Dissection of the protein G B1 domain binding site for human IgG Fc fragment

DAVID J. SLOAN¹ AND HOMME W. HELLINGA²

¹Department of Pharmacology and Molecular Cancer Biology, Duke University Medical Center,
Box 3711, Durham, North Carolina 27710

²Department of Biochemistry, Duke University Medical Center, Box 3711, Durham, North Carolina 27710

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Abstract

The contribution to the free energy of binding of each of the residues forming the binding site for a human IgG Fc fragment on the surface of the B1 domain of protein G was determined by alanine-scanning mutagenesis. The interface between these two proteins is atypical in that it is smaller than usual, polar in character, and involves two well-defined “knobs-into-holes” interactions. The bulk of the free energy of binding is contributed by three central residues, which make hydrogen bonds across the interface. Of these, the most critical interaction is formed by Glu27, which acts as a charged knob on the surface of the B1 domain, inserting into a polar hole on the Fc fragment. A single alanine mutation of this residue virtually abolishes stable complex formation. Formation of a stable interface between these two proteins is therefore dominated by a small, polar “hot spot.”

Keywords: alanine scanning mutagenesis; B1 domain of protein G; disassociation constant; environmentally sensitive fluorophore; human Fc IgG

Protein–protein interactions play an essential role in many biological processes. Understanding the energetics of such interactions is of great importance because it defines the necessary concentrations of interacting partners, the rates at which these partners are capable of associating, and the relative concentrations of bound and free proteins in the solution (Stites, 1997). Well-studied classes of protein–protein interactions (Jones & Thornton, 1996; LoConte et al., 1999) include hormone receptor binding and activation (Wells & deVos, 1996), antibodies with protein antigens (Davies & Cohen, 1996), enzyme inhibitor complexes (Tsunogae et al., 1986), and protein oligomerization (Argos, 1988). Here, we present a study of the contributions to the free energy of binding of the residues forming the site on the surface of the B1 domain *Streptococcal* protein G for binding of human Fc fragment of IgG (hFc). B1 is one of the domains of protein G, a member of an important class of proteins which form IgG-binding receptors on the surface of certain *Staphylococcal* and *Streptococcal* strains (Boyle, 1990; Frick et al., 1992). It has been suggested that these proteins allow the pathogenic bacterium to evade the host immune response by coating the invading bacteria with host antibodies (Goward et al., 1993), thereby contributing significantly to the pathogenicity of these bacteria. Furthermore, the B1 domain has found numerous

application in biotechnology as a reagent for affinity purification of antibodies (Ståhl et al., 1993), since it binds to IgGs of many different species and subclasses (Stone et al., 1989). A deeper understanding of the sequence determinants that contribute to IgG binding may therefore lead to new therapeutics for *Streptococcal* infections and novel immunochemical reagents.

B1 is a 56-residue domain that folds into a four-stranded β -sheet and one α -helix, as shown by NMR and X-ray crystallography. Despite its small size, the B1 domain has two separate IgG-binding sites on its surface, each interacting respectively with specific, independent sites on the Fab or Fc fragments of the antibody. The structure of a B1-Fab complex (Derrick & Wigley, 1992) has revealed that the Fab-binding site is mediated almost entirely through backbone contacts between the edge of the β -sheet of the B1 domain and the last β -strand of the C_H1 domain of the Fab fragment, thereby forming a continuous β -sheet spanning the interface between the two partners. In contrast, a B1-Fc complex (Sauer-Eriksson et al., 1995) has shown that the Fc-binding site is mediated primarily by side-chain contacts between the two proteins. This is further supported by competition experiments in which an 11-residue peptide corresponding to the C-terminus of the α -helix and the N-terminal part of the third β -strand competitively inhibits binding of B1 to hFc (Frick et al., 1992). NMR experiments also confirm the general location of the Fc-binding site on the surface of the B1 domain (Gronenborn & Clore, 1993; Kato et al., 1995).

Reprint requests to: Homme W. Hellinga, Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, North Carolina 27710; e-mail: hellinga@linnaeus.biochem.duke.edu.

Compared to most other protein–protein interactions, the Fc-binding site on the B1 protein is somewhat atypical. First, it is predominantly polar rather than hydrophobic in character (Stites, 1997); second, the interfacial area is on the lower end of the observed range, $\sim 700 \text{ \AA}^2$ rather than the average $1,200 \text{ \AA}^2$ (Jones & Thornton, 1996); third, rather than a planar interaction surface typically observed in heterodimers, this interface is formed by a double “knobs-into-holes” interaction (Crick, 1952, 1953) in which a knob from the B1 protrudes into a hole in the hFc, and vice versa. This study defines the energetic contributions of each of these elements on the B1 domain binding surface for hFc.

Results

Mutagenesis of the Fc-binding site on the B1 domain

The X-ray structure of the complex between the B1 and a human IgG Fc fragment (hFc) (Sauer-Eriksson et al., 1995) was used to identify the residues located in the interface between these two proteins. The static solvent-accessible surface (Richards, 1977) was calculated for the B1 domain in the presence and absence of the hFc fragment. Eleven residues showed a significant decrease in solvent accessibility in the structure of the complex (Table 1). Single alanine mutants of each of these residues were constructed. These are located in the alpha helix, the third β -strand, and the loop between this helix and strand (Fig. 1).

Binding studies

The interaction of the mutant B1 domain with the hFc was measured using a fluorescence method previously developed by us (Sloan & Hellinga, 1998), which relies on the attachment of an environmentally sensitive fluorophore placed at the rim of the binding site in a location such that its fluorescence changes upon formation of the complex by virtue of alterations in conformational degrees of freedom and solvent accessibility, without ad-

versely affecting the binding constant. Binding constants for hFc were determined in a direct titration experiment in which purified hFc was added to a solution of a Q32C B1 mutant to which the fluorophore acrylodan was covalently coupled at position 32C (Fig. 2A). The binding constants for all the B1 mutants were determined in the Q32C background (Table 1). For those mutants whose K_d showed a greater than 50-fold increase, the binding constants were determined in a competition experiment in which a complex was preformed between fluorescently labeled wild-type B1 (Q32C) and hFc, which was then titrated with an unlabeled, mutant B1 domain whose free cysteine at position 32 had been blocked with iodoacetamide (Fig. 2B). This approach was used to avoid having to use large quantities of pure hFc.

To verify that large decreases in the binding constants were not due to loss of structure, we took advantage of the fact that B1 has a separate binding site for the Fab fragment, which does not overlap with the Fc binding site. Fab binding was determined semi-quantitatively by phage display in M13 (Smith & Scott, 1993) using a genetic fusion between gene III and B1, expressed as a second copy in the phage genome (Armstrong et al., 1996) to obtain uni- rather than polyvalent display of the B1 domain (McConnell et al., 1994). In all cases, the mutants constructed in the Fc-binding site retained the ability to bind Fab (data not shown), indicating that there was no significant loss in overall structure.

The results show that 5 out of the 10 residues in the binding site had a significant effect on hFc binding (K_d increased at least 10-fold), whereas the other 5 showed little or no effect (less than twofold change in K_d). A single mutant, E27A, virtually abolished binding ($>4,000$ -fold increase in K_d). The five residues that most affected binding form a contiguous patch on the surface of B1, surrounded by a ring of the other residues (Fig. 1).

Discussion

The experiments described in this paper report the energetic contributions of the residues forming the binding site between the B1 domain of protein G and human IgG Fc fragment. The X-ray structure of a complex between the B1 domain and hFc show that about 13 residues on the surface of B1 are directly involved in the binding site as defined by a change in their solvent-accessible area upon complex formation. Three of these residues are alanines, and we did not perform glycine scanning mutagenesis to determine the energetic contribution of these residues. Alanine mutations of only about half of the 10 mutated residues significantly affect the binding free energy (at least 10-fold increase in K_d). Within these five residues the effect of binding varies sharply, ranging from 10-fold to more than 4,000-fold increases in K_d , indicating that the determinants contributing to the free energy of binding are highly localized. The localization of binding energy to such “hot spots” has also been observed in other heterodimeric interfaces (Bogan & Thorn, 1998; Wells, 1991).

The binding hot spots on the surface of the B1 domain are associated with clear structural motifs. The binding site is formed by two “knobs-into-holes” interactions. E27 of the B1 domain fits into a hole formed by I253 and S254 on the surface of the Fc fragment, where the carboxylate forms hydrogen bonds with the backbone amides of these residues as well as the O_γ of S254. Mutation of this charged knob on the surface of the B1 domain virtually completely abolishes binding. The carboxylate of E27 is held in position by a hydrogen bond from the amino group of K31, the neighboring residue, on the surface of B1. Removal of this

Table 1. Binding properties

Mutant	K_d^a (μM)	$\Delta\Delta G^b$ (kcal/mol)	H— bonds ^c	f_{SASA}^d
Q32C ^e	0.24	0	1	0.53
T25A	0.36	0.24	0	0.6
E27A	$>1,000^f$	$>4.9^f$	3	1
K28A	2.0	1.3	1	0.95
K31A	85	3.5	0	0.94
N35A	13	2.4	2	0.79
D40A	0.38	0.3	0	0.40
E42A	0.46	0.4	1	0.30
W43A	140	3.8	1	0.89
T44/Y45A	6.5	2.0	0	0.43

^a K_d calculated from Equation 1.

^b $\Delta\Delta G = -RT \ln(K_d \text{ wild-type}/K_d \text{ mutant})$; $T = 298 \text{ K}$.

^cNumber of hydrogen bonds formed by the residue and the Fc as determined by inspection of the crystal structure.

^d f_{SASA} is fractional change in solvent accessible surface area: $f_{\text{SASA}} = \text{SASA}(\text{complex})/\text{SASA}(\text{free})$.

^eThe wild-type construct relative to which all measurements are made.

^fBinding is too weak to be quantified.

Fig. 1. The interaction between the B1 domain and a human Fc fragment. All structures were drawn from the coordinates of the B1-hFc complex (Sauer-Eriksson et al., 1995) (Brookhaven accession number 1fcc). **A:** Ribbon representation with the two partners pried apart. The residues on the surface of the B1 domain are colored according to the loss in binding free energy when mutated to alanine: yellow, >500-fold; orange, >300-fold; red, >50-fold; brown, >10-fold; grey, <2-fold. The approximate position of the reporter fluorophore covalently attached to a cysteine at position 32 is indicated by the purple sphere. **B:** Surface rendering of the interaction, highlighting the position of the knobs and holes on the two proteins. **C:** Stereopair showing details of the interaction between the B1 domain (heavy lines) and the C γ 2-C γ 3 region of the Fc fragment (light lines).

Fig. 2. The interaction of B1 mutants with a human Fc fragment. Binding was monitored by changes in fluorescence of an acrylodan reporter group site-specifically attached to a cysteine mutation at position 32 of the B1 domain. Each point represents the average of three independent titrations, with experimental errors as indicated. **A:** The unlabeled Fc fragment was titrated into a solution of 250 nM of the T25A mutant B1 domain. The data were fit to Equation 1. Experimental error bars are smaller than symbols on graph. **B:** Determination of the free energy of binding of a weakly binding B1 mutant, N35A, by a competition experiment in which unlabeled mutant protein is titrated into a 250 nM preformed complex of fluorescently wild-type B1 and human Fc. The data were fit to Equation 2.

interaction in the K31A mutant significantly decreases affinity (350-fold increase in K_d). The second knob-into-hole interaction is formed by the protrusion of N434 from the surface of the Fc into a hole in B1 bordered by N35, D36, D40, E42, and W43. The indole nitrogen of W43 forms a hydrogen bond with N434. Of all the bordering residues forming the hole on B1, the W43A mutant has the most profound effect on the interaction (580-fold increase in K_d), presumably as a direct consequence of this interaction. Of the other residues, only N35A significantly affects binding (50-fold increase in K_d). Interestingly, it makes a hydrogen bond with H433 on the hFc.

These results quantify the contribution of the individual residues identified in the high-resolution structure of the B1-hFc complex (Sauer-Eriksson et al., 1995). It was noted in the description of this structure that all of these residues are involved in contacts between

the two proteins. From this study it is clear, however, that only a small subset makes significant contribution to the free energy of binding, illustrating the importance of combining structural information with thermodynamic data. It should be emphasized that in an alanine-scanning mutagenesis experiment side chains are deleted. Lack of a large effect upon loss of an interaction does not imply that the choice of a particular amino acid is unimportant, since replacement with residues that result in incorrect steric packing or charge complementarity may adversely affect proper formation of the interface. Furthermore, an alanine scanning experiment does not address the issue of main-chain interactions. Two B1 domain residues potentially form main-chain interactions with hFc residues, those being the nitrogen of G41 and the carbonyl oxygen of V39 (Sauer-Eriksson et al., 1995).

The four critical binding residues within the B1 domain are glutamate, tryptophan, lysine, and asparagine. Tryptophan is frequently found within interfaces, whereas lysine and asparagine are not normally enriched, and glutamate is underrepresented in heterodimer interfaces (Bogan & Thorn, 1998). It has been suggested that hot spots located in planar interfaces need to be surrounded by a ring of residues that exclude bulk solvent (the O-ring hypothesis), aiding in the formation of polar hydrogen bonds (Bogan & Thorn, 1998). The polar knobs-into-holes binding motif observed in the B1 domain provides another mechanism to form buried, polar hydrogen bonds.

Staphylococcal protein A competitively binds to a similar site on hFc as the B1 domain, involving in both cases hFc residues 252–254, 433–435, and 311 (Deisenhofer, 1981; Sauer-Eriksson et al., 1995). Whereas the interactions between B1 and hFc are predominantly polar, half of the protein A interactions are polar and half are hydrophobic. These proteins present an example of two distinct folds that have evolved different structural features to achieve binding at very similar sites on the same target molecule.

The existence of a well-defined hot spot suggests that it may be possible to develop a low molecular weight reagent that could disrupt this interface. Such a reagent could have applications in immunochemistry as well in treatment of Streptococcal infections by potentially unmasking bacteria cloaked in a coat of antibodies.

Materials and methods

Mutagenesis

The construction of the B1 domain construct (Q32C) has been reported previously (Sloan & Hellinga, 1998). All mutations in this study were constructed in that background by polymerase chain reaction (PCR) mutagenesis (Ho et al., 1989). A C-terminal oligohistidine (His_5) affinity tag was used to allow the purification of the mutant proteins by immobilized metal affinity chromatography (Hochuli et al., 1988). The recombinant genes were flanked by *EcoRI* sites that allowed facile cloning into either the pKK223-3 vector for protein expression or the M13 construct for phage display using gIII fusions.

Protein expression and purification

The expression and purification of mutant B1 domains has been previously described (Sloan & Hellinga, 1998). A typical expression experiment produced 15 mg of pure mutant protein from 1 L of culture.

Fluorophore coupling and iodoacetamide blocking

All of the above mutations were constructed into the Q32C background to allow site-specific covalent coupling with acrylodan for fluorescent binding assays as described previously (Sloan & Hellinga, 1998). In competitive binding experiments between fluorescently labeled wild-type B1 and unlabeled mutants, the free thiol in the mutant protein was blocked by reaction with iodoacetamide.

Binding of B1 mutants to human IgG Fc fragment

The binding constants for each of the alanine mutants were measured as an increase in fluorescence by direct titration of Fc fragment of human IgG (ICN Biomedicals, Costa Mesa, California) into a solution containing 250 nM acrylodan-conjugated B1 domain mutant in 20 mM KPO₄ (6.0) at 25 °C (excitation 392 nm; emission 500 nm; slit widths of 4 and 16 nm, respectively). After each addition of protein, the solution was allowed to equilibrate for 30 s before the final fluorescence value was recorded. Each titration consists of 20 points and was performed in triplicate. The data were fit to a binding isotherm that described a single binding site and takes into account all of the species present (Segel, 1975):

$$F = F_0 + (F_{\max} - F_0) \times \frac{([B1]_t + [hFc]_t + K_d - \sqrt{([B1]_t + [hFc]_t + K_d)^2 - 4[B1]_t[hFc]_t})}{2[B1]_t} \quad (1)$$

where F is the measured fluorescence with F_0 and $F_{\max}$ representing the initial and final values, respectively. $[B1]_t$ and $[hFc]_t$ are the concentrations of B1 and hFc, respectively, and the K_d is the disassociation constant.

The binding constants of weak binders (greater than 50-fold increases in K_d) were measured in a competition assay with a pre-formed complex (250 nM) of wild-type Q32C construct conjugated to acrylodan and hFc. Binding constants were determined by measuring the decrease in fluorescence of the complex by the addition of iodoacetamide blocked mutant B1 domains. From these competition binding curves, the K_d for each of the mutant B1 domains was calculated from the binding isotherm (Fierke et al., 1991):

$$F = \frac{F_0}{1 + \left(\frac{K_{DWT}}{[B1_{WT}]} \right) \times \left(1 + \frac{[B1_{mut}]}{K_{Dmut}} \right)} + F_f \quad (2)$$

where F is the measured fluorescence with F_0 and F_f representing the initial and final values, respectively. $[B1_{WT}]$ and $[B1_{mut}]$ are the concentrations of B1 wild-type and mutant, respectively, and K_d is the disassociation constant of the mutant or wild-type.

Phage display

A gly-gly-gly-ser-gly-gly-gly-ser linker was inserted into the *NotI* site of pCANTAB5 (Pharmacia, Uppsala, Sweden) to reduce the interactions between the displayed protein and PIII (Smith & Scott, 1993). The resulting gIII gene fusion was subcloned from pCANTAB5 into the multiple cloning site of M13mp18 (Messing, 1991). A unique *NheI* site was introduced into the gIII fusion by site-directed mutagenesis (Kunkel, 1985) into which the B1 gene

was cloned. Alanine mutations were constructed directly into this vector. Biopanning (Smith & Scott, 1993) was used to determine the Fab-binding properties of the fusion constructs, using 100 ng biotinylated IgG, 5×10^9 pfu of each B1 bacteriophage, high protein binding ELISA wells (Greiner, Frickenhausen, Germany), and 2% nonfat dry milk blocking solution. Binding between mutant B1 and IgG was detected by anti-M13 antibody-HRP conjugate (Pharmacia; manufacturers protocol). Control experiments in which the Fab fragment was left out were done in parallel to rule out nonspecific binding.

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