Multisite association by recombinant proteins can enhance binding selectivity

Preferential removal of immune complexes from serum by immobilized truncated FB analogues of the B domain from staphylococcal protein A

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INTRODUCTION

A common goal of protein engineering is to enhance recombinant protein binding to a cell or to another macromolecule. A typical strategy involves modifying individual protein binding sites to increase their affinity for target molecules, but this may not improve specificity of binding. Significantly enhanced binding selectivity may arise, in principle, from multisite binding interactions of low individual affinity. Binding species that generally meet these criteria may utilize engineered antibody binding sites, such as modified single-chain Fv (sFv) proteins (1–4), directed against any antigen of interest. The present research sought to remedy a practical biomedical problem: disease-related immune complexes (ICs) can be removed from human serum by immobilized staphylococcal protein A, but only with accompanying depletion of free IgG, thereby compromising patients' humoral immunity. As the basis for an affinity separation device that would exclusively bind ICs in normal serum, we devised truncated analogues of the B domain of staphylococcal protein A (5) that bound monomeric IgG with greatly reduced affinity. Other workers studied an immobilized peptide from C1q which exhibited selectivity for immune complexes (6). However, our experiments were designed to test this multisite binding hypothesis by comparing the binding of ICs and IgG in serum by immobilized FB species of high and low affinity.

MATERIALS AND METHODS

Several truncated fragment B analogues were prepared, which are designated by the number of residues retained from the 58 residue sequence of the parent B domain in staphylococcal protein A (Table 1). The FB 58 and FB 29 genes were prepared from synthetic oligonucleotides (1, 5), encoding the protein sequences given in Table 1. The FB 58 protein and its analogues were produced by recombinant DNA methodology with direct or fusion protein expression in *Escherichia coli* (1–5), and FB 51 was prepared by tryptic cleavage of FB 58. Cysteine was added to these sequences for purposes of immobilization; the FB genes were modified so that the FB 58 sequence was preceded by Met-Ala-Cys, while the FB 29 sequence was preceded by Met-Pro-Ala-Cys. The MAC-FB 58 was expressed directly in *E. coli,* whereas tandem repeats of 3 or 4 MPAC-FB 29 genes were expressed as fusion proteins that formed inclusion bodies. The MPAC-FB 29 fusion protein was treated with cyanogen bromide (0.25 g/g cell paste) in 0.1 N HCl and was purified by HPLC (5), resulting in mixtures of species with and without COOH-terminal homoserine lactone; the PAC-FB 29 binding properties appeared insensitive to this slight heterogeneity. Cysteine-containing proteins were immobilized on diaminodipropylamine-Sepharose CL-4B that was first modified with m-maleimidobenzoyl sulfosuccinimide (sulfo-MBS; Pierce Chemical Co., Rockford, IL) (5).

The relative affinities of FB analogues (Table 1) were determined by competition radioimmunoassays in which a dilution series of a given FB analogue was incubated with HulgG-coated polyvinyl chloride microtiter plates; after washing, a constant quantity of ^{125}I-labelled FB 58 was incubated with each well to determine the percent saturation by analogue compared to control wells devoid of analogue; the relative percent inhibition by a given analogue was plotted as a function of its concentration. Using an experimental estimate for recombinant FB 58 binding to HulgG of $K_{a,app} = 2.5 \times 10^7\ M^{-1}$ (1), the unknown binding constant was calculated as the product of this $K_{a,app}$ value times the quotient of the 50% inhibition concentration of FB 58 divided by that of the truncated FB analogue.

Chemically aggregated IgG (denoted CAG in Table 2) was prepared by carbodiimide cross-linking, and the tetrameric M, 600,000 protein fraction was isolated by size exclusion chromatography; experiments also involved heat aggregated IgG as model ICs. Concentrations of CAG as a model immune complex were measured by a circulating immune complex assay kit from Cytotech (San Diego, CA), wherein concentrations of an unknown IC are related to a reference curve based on a heat aggregated IgG standard, and IC levels are given in μg equivalents of the standard per ml (μg Eq/ml). Free human IgG (HuIgG) concentrations were measured by radioimmunoassay (5) in normal human serum (NHS) or in CAG-NHS mixtures before and after resin treatment. In the IC binding experiments, 100 μl of CAG/NHS or NHS were added to 100 μl of resin (varied as noted in Table 2) pelleted by centrifugation in an Eppendorf tube (Table 2); following incubation, the suspension was respun in an Eppendorf centrifuge, the supernatant solution was saved and pooled with an additional 100 μl wash of each resin pellet, and subsequently assayed.

RESULTS AND DISCUSSION

The rationale for generating the low affinity FB analogues was primarily based on the crystal structure of FB 58 complexed with human Fc (7). Deisenhofer found that FB residues 5–47 were identifiable as a core bound to the Fc fragment, but residues 1–4 and 48–58 were not

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TABLE 1 Sequences of truncated FB analogues and their $K_{a,app}$ values for binding to human IgG

Helix designation:	I	II	III	
	1 4 7 8	36	48 58	
FB 58	ADNKFNK	EQQNAFYEILHLPNLNEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK	$K_{a,app} = 2.5 \times 10^7\ M^{-1}$
	8	36		
FB 29		EQQNAFYEILHLPNLNEEQRNGFIQSLKD		$2 \times 10^2 < K_{a,app} < 1 \times 10^3\ M^{-1}$
	1	36		
FB 36	ADNKFNK	EQQNAFYEILHLPNLNEEQRNGFIQSLKD		$K_{a,app} = 1 \times 10^3\ M^{-1}$
	8		47	
FB 40		EQQNAFYEILHLPNLNEEQRNGFIQSLKD	DPSQSANLLAE	$3 \times 10^3 < K_{a,app} < 1 \times 10^4\ M^{-1}$
	1		47	
FB 47	ADNKFNK	EQQNAFYEILHLPNLNEEQRNGFIQSLKD	DPSQSANLLAE	$K_{a,app} = 1 \times 10^3\ M^{-1}$
	8		58	
FB 51		EQQNAFYEILHLPNLNEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK	$K_{a,app} = 2.5 \times 10^6\ M^{-1}$

Polypeptide sequence is given in single letter code; underlined helices I and II were defined in crystals (7) and III in solution (11).

TABLE 2 Levels of model immune complexes and human IgG after resin adsorption

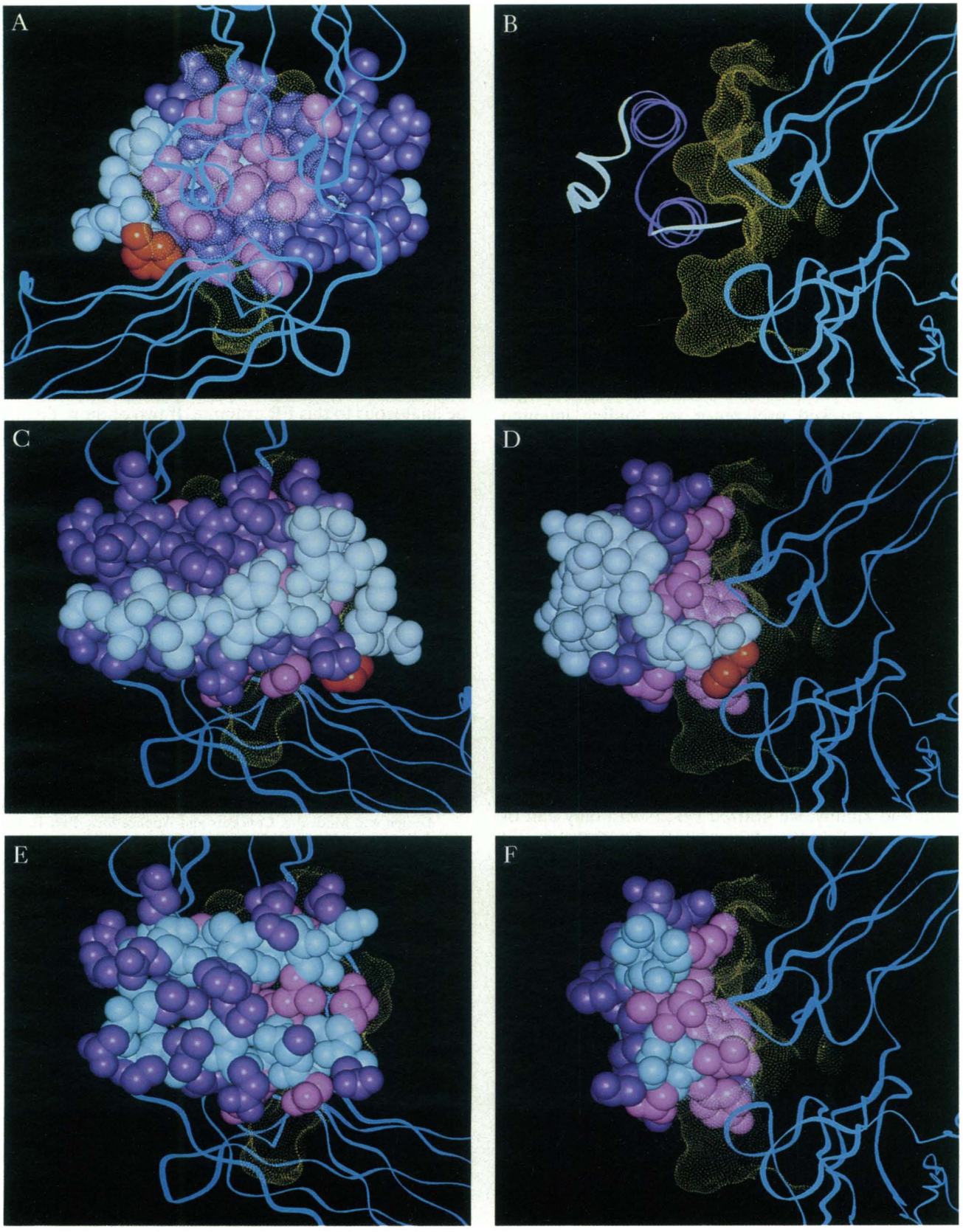
Resin conjugate	Ig source	Measured species	Immune complex or human IgG concentration after resin adsorption					
			No resin	Control resin	1 mg FB/ml resin	2 mg FB/ml resin	3 mg FB/ml resin	4 mg FB/ml resin
FB 29	CAG/NHS	IC*	21.0	18.3	12.7	10.3	8.0	7.0
		HuIgG‡	4.03	3.70	3.73	3.04	3.72	3.55
FB 58	CAG/NHS	IC	21.0	18.3	10.2	9.1	7.0	8.0
		HuIgG	4.03	3.70	2.11	1.49	0.99	0.90
FB 29	NHS	IC	2.2	1.6	n.d.§	n.d.	n.d.	0.8
		HuIgG	3.82	3.10	2.78	3.46	3.26	3.39
FB 58	NHS	IC	2.2	1.6	n.d.	n.d.	n.d.	0.2
		HuIgG	3.82	3.10	1.97	1.07	1.04	0.92

*Concentration of immune complexes is expressed in μg Eq/ ml (note Materials and Methods). ‡Concentration of human IgG is given in mg/ml. §n.d. Not determined.

accounted for in the FB-Fc electron density maps. This core comprises two antiparallel α-helices that are denoted I and II in Table 1. Thus, our strategy was to preserve most of this core so that it could maintain its specificity of binding to the Fc, and we progressively deleted parts of the flanking polypeptide sequence as a means for reducing its Fc-binding affinity. This approach generated analogue affinities ranging from $\sim 10^2\ M^{-1}$ to

FIGURE 1 Structural views of the fragment B and its contacts with the Fc region, based on Deisenhofer's crystallographic analysis of FB 58 complexed with human Fc fragments (7). FB 58 (represented by space-filling models in all panels except *B*) binds to Fc at the junction of its C_H2 and C_H3 domains (shown as blue ribbons tracing the peptide backbone). The approximate Fc interactions with FB 58 are displayed as a yellow dot surface showing contours of Fc atoms within 4 Å of the FB 58. The FB helix axes are parallel to the page in the left series of pictures, while the FB helix axes are orthogonal to the page in the right series. X-ray diffraction studies localized only 43 residues of the FB 58 polypeptide in electron density maps (7), and therefore, only residues 5–47 are displayed in *A–D*; *E* and *F* display residues 8–36, representing the FB 29 portion of FB 58 in the parent structure. (*A*) FB 58 oriented with its Fc interaction surface visible through the Fc ribbon profile. Color scheme for *A*, *C*, and *D*: atoms in residues 5–7 and 37–47 are light gray, except for the red sidechain of Phe-5, which contacts the Fc; atoms in residues 8–36 corresponding to FB 29 are purple, except for lavender side-chains, which contact the Fc region. (*B*) Side view of the FB-Fc complex with both displayed as ribbon profiles; the chain termini of FB 29 (*purple ribbon*) are facing toward the reader; the crystallographically defined residues in FB 58 that flank FB 29 are shown as white ribbons (residues 5–7 on the NH_2-terminal side, and partially helical residues 37–47 on the COOH-terminal side). (*C*) Solvent accessible side of FB 58 complexed to Fc (a two-fold rotation of view *A*; color scheme is the same as for *A*). (*D*) Right side of structure in *C*, viewed along the axes of helices I and II; color scheme is the same as for *A*. (*E*) FB 29 portion of the FB 58 crystal structure shown in *C*; peptide backbone is light blue, side-chains contacting Fc are lavender, and other side-chains are purple. (*F*) Right side of structure in *E*, viewed along the axes of helices I and II; color scheme is identical to that for *E*.

Molecular modeling was conducted on a Silicon Graphics 4D-70 GTX Superworkstation (Silicon Graphics, Mountain View, CA) using coordinates from the 1FC2 coordinates (version: January 1990) for the FB-Fc structure (7) obtained from the Brookhaven Protein Data Bank (9) and modeled with the Insight II Program (Biosym Technologies, Inc., San Diego, CA).


A
B
C
D
E
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10^6 M^{-1} (Table 1), which at the lower extreme corresponds to a dissociation constant of 10^{-2} M that virtually precludes single-site binding to IgG at its normal serum concentration of $\sim 5 \times 10^{-5}$ M.

FB analogues immobilized at 1–4 mg/ml of resin provided sufficient binding site density to demonstrate validity of the multisite binding hypothesis. As the density of immobilized FB 29 protein was increased, the ICs were preferentially removed from CAG/NHS and NHS in a dose dependent manner while leaving IgG at control levels (Table 2). In relation to control resin devoid of FB, the FB 29 resins bound 41–60% of the CAG with negligible binding of monomeric IgG, whereas the FB 58 resin bound the CAG similarly but its resin conjugates reduced the HuIgG level by 43–76% (Table 2).

This research has demonstrated that immobilized FB 29 having a low intrinsic affinity for the Fc region of IgG exhibited a marked preference for binding immune complexes over free IgG, whereas immobilized FB 58 exhibited no such preferential binding. The interactions involved in this experiment can be readily interpreted in terms of the unitary free energy change (8) resulting from a single binding interaction (ΔG_u) versus multisite interaction at n sites ($n\Delta G_u$). Corresponding K_0 values, calculated using molal concentrations, would then be K_0 for IgG versus K_0^4 for the $(IgG)_4$ model ICs noted above; for FB 29 binding to monomeric IgG with a K_0 of 10^2 m^{-1}, it would bind four sites on the IC with a maximum K_0 of 10^8 m^{-1}.

The specificity shown by FB 29 in binding IgG multimers (Table 2) is consistent with its retention of essential Fc-complementarity exhibited by the FB 58 (Fig. 1). Given the practical advantages of working with a functional B domain analogue of the lowest molecular weight and affinity, we utilized FB 29 with only half of the parent B domain polypeptide (residues 8–36) and $K_{a,app}$ between 2×10^2 M^{-1} and 1×10^3 M^{-1}. However, the dramatic reduction of Fc-binding affinity achieved by deleting COOH-terminal residues (Table 1) raises significant question about the physical basis for Fc binding by intact B domain. Most of this affinity change was effected by elimination of the COOH-terminal 11 residues of the parent FB 58, leading to FB 47 (residues 1–47) with $K_{a,app}$ of 1×10^3 M^{-1}. Although this COOH-terminal segment was not crystallographically defined in the FB-Fc complex (7), it was shown by solution NMR to contribute to helix III formation in the Fc-free state (helix III consists of residues 41–54 in our FB 58 sequence) (10, 11). Deletion of 7 residues at the amino-terminus in FB 51 only lowered $K_{a,app}$ to 2.5×10^6 M^{-1}, which emphasizes the importance of this COOH-terminal segment to FB 58 association with the Fc region. Simple electrostatic effects do not provide a rationale for the observed order of FB analogue Fc-affinities (Table 1), as the net charge calculated at neutral pH showed the Fc to be positive and various truncated FB forms to be negative, with weak binding FB 47 and FB 29 analogues both more negative than FB 58. This high affinity binding phenomenon appears to involve functional linkage (12) between the COOH-terminal helix-coil transition and the FB 58-Fc interaction (10, 11). Truncated forms of the B domain appear to hold promise for the removal of circulating immune complexes by extracorporeal perfusion (13) and for biophysical analysis of FB-Fc association.

This approach may be extended to multisite binding with other recombinant molecules, such as single-chain Fv analogues and fusion proteins (2). These may be individually immobilized on a suitable matrix for affinity fractionation or fused to each other for solution targeting (2, 4). Use of a single form of low-affinity sFv would be analogous to this FB example. If two or more distinct sFv species were used together, specificity could be enhanced further for multi-determinant antigens or multi-antigen cell surfaces.

Molecular modeling and single-chain Fv research were supported in part by National Institutes of Health grants CA39870 and CA51880.

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