it cleaves off from the protein of interest have been described. This facilitates simultaneous capture of enzyme and cleaved tag in a single step that leaves pure target protein. Such systems are exemplified by 3C protease in the PreScission system (Walker et al. 1994) and a similar approach was developed for use with the ABP-tag fused to both the protease and the target protein (Graslund, T. et al. 1997). Recent developments include the Profinity Exact system where tagged proteins are captured by a modified subtilisin that is subsequently activated to specifically cleave off, but retain, the tag and release the pure target protein (Bio-Rad Laboratories, Hercules, CA).

A maintained tag can sometimes be a way to achieve directed immobilization or detection. This has been demonstrated for several formats involving different parts of the albumin-binding regions of SPG (Baumann et al. 1998; Konig & Skerra 1998; Stahl et al. 1989). Albumin-binding fusions have also found interesting applications *in vivo* for delivery of subunit vaccines or protein therapeutics (Nilsson, J. et al. 1997b; Sjolander et al. 1997; Stahl & Nygren 1997). Fusion of the BB-fragment of SPG to CD4 resulted in stabilization of the protein *in vivo* in mice as well as in macaques (Nygren 1991, as cited in Sjolander et al. 1997). The same concept has been evaluated in rats for the human soluble complement receptor type 1 (Makrides et al. 1996). Those early attempts suggested that the minimal binding motif ABD might also be useful to improve the *in vivo* stability of proteins. Several recent studies have indeed demonstrated that fusions to albumin-binding domains efficiently prolong the half-life of the fused protein *in vivo* (Andersen et al. 2011; Hopp et al. 2010; Nilsson, F. Y. & Tolmachev 2007; Stork et al. 2009). Albumin-binding proteins may also have immunopotentiating properties when used as carriers for a fused immunogen. This concept was originally evaluated for malaria antigens by Sjölander (Sjolander et al. 1995; Sjolander et al. 1997; Sjolander et al. 1993) and has been observed for other antigens as well, for example an antigen derived from the syncytical virus subgroup A (Libon et al. 1999). However, it is not clearly elucidated whether those effects result from prolonged half-life, occurrence of T-cell epitopes or a combination of both (Stahl & Nygren 1997). Some B- and T-cell epitopes have been identified in the albumin-binding region of SPG (Goetsch et al. 2003) and an immunogenicity mapping of the albumin-binding protein has been undertaken (Steen et al., manuscript 2011). Related work has shown that the Z-domain can stimulate B-cells and therefore act as an adjuvant. This has been demonstrated using ZZ as a fusion partner for immunization (Lowenadler et al. 1987; Stahl et al. 1989). A dual expression system for immunogens expressed as either a fusion to ZZ or BB was also devised (Stahl et al. 1989). This strategy facilitated immunization with an immunogen tagged in one way followed by evaluation of the antibody response using a differently tagged antigen, which eliminates the background response raised against the fusion partner.

Gene fusions are not limited to only one fusion partner or one end of the gene of interest. Several studies exist where two or several tags have been used in combination. A dual affinity fusion strategy where the gene of interest is expressed flanked by immunoglobulin and albumin-binding regions was shown to be able to provide active protein after two sequential affinity purification steps, as exemplified with human insulin-growth factor II (Hammarberg et al. 1989). The cell lysate was initially passed through an IgG column and secondly through an HSA column. This system will not prevent truncated forms of the protein from being produced, but they will not be collected in both purification steps. Interestingly, it was observed that the proteolytic stability of the recombinant target protein was increased when expressed between two tags compared to an N-terminal ZZ-tag alone (Hammarberg et al. 1989). This indicates that the C-terminal affinity tag confers increased overall stability to the fusion protein. Those findings motivated another study where this phenomenon was investigated in more detail (Murby et al. 1991). Here, the dual-affinity fusion approach was used to assess the degradation of the albumin-binding region of SPG in *E. coli*, when fused to different recombinant partners with an N-terminal ZZ-protein (Murby et al. 1991). The tagging made independent recovery of the C- and N-terminal regions possible, thereby providing a means for characterization of proteolytic events. Proteins were captured on an HSA column and proteolysis products in the flow through were recovered by a subsequent IgG-affinity chromatography step. In addition to demonstrating that susceptibility to proteolysis can be addressed by gene fusion strategies, this study also showed that small fragments around 6 kDa with functional HSA-binding activity could be recovered. A related system has been used to stabilize various mammalian proteins from degradation when expressed in *E. coli* (Murby et al. 1991). A similar approach utilized dual tagging with albumin- and metal-binding gene fusions in the termini to recover unstable derivatives of an IgG-binding domain from SPA (Jansson et al. 1990). Taken one step further, a tri-functional Bio-His-ABP tag has been evaluated (Nilsson, J. et al. 1996). This multi-functional tag combines properties of different tags to facilitate detection or immobilization (by streptavidin binding to the *in vivo* biotinylated bio-tag), refolding and solubility enhancing properties of ABP and possibility to purify under both native and denaturing conditions by immobilized metal ion affinity chromatography through the hexahistidine tag. In the same study, an alternative format for multiple tagging; including a FLAG-epitope tag, a His-tag, a strep-tag and an IgG-binding Z-domain; was evaluated. The main advantage with the former version is the lack of reactivity against antibodies, which broadens its applicability (Nilsson, J. et al. 1996). Tags based on SPA or SPG have also been used in tandem affinity purification strategies to efficiently recover low abundant target proteins and acquire very high purity (Burckstummer et al. 2006; Rigaut et al. 1999).

4. Engineered protein domains derived from SPA and SPG

The robustness of the individual domains of SPA and SPG, together with the knowledge from previous studies of their various properties and applications, has motivated novel protein engineering efforts. Using the small and stable domains as starting points, a wide range of new proteins has been developed for various purposes. Several rational and combinatorial approaches have been attempted to provide small proteins with novel or improved properties that advance the field of protein purification. In this section we summarize some strategies for stabilization, miniaturization, surface engineering and combination of various domains or modified variants of domains derived from SPA and

SPG. The focus lies on modification of the Z-domain derived from SPA and the third albumin-binding domain derived from SPG (figure 8), some associated examples based on related domains are also discussed.

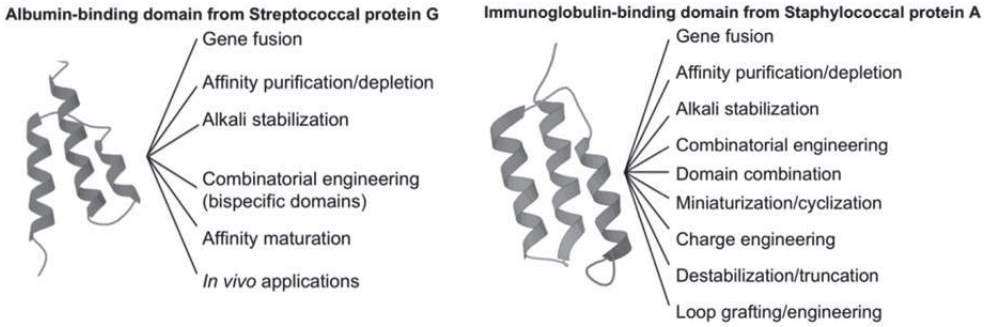

Fig. 8. Engineered protein domains. Several strategies have been devised to engineer new or modified properties into protein domains to facilitate for example affinity purification, increase or decrease stability or introduce novel binding sites.

4.1 Protein engineering to address stability and its implications on affinity

Several innovative strategies have been devised for stabilization of domains derived from the bacterial surface proteins and some examples are reviewed below. The Z-domain represents one of the first rational engineering efforts of a protein domain for increased stability, however at the expense of Fab-binding ability (Nilsson, B. et al. 1987). The C1-domain of SPG has also been engineered for increased stability by an approach that first identified candidate positions for stabilizing mutations and then selected variants from a combinatorial library encompassing combinations of potentially stabilizing mutations (Wunderlich & Schmid 2006). An ingenious system that links the stability of a particular protein variant to the infectivity of a phage particle carrying the same mutant was devised for this purpose. Earlier attempts to stabilize C1 using computational design tools (Dahiyat & Mayo 1997; Malakauskas & Mayo 1998) only resulted in modest improvements compared to this strategy. Stability improvements of C1 have also been achieved by screening of a small combinatorial library built on the domain with a fragment complementation system (Lindman et al. 2010). In this method, fluorescence functions as a reporter for increased affinity between split fragments of green fluorescent protein, which in turn is linked to stability of the C1-fusion. Other applications of the C1-domain include stabilization of a fusion partner to facilitate for example NMR-based studies (Cheng & Patel 2004). Contrarily, to decrease the stability of C1, a screen for destabilized but active variants of the same domain by phage display has been employed (O'Neil et al. 1995). Engineered variants of the C1-domain with a pH-sensitive binding to Fc have also been described recently (Watanabe et al. 2009). Common for those strategies is that they have used C1, which is one of the best-studied models for protein folding, stability and dynamics and therefore a good model to evaluate stabilization approaches (Magliery & Regan 2004). In general, combinatorial library approaches have provided new tools to address the relationship between sequence and folding, stability and function (Forrer et al. 1999; Hoess 2001; Kotz et al. 2004; Magliery & Regan 2004).

Native SPA and SPG are remarkably stable to challenging chemical or physical conditions (Boyle 1990; Girot et al. 1990; Hale et al. 1994). The alkaline tolerance of the immunoglobulin-binding domains of SPG is not as good as for SPA, however resistance of proteins to environmental challenges can be substantially improved by protein engineering. In a strategy that was first proven effective for the albumin-binding domain of SPG, all four asparagine residues susceptible to base catalyzed deamidation were replaced with other residues (Gulich et al. 2000a). This approach resulted in a molecule that could withstand repeated cycles of cleaning with high concentrations of sodium hydroxide, without any considerable loss in performance when linked to a chromatographic resin. An increased thermal stability was also achieved without significant alteration in structure or function (Gulich et al. 2000a). To improve the performance of this ligand even further, different linker regions and multimeric formats have been evaluated for tolerance to extended time periods of alkali exposure (Linhult et al. 2003). The alkali stabilized albumin-binding domain provides a robust affinity ligand for purification or depletion of albumin from for example serum to facilitate detection of low abundant biomarkers (Eriksson et al. 2010; Linhult et al. 2003). Furthermore, the strategy for increasing tolerance to alkali has proven effective also when applied to the Z-domain (Linhult et al. 2004) and the C2-domain (Gulich et al. 2002). The strategies employed to those domains evaluated, apart from asparagine residues, also replacements of glutamine residues, and showed that all replacements have to be evaluated on a case-by-case basis. Some residues may be crucial for binding or stability and thereby impossible to alter, and one can also take homologous sequences into account to decide on suitable replacements. When applying the stabilization strategy to the originally very stable Z-domain, a destabilized point mutant where a core phenylalanine was substituted for an alanine (Cedergren et al. 1993), was used as a model to assess the influence of various mutations (Linhult et al. 2004). Finally, the improvements suggested by the bypass mutagenesis approach were grafted back onto the original domain to verify the result (Linhult et al. 2004). A multimeric variant of this alkali stabilized Z-domain is now utilized as a ligand on a commercially available resin for purification of antibodies or Fc-fused proteins (MabSelect SuRe, GE Healthcare). Furthermore, use of only this domain for purification of antibodies instead of native SPA results in negligible binding to the variable region and therefore a more homogenous pH elution profile (Ghose et al. 2005). A truncated variant of the C2-domain with increased alkaline stability has been described (Goward et al. 1990), however the mutational strategy described above yielded additional tolerance (Gulich et al. 2002). The alkaline stability of SPG was further evaluated and improved in a recent study (Palmer et al. 2008). The mutational strategy was expanded to consider thermodynamic stabilization that retains the tertiary structure and modification of surface electrostatics as well. These examples illustrate the potential of both a relatively straightforward approach to improve the tolerance to alkaline conditions of proteins and a strategy based on more detailed structural understanding. In theory, combinatorial approaches should also be feasible to address this issue, provided that a sensitive enough selection protocol, which can distinguish between differences in tolerance to a specific challenge, can be set up.

Strong interactions between the native domains and their target molecules often require harsh conditions for elution. This may potentially be harmful for the target protein, for example recombinant proteins fused to an affinity tag. To address this problem, modified variants of the B- or Z-domain of SPA have been developed. Variants of the B-domain of

SPA with different C-terminal truncations have been used for affinity chromatographic purification of human IgG (Bottomley et al. 1995). Several variants with decreased affinities were produced and shown to enable elution at elevated pH-values compared to the full-length protein A. The objective of another approach was to replace or extend the loop linking the second and third helix of the Z-domain, thereby yielding destabilized variants of the molecule that facilitate milder elution (Gulich et al. 2000b). Destabilized Z-variants that dissociate from IgG more rapidly were coupled to columns and shown to elute bound immunoglobulin at pH 4.5, which is more than one unit higher than what in commonly used (Gulich et al. 2000b). Potential use of such destabilized immunoglobulin-binding molecules includes utilization as affinity tags for pH sensitive proteins or, when used on a solid support, purification of antibodies using milder conditions. The C1-domain has also served as a more general model to understand how introduction of various loops can affect stability (Zhou et al. 1996). The modified variants have however not been used in chromatographic systems.

4.2 Miniaturization and protein mimicking

Several attempts have aimed at miniaturizing the binding units of protein A, and a few studies have investigated smaller variants of SPG-derived domains. Smaller proteins can more easily be produced and modified by peptide synthesis, they may be useful as starting points for design of smaller organic mimetics or aid in the understanding of protein stability and structure (Braisted & Wells 1996). Analogues of the B-domain of SPA that were truncated in both termini, which are not directly involved in the domain core packing (Deisenhofer 1981), have been produced (Huston et al. 1992). The modified proteins were shown to have lowered affinity against IgG. This was also observed for C-terminally truncated variants of the B-domain that were used as affinity ligands for the removal of immune complexes from blood (Bottomley et al. 1995). Similar results indicating that constructs with a truncated C-terminus are less stable have been reported for dual-tagged variants of the Z-domain (Jansson et al. 1990). Other studies have also addressed folding and stability of mutant variants of the B-domain (Bottomley et al. 1994; Popplewell et al. 1991; Sato et al. 2006). A more systematic approach including iterative structure based design and phage display selections resulted in a miniaturized Z-domain that only comprises 38 residues (Braisted & Wells 1996). This was achieved by stabilizing the first two helices so that IgG-binding would be retained even without the third helix (Braisted & Wells 1996). Structural evaluation of the Z38-variant by NMR (Starovasnik et al. 1997) further strengthened the conclusion that the conformational paratope responsible for IgG-binding was shared with the original Z-domain. Guided by the structural data, a disulphide bonded 34 amino acid truncated variant was designed, synthesized and structurally characterized (Starovasnik et al. 1997). Both this variant and the 38 residue version formed two anti-parallel helices similar to the topology of the corresponding parts in the Z-domain and both demonstrated retained binding to IgG with affinities comparable to the original scaffold (Starovasnik et al. 1997). In addition, an X-ray structure of the miniaturized cyclic Z-domain in complex with Fc demonstrated that the fold and fundamental interactions were preserved in the interaction (Wells et al. 2002). This cyclization strategy has since then been modified to a backbone cyclization through a native chemical ligation reaction (Jarver et al. 2011). The resulting miniaturized molecule was shown to be able to capture human polyclonal IgG when immobilized onto a solid support. In an interesting modification of the original two-

helix version of the Z-domain, an elastin sequence was inserted in the inter-helix turn (Reiersen & Rees 1999). This modification dramatically altered the helical structure of the resulting protein. However, in contrast to the starting molecule, the elastin-turn mutant exhibited a more than 20-fold improvement of Fc-binding affinity when the temperature was increased. This effect is hypothesized to arise through a temperature- or salt-induced formation of a ß-turn that stabilizes the alignment of the Fc-binding helices and represents a modular switch to alter structure and activity (Reiersen & Rees 1999, 2000). For small protein domains, synthesis provides a straightforward means for site-specific labeling, chemical cross-linking or introduction of non-natural building blocks to make novel variants available for different applications. Deeper understanding of interaction interfaces between proteins may also facilitate rational design of small molecular weight mimics (Wells et al. 2002); miniaturized proteins only represent intermediates for the challenging task of designing small molecule mimetics.

In rational molecular design, starting from a structurally defined scaffold and a binding surface rather than a sequential stretch of amino acids such as a loop region, usually results in a more defined binding molecule (Stahl & Nygren 1997). For example, the key determinants of the interaction site between the IgG-binding domains of SPA and Fc have stood model for the generation of several small protein mimetic organic molecules. This concept was beautifully demonstrated for the interaction between the B-domain of SPA and the Fc-part of IgG (Li et al. 1998). Using the hydrophobic core dipeptide Phe132-Tyr133 as a starting point, a novel triazine mimetic was rationally designed, synthesized and utilized for purification of antibodies (Li et al. 1998). Since then, mimetics have also been developed for other antibody-binding proteins using modified synthetic molecular scaffolds and chemistries (Haigh et al. 2009; Lowe 2001; Roque et al. 2005). The SPA mimetic peptide PAM is another example of a protein A mimetic ligand. PAM was selected from a combinatorial peptide library, and to further increase the stability of this molecule D-amino acids have been used to hinder degradation of the molecule by proteases (Verdoliva et al. 2002). A synthetic protein called MAbsorbent®A2P (ProMetic BioSciences), which binds all subclasses of human IgG, has also been described (Newcombe et al. 2005). One can also use thiophilic ligands for antibody purification, the most common is called "T-gel", which carries linear ligands with two sulfur atoms and displays good selectivity for antibodies in the presence of high concentrations of lyotropic salts (Boschetti 2001). These small molecule imitations may provide a competitive, robust, scalable and chemically resistant alternative to SPA, SPG or domains thereof for purification of antibodies or Fc-fused proteins. They may achieve increased stability compared to proteinacious ligands, but may however be limited to lower flow-rates since the binding is normally not as fast as for the protein-based ligands.

4.3 Engineering and improving new binding surfaces

Protein engineering may also be applied to modify or evaluate larger binding areas (Sidhu & Koide 2007). Surface exposed amino acids of the Z-domain have been replaced with charged amino acids to generate modified variants of the molecule that carry an excess of positive or negative charge (Graslund, T. et al. 2000; Hedhammar et al. 2004). These molecules, Z_{basic} and Z_{acid}, have efficiently been employed as affinity fusion tags for the purification of recombinant target proteins by cation- or anion-exchange chromatography.

Target protein capture through the Z_{basic}-tag has also been exploited for solid-phase refolding of denatured proteins purified from solubilized inclusion bodies (Hedhammar et al. 2006), capture of fusion proteins by cation-exchange chromatography in an expanded bed adsorption mode (Graslund, T. et al. 2002b) and for high-throughput protein expression and purification (Alm et al. 2007). Those examples illustrate that compact, stable protein domains may be extensively engineered and still retain the beneficial characteristics of the original domain.

Another engineering approach related to the concept of affecting stability through modification of loops has been reported. Here, a biologically active peptide that was selected by phage display to inhibit cathepsin L, was grafted into the loop between the second and third helix of the Z-scaffold (Bratkovic et al. 2006). Loop grafting, and thereby transfer of a novel biological function, could be achieved without loss of structure, as evaluated by CD spectroscopy. Moreover, all constructs also retained their IgG-binding ability (Bratkovic et al. 2006). Consequently, the Z-domain could be utilized as a stable carrier for a new functional entity without loosing its structure or inherent Fc-binding capability.

Combinatorial approaches using robust protein domains can be a valuable tool for the development of tailored purification strategies for native biomolecules (Jonasson et al. 2002; Nygren & Uhlen 1997). Engineering protein surfaces to accommodate novel binding regions provides a means to produce proteins with new functions. On the Z-domain, 13 discontinuous surface-exposed amino acids on the same two helices that mediate the interaction with Fc have been targeted for randomization (Nord et al. 1995). The amino acids involved in the Fc-binding, as identified in the crystal complex of the B-domain and Fc (Deisenhofer 1981), are situated on the outer surfaces of the first and second helix and are not involved in the packing of the core. The Fc-binding surface covers an area of roughly 600 $Å^2$, which is comparable to interfaces observed in antibody-antigen interactions (Lo Conte et al. 1999; Nygren 2008). This targeted randomization approach provides a combinatorial library from which so called Affibody molecules with novel binding specificities may be selected (Nord et al. 1997; Nord et al. 1995). To enable selection of variants with desired specificities, the combinatorial library was fused to the gene encoding phage coat protein III and fusions were expressed on filamentous phage. Post selection output was subsequently expressed as fusions to an albumin-binding domain to facilitate evaluation (Nord et al. 1997; Nord et al. 1995). Currently, a large number of alternative display and selection systems are available, many of which have been utilized for selection of Affibody molecules as well as other scaffold proteins (Binz et al. 2005; Lofblom et al. 2010; Nygren 2008; Nygren & Skerra 2004). Early targets for selection of Z-based binding molecules include Taq DNA polymerase, human insulin and a human apolipoprotein variant (Nord et al. 1997) and as of today Affibody molecules have been selected against a large number of targets for use in a variety of applications (Lofblom et al. 2010; Nygren 2008). Several variants have found use within protein purification applications. The before mentioned molecules specific for Taq DNA polymerase or human apolipoprotein A were, in the form of dimers, successfully utilized as affinity ligands for the capture of their respective targets from *E. coli* lysates (Nord 2000). Repeated cycles were performed with elution at low pH, without any observed loss in capacity or selectivity of the Affibody-coupled columns. Furthermore, *in situ* sanitation of columns with 0.5 M NaOH did not result in any significant loss of performance

(Nord et al. 2000). Affibody-mediated capture has also been demonstrated for many proteins, including for example human Factor VIII produced in Chinese hamster ovary cells (Nord et al. 2001), depletion of transferrin (Gronwall et al. 2007b), human IgA (Ronnmark et al. 2002a), amyloid-ß-peptide (Gronwall et al. 2007a), human IgG (Eriksson et al. 2010) or combinations of proteins (Ramstrom et al. 2009). Affibody molecules are available, together with several other capture agents, in commercial multiple affinity removal systems (MARS) (MARS-7, MARS-14 columns, Agilent Technologies). Those kits and utilization of non-antibody based capture proteins have been shown to have advantages compared to utilization of native SPA or SPG for depletion (Coyle et al. 2006; Echan et al. 2005; Eriksson et al. 2010). In addition to protein capture on columns, binding molecules based on the Z-domain have also been utilized for capture in protein microarray applications (Renberg et al. 2007; Renberg et al. 2005).

Another recent example of how novel specificity may be incorporated in small protein domains is illustrated by selection of Affibody molecules with increased affinity to mouse IgG1 (Grimm et al. 2011). The original Z-domain has practically non-existing affinity against mouse IgG, which represents the most widely used within biotechnology. The new specificity possessed by the mouse IgG1-specific binding molecule facilitates specific recovery of monoclonal mouse antibodies from hybridoma supernatants rich in bovine immunoglobulin that may cross-react with alternative capture agents (Grimm et al. 2011). Furthermore, anti-ideotypic Affibody molecules have been generated using other affinity ligands or SPA itself as the target in the selections (Eklund et al. 2002; Wallberg et al. 2011). One such molecule was recently used to facilitate the recovery of untagged Affibody molecules, aimed for imaging studies of human epidermal growth factor receptor 2 over-expressing tumor xenografts, from *E. coli* lysates (Wallberg et al. 2011). An interesting related approach utilized an Affibody molecule specific for SPA as affinity fusion for purification of fusion proteins on readily available protein A media (Graslund, S. et al. 2002a). Similarly, purification of Fc-fused Affibody molecules in an artificial antibody format on protein A Sepharose has been described (Ronnmark et al. 2002b). Together, those examples demonstrate the usefulness of custom-made affinity molecules in various applications. Several structures of Affibody molecules alone or in complex with their targets have been solved, which further expands the understanding of structure- and function-relationships in engineered binding molecules and provides detailed insights for the interactions (Eigenbrot et al. 2010; Hogbom et al. 2003; Hoyer et al. 2008; Lendel et al. 2006; Nygren 2008; Wahlberg et al. 2003). Some applications however demand higher binding affinities than is normally achieved by a single selection from a naïve library. Different approaches to affinity mature Affibody molecules have been devised. For example helix shuffling, error-prone PCR (Grimm et al. unpublished results) or construction of targeted libraries with more focused diversification based on first generation binding molecules have been developed (Gunneriusson et al. 1999; Nord et al. 2001; Orlova et al. 2006). Alternatively, multimeric formats may provide a sufficient gain in apparent affinity for more demanding applications (Nord et al. 1997).

The same miniaturizing strategies that were originally applied to the Z-domain have now also been demonstrated on Affibody molecules with novel binding specificities (Ren et al. 2009; Webster et al. 2009). Those studies demonstrated that the two-helix format could provide a starting template for the design of miniaturized binding molecules, nonetheless

some specific optimization may be required to yield a molecule fit for use. Another study has also shown that truncation of a binding molecule based on structural data, here an Affibody molecule specific for the amyloid-ß-peptide, can provide improved variants (Lindgren et al. 2010). This may however require case-by-case optimization and only be applicable when detailed structural data is available. The prospect of producing binding molecules by solid phase peptide synthesis has also motivated an optimization of the Z-scaffold for synthesis. This has been accomplished by utilizing a well-characterized human epidermal growth factor receptor 2-binding molecule as a template (Feldwisch et al. 2010). In addition, the recent scaffold optimization resulted in increased thermal and chemical stability as well as improved solubility. A successful grafting of binding-surfaces for a selection of molecules with other target specificities onto the new scaffold was also demonstrated (Feldwisch et al. 2010). Taken together, a wide range of technologies are now available for the construction of combinatorial libraries, selection of molecules with desired properties, affinity maturation and even miniaturization to provide novel or improved affinity reagents for bioseparation as well as many other applications (Binz et al. 2005; Nygren 2008; Nygren & Skerra 2004). Several synthetically produced and modified variants have so far been described for the Z-domain and C1-domain (Boutillon et al. 1995; Ekblad et al. 2009; Engfeldt et al. 2005). Robust and tailor-made target-specific affinity ligands provide an interesting approach to recover recombinant or naturally occurring proteins in their native forms and will certainly find even broader use in the future. Recent development of new orthogonal aminoacyl-tRNA synthetase/tRNA pairs, which allows for addition of various unnatural amino acids to recombinantly expressed proteins, may aid the further advancement of this expanding field of protein engineering (Liu & Schultz 2010). The addition of building blocks with novel properties to the 20 amino acids chosen by nature may further expand the fitness landscape in which proteins evolve to fulfill novel or enhanced functions. Recent progress includes phage-based *in vitro* evolution systems that utilize bacteria designed to read a 21 amino acid code (Liu et al. 2008).

In a similar fashion as explored for the Z-domain derived from SPA, the albumin-binding domain of SPG has been used as a scaffold for the design of a combinatorial library (Alm et al. 2010). From this library, bispecific binding molecules with retained binding to albumin and an additional acquired affinity to a novel target molecule have been selected by phage display (Alm et al. 2010). In a proof-of-principle study, target proteins with different characteristics were genetically fused to a bispecific ABD-molecule that had been identified through biopanning against the Z-domain. Following expression in bacterial hosts, the target proteins could efficiently be purified to high homogeneity by a two-step affinity purification protocol utilizing the two binding specificities of the tag for the Z-domain and HSA. Affinity maturation of ABD-based, bispecific molecules have also been demonstrated exploiting a cell-displayed library, designed for targeted randomization based on phage display-selected TNF-α-binding molecules (Nilvebrant et al., manuscript 2011). Furthermore, the affinity of the ABD-molecule itself has been addressed in a combinatorial engineering approach (Jonsson et al. 2008). Through several rounds of affinity maturation and rational design where 15 of the 46 amino acids that constitute the domain were randomized, a molecule with an extremely strong affinity against HSA was achieved. Both this molecule and the original albumin-binding domain have successfully been used as gene fusions with for example antibody fragments (Kontermann 2009) or Affibody molecules (Tolmachev et al. 2009) to provide improved persistence *in vivo*, mediated by the binding to

serum albumin. Moreover, a recent protein engineering effort was aimed at de-immunizing the affinity-matured albumin-binding domain described above. Identified T-cell epitopes could be removed without influencing the stability, solubility or high affinity of the protein domain (Affibody AB, unpublished results).

Phage display has also been used in an attempt to evolve albumin-binding domains with different species specificities and gain understanding about their mode of interaction, biophysical properties and structural basis for specificity (He et al. 2007; He et al. 2006). A GA-domain derived from *F. magna* with affinity against two phylogenetically distinct serum albumins was successfully selected (Rozak et al. 2006). The binding mode of the resulting molecule, referred to as phage-selected domain-1, to albumin of different species has been further characterized by chemical shift perturbation measurements (He et al. 2007) and structural evaluation (He et al. 2006). The results demonstrate that increased flexibility is not a requirement for broadened specificity (He et al. 2006) and also indicate that a core mutation stabilizes the backbone in a conformation that more closely resembles the structure found in the complex between the GA-module and HSA (He et al. 2007; Lejon et al. 2004). This core residue, a tyrosine, is therefore the main reason for the broader species specificity of the albumin-binding domain from SPG compared to the GA-module derived from *F. magna*. Those efforts illustrate how homologs of a naturally evolved protein scaffold can be used as a starting point to alter the binding specificities through minor modifications of the binding surface. The *in vitro* recombination technique used in those experiments, offset recombinant polymerase chain reaction (Rozak & Bryan 2005), may also be a useful tool to further evaluate or evolve other homologous small protein domains.

Most of the modifications reported for the C1-C3 domains of SPG relate to structural or biophysical questions that lie outside the scope of this chapter (Gronenborn et al. 1991; Gronenborn et al. 1996; Malakauskas & Mayo 1998). However, one interesting example that relates to engineering of novel binding surfaces is the computational *de novo* design of a protein-protein heterodimer based on the C1-domain (Huang et al. 2007). Through rational design, molecules that spontaneously formed heterodimers could be produced. This demonstrates a step forward, among many other examples, on the path to envision a link between design of a primary sequence and a desired structure and function.

4.4 Generation of hybrid proteins

In order to broaden the class- and subclass specificity of immunoglobulin-binding proteins, several hybrid proteins have been compiled from domains of various bacterial surface proteins. The first hybrid protein was developed as a fusion between domains of protein A and G (Eliasson et al. 1988). Four constructs encoding either five domains from SPA, two domains from SPG, or combinations of domains from both, as well as the synthetic Z-variant instead of the native SPA-domains, were evaluated. It was shown that binding specificities from different immunoglobulin-binding proteins could successfully be combined in the hybrid proteins (Eliasson et al. 1989; Eliasson et al. 1988). In a similar approach, immunoglobulin-binding domains from SPA and SPG were combined and expressed in fusion to β-galactosidase to provide a novel enzymatic tool for immunoassays with broad antibody specificity (Strandberg et al. 1990). Similar concepts have since then been applied to produce hybrid molecules of protein L from *F. magna* and protein G

(Kihlberg et al. 1992) as well as protein L and A (Svensson et al. 1998). Immunoglobulin-binding domains of protein L have a fold that resembles the immunoglobulin-binding domains of SPG and interact with the light chain of many antibodies, which provides potential for broadened specificity of the hybrid proteins (Bjorck 1988; Wikstrom et al. 1994). Protein LG was constructed from four domains of protein L combined with two domains from protein G (Kihlberg et al. 1992). Protein LA was assembled from four domains each of the primary proteins (Svensson et al. 1998). The hybrid protein with the broadest combination of specificities has been further minimized in the form of a fusion of a single domain from protein L with one domain from protein G (Harrison et al. 2008). The fused domains were shown to be able to fold and interact with their respective target proteins in an independent manner. A combinatorial approach has also been described to combine individual domains of protein A, G and L (Yang et al. 2008). Randomly arranged domains were displayed on phage and selected against four different immunoglobulin-baits. Powerful library and selection technologies may provide a means to further improve or fine-tune the available range of hybrid proteins to tailor-make new ligands for specific purification or detection of antibodies, antibody fragments as well as many other target proteins.

5. Conclusions

For a few decades, SPA and SPG have been widely investigated to provide the deep understanding we have today about the evolution of the proteins, the structure of the domains and their binding specificities. This, in turn, has enabled us to find many applications for the proteins in a wide range of areas, the most common being ligands for antibody purification or depletion of abundant proteins from complex samples. As structural studies show that individual domains of SPA and SPG fold individually, it is possible to use single domains of the proteins, which have obtained especially good applicability as fusion proteins for production of recombinant proteins. Recombinant DNA technology enables simple construction of expression vectors where a domain of SPA or SPG is fused to a protein of interest. The domains not only simplify the purification procedure, but may also act as solubilizing and stabilizing agents.

Moreover, protein engineering has been applied to improve or combine properties of the stable domains derived from the bacterial surface proteins. Those efforts have resulted in new refined proteins with wide applicability. Furthermore, those techniques have been demonstrated to provide new insights in protein folding and dynamics as well, using small and stable protein domains as models to deepen the understanding of complicated biophysical processes. In summary, small, stable scaffolds have already proven their value in the biotechnological field in many ways and new, innovative applications are currently being investigated. Those rational and combinatorial engineering concepts have the potential to generate alternatives to antibodies as affinity capture agents in demanding, large-scale applications and thereby expand the applicability of affinity chromatography to a wider range of target proteins.

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7. References

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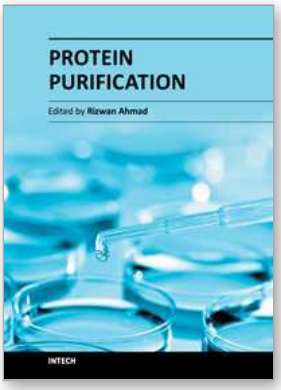

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