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A Bifunctional Fusion Protein Containing Fc-Binding Fragment B of Staphylococcal Protein A Amino Terminal to Antidigoxin Single-Chain Fv†

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Received May 21, 1990; Revised Manuscript Received July 5, 1990

ABSTRACT: A bifunctional molecule was genetically engineered which contained an amino-terminal effector domain that bound immunoglobulin Fc (fragment B of staphylococcal protein A) and a carboxyl-terminal domain that bound digoxin [a single-chain Fv (sFv)]. Effector and sFv binding properties were virtually identical with those of the parent molecules, despite the proximity of the FB to the sFv combining site. This finding is unprecedented since in all molecules of the natural immunoglobulin superfamily, the antigen binding domain is amino terminal to the effector domain. The FB-sFv sequence was encoded in a single synthetic gene and expressed as a 33 106 molecular weight protein in *Escherichia coli*. After purification, renaturation, and affinity isolation, yields of active fusion protein were 110 mg/L of fermented cells (18.5-g cell paste). Bifunctionality was confirmed by the ability of FB-sFv to cross-link IgG to digoxin–bovine serum albumin, as measured by plate assays and by Ouchterlony analysis. Analysis of the expressed fusion protein suggests that the sFv holds promise for the development of multifunctional, targetable single-chain proteins.

The ability to target therapeutic agents with antibodies has been a long-term goal of medical research. The most elegant targetable proteins would consist of the minimum structures needed for selective delivery and effector function. Here we combined the minimal antigen binding site, a single-chain Fv,[1] with an individual Fc binding domain of staphylococcal protein A into a single polypeptide that expresses both digoxin and Fc-binding properties.

In immunoglobulins, separate heavy and light chains contribute the V_H and V_L variable domains that constitute the Fv region (Inbar et al., 1972) and form the antibody combining site. Recently, protein engineering methods have been used to link the V_H and V_L, creating functional single-chain Fv proteins (Huston et al., 1988a; Bird et al., 1988). The resulting

† This research was supported in part by NIH through Small Business Innovation Research Grant CA 39870 and Program Project Grant HL 19259.

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[1] Abbreviations: BSA, bovine serum albumin; CDR, complementarity-determining region; EDTA, ethylenediaminetetraacetic acid; Fab, antigen binding fragment derived from IgG by papain cleavage; FB, fragment B, a 58-residue domain of staphylococcal protein A; FB-sFv$^{26\text{-}10}$, fusion protein that comprises FB and 26-10 single-chain Fv; Fc, complement binding region of IgG that associates with the FB domain; FR, framework region; Fv, variable-region fragment consisting of noncovalently associated V_H and V_L domains; GaMFab, goat anti-mouse Fab antibody; Gdn-HCl, guanidine hydrochloride; hIgG, human immunoglobulin G; mIgG, murine immunoglobulin G; PBSA, 0.15 M NaCl + 0.05 M potassium phosphate, pH 7.0, + 0.03% NaN_3; rIgG, rabbit immunoglobulin G; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sFv (single-chain Fv), biosynthetic Fv analogue comprising both variable domains on a single polypeptide chain; V_H, heavy-chain variable region; V_L, light-chain variable region.

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sFv offers advantages for design and production of both individual binding proteins and fusion proteins, as exemplified by single-chain Fv immunotoxins constructed with a toxin attached to the sFv carboxyl terminus (Chaudhary et al., 1989, 1990). Moreover, the unimolecular folding reaction of the sFv leads to a native structure that is independent of concentration, whereas heterodimeric Fv can dissociate at low concentration into separate V_H and V_L domains (Glockshuber et al., 1990; Riechmann et al., 1988). The principle of a single-chain Fv (Huston et al., 1988a; Bird et al., 1988) has been successfully used in several subsequent sFv constructions[2] (Chaudhary et al., 1989, 1990; Condra et al., 1990; Glockshuber et al., 1990), suggesting general utility for this approach.

We undertook the present experiments to probe the design of sFv fusion proteins and to assess the fidelity of the sFv antigen binding site. Ideally, sFv fusion proteins should exhibit the unperturbed activities of their component domains. We developed FB-sFv$^{26\text{-}10}$ as a model that could be thoroughly analyzed to assess the nativeness of its two binding regions. The sFv was based on monoclonal antibody 26-10, for which highly sensitive assays allow a precise measure of its digoxin-binding properties[3] (Mudgett-Hunter et al., 1982; Huston et al., 1988a). The effector domain was Fc-specific fragment B (FB) of staphylococcal protein A, for which Fc-binding interactions have been well characterized (Lancet et al., 1978; Wright et al., 1978; Deisenhofer, 1981). In the present investigation, the FB sequence was fused to the amino terminus of the 26-10 sFv by recombinant DNA methods, and the fusion protein was bacterially expressed and refolded in vitro. The digoxin-binding properties of both FB-sFv$^{26\text{-}10}$ and 26-10 sFv were determined in comparison with those of the parent 26-10 antibody and Fab fragment, and Fc binding to the fusion protein was characterized as well.

Experimental Procedures

Protein Design. The design of the 26-10 sFv has been described (Huston et al., 1988a), the architecture of the amino-terminal FB sequence can be predicted from crystallographic data,[4] and additional considerations that relate to the FB-sFv$^{26\text{-}10}$ are displayed in Figure 1.

Gene Synthesis. The 26-10 sFv gene was joined with a gene encoding the FB to make a 927-base sequence encoding the 33-kDa FB-sFv$^{26\text{-}10}$ polypeptide (Figure 1A). The FB-sFv$^{26\text{-}10}$ gene was constructed entirely from synthetic oligonucleotides. Assembly of 26-10 sFv has been detailed (Huston et al., 1988a), and the FB gene was assembled from synthetic 15-mers, essentially as described previously (Roberts et al., 1985). The two genes were fused by a pair of synthetic oligonucleotides spanning suitable restriction sites on each of the genes, which resulted in the addition of three extra amino acids (-Ser-Asp-Pro-) between the FB and V_H sequences.

Expression Vector Construction. The assembled gene was incorporated into an expression vector derived from pKK223-3 (Brosius & Holy, 1984) (Figure 1B), and the expression plasmid was used to transform *Escherichia coli* strain RB791. The entire FB-sFv gene was inserted between the *Eco*RI and *Pst*I sites of the modified pKK expression vector, and this pC105 plasmid was used to transform *E. coli* strain RB791 (lac Iq). The fusion protein was expressed under control of the tac promoter by addition of isopropyl thiogalactoside (IPTG). The modified pKK, conferring resistance to tetracycline and ampicillin, was assembled in four stages: (1) The tet gene of pBR322, from *Eco*RI to *Ava*I, was inserted into pUC8 between *Eco*RI and *Ava*I (*Sma*I). Clones were chosen that contained the *Ava*I site digestible with *Sma*I. Subsequently, the single *Eco*RI site was converted to an *Xmn*I site by Klenow polymerase-mediated filling-in. The orientation of the tet gene in this plasmid, between *Eco*RI and *Sma*I, is collinear with that of the amp gene. (2) The *Bam*HI site upstream of the tac promoter in pKK223-3 was modified to *Stu*I by using a synthetic oligonucleotide. (3) The tac promoter on a *Stu*I to *Pst*I fragment, including the downstream polylinker found in pKK223-3, was inserted into the tet-resistant pUC8 of step 1, between *Sma*I and *Pst*I. (4) The FB-sFv gene was inserted near the tac promoter between the proximal *Eco*RI site and the more distal *Pst*I site.

Fusion Protein Folding and Isolation. E. coli cell paste was suspended in 25 mM Tris-HCl, pH 8, and 10 mM EDTA (10 mL/g) and treated overnight with lysozyme (0.1 mg/mL) in the cold, followed by sonication to ensure complete cell lysis. Inclusion bodies were centrifuged from lysed cells, washed with buffer containing 3 M urea, 25 mM Tris-HCl, pH 8, and 10 mM EDTA, recentrifuged, and dissolved in a solution containing 6 M Gdn-HCl, 0.2 M Tris-HCl, pH 8.2, and 0.1 M 2-mercaptoethanol. After 1.5 h at room temperature, the solution was dialyzed against 6 M urea, 2.5 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5 mM dithiothreitol and chromatographed on a column of Whatman DE 52 equilibrated with this 6 M urea buffer. Pure FB-sFv$^{26\text{-}10}$ passed directly through the column, eluting ahead of impurities that were retarded by the resin. The pure FB-sFv$^{26\text{-}10}$ fractions were pooled and diluted at least 10-fold into redox refolding buffer (3 M urea, 25 mM Tris-HCl, pH 8, 10 mM EDTA, 1 mM oxidized glutathione, and 0.1 mM reduced glutathione), with a final protein concentration below 0.1 mg/mL. The refolding solution was kept at room temperature for 16 h and then dialyzed at 4 °C into 0.05 M potassium phosphate, pH 7.0, and 0.25 M urea to stop the redox reaction. The dialyzed protein was applied to a ouabain–Sepharose column for affinity purification, and the column was washed sequentially with dialysis buffer, 1 M NaCl in PBSA, and PBSA. The active fusion protein was eluted with 20 mM ouabain in PBSA and dialyzed against several changes of PBSA buffer to yield the material that was characterized in subsequent experiments. The concentration of FB-sFv$^{26\text{-}10}$ in PBSA was measured spectrophotometrically by using an extinction coefficient determined by dry weight measurements ($E^{0.1\%}_{280nm}$ = 1.3 mL mg^{-1} cm^{-1}). N-Terminal amino acid sequencing of the fusion protein and its cyanogen bromide fragment mixture was conducted with a gas-phase sequencer (Applied Biosystems), as described previously (Huston et al., 1988a).

sFv Refolding and Isolation. The 26-10 sFv was isolated as noted previously through the DE 52 fractionation (Huston et al., 1988a). The denatured 26-10 sFv was then incubated

[2] Initial reports of the present work appeared as meeting abstracts (Huston et al., 1988b, 1989).

[3] Intravenous administration of antidigoxin Fab fragments has been shown to be an effective treatment for digoxin toxicity, as the Fab binds digoxin and the complexes are eliminated through the kidney (Smith et al., 1982). The 26-10 monoclonal antibody and Fab fragment similarly reverse lethal digoxin toxicity, as shown in guinea pigs (Lechat et al., 1984).

[4] Fragment B of staphylococcal protein A is a 58-residue polypeptide that binds specifically to the Fc region of rabbit IgG. Its three-dimensional structure in a complex with human Fc was determined by X-ray crystallography (Deisenhofer, 1981), which defined the positions of FB residues 5–47 by electron density maps. The remaining amino acids were not accounted for in the maps, suggesting that 4 residues at the amino terminus and 11 at the carboxyl terminus may be mobile within the crystal or may at least occupy several different orientations that cannot be distinguished above background electron density in the crystallographic data. There can be a maximum of two FB molecules bound per Fc region.

A

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                                    FB-10                                                   FB-20                                                   FB-30
Ala Asp Asn Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe
GCT GAC AAC AAA TTC AAC AAG GAA CAG CAG AAC GCG TTC TAC GAG ATC TTG CAC CTG CCG AAC CTG AAC GAA GAG CAG CGT AAC GGC TTC
                                    FB-40                                                   FB-50                                                   FB-60
Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Ser Asp
ATC CAA AGC TTG AAA GAC GAC CCG TCT CAG AGC GCT AAC CTG CTG GCA GAG GCC AAG AAA CTG AAC GAC GCT CAG GCG CCG AAG AGT GAT

                                    10                                                      20                                                      30
Pro Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala Ser Val Arg Met Ser Cys Lys Ser Ser Gly Tyr Ile Phe
CCC GAA GTT CAA CTG CAA CAG TCT GGT CCT GAA TTG GTT AAA CCT GGC GCC TCT GTG CGC ATG TCC TGC AAA TCC TCT GGG TAC ATT TTC
                                    40                                                      50                                                      60
Thr Asp Phe Tyr Met Asn Trp Val Arg Gln Ser His Gly Lys Ser Leu Asp Tyr Ile Gly Tyr Ile Ser Pro Tyr Ser Gly Val Thr Gly
ACC GAC TTC TAC ATG AAT TGG GTT CGC CAG TCT CAT GGT AAG TCT CTA GAC TAC ATC GGG TAC ATT TCC CCA TAC TCT GGG GTT ACC GGC
                                    70                                                      80                                                      90
Tyr Asn Gln Lys Phe Lys Gly Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu
TAC AAC CAG AAG TTT AAA GGT AAG GCG ACC CTT ACT GTC GAC AAA TCT TCC TCA ACT GCT TAC ATG GAG CTG CGT TCT TTG ACC TCT GAG
                                    100                                                     110                                                     120
Asp Ser Ala Val Tyr Tyr Cys Ala Gly Ser Ser Gly Asn Lys Trp Ala Met Asp Tyr Trp Gly His Gly Ala Ser Val Thr Val Ser Ser
GAC TCC GCG GTA TAC TAT TGC GCG GGC TCC TCT GGT AAC AAA TGG GCC ATG GAT TAT TGG GGT CAT GGT GCT AGC GTT ACT GTG AGC TCT
                                    130                                                     140                                                     150
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Val Val Met Thr Gln Thr Pro Leu Ser Leu Pro Val Ser Leu
GGT GGC GGT GGC TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA TCT GAC GTC GTA ATG ACC CAG ACT CCG CTG TCT CTG CCG GTT TCT CTG
                                    160                                                     170                                                     180
Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser Leu Val His Ser Asn Gly Asn Thr Tyr Leu Asn Trp Tyr Leu Gln Lys Ala
GGT GAC CAG GCT TCT ATT TCT TGC CGC TCT TCC CAG TCT CTG GTC CAT TCT AAT GGT AAC ACT TAC CTG AAC TGG TAC CTG CAA AAG GCT
                                    190                                                     200                                                     210
Gly Gln Ser Pro Lys Leu Leu Ile Tyr Lys Val Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
GGT CAG TCT CCG AAG CTT CTG ATC TAC AAA GTC TCT AAC CGC TTC TCT GGT GTC CCG GAT CGT TTC TCT GGT TCT GGT TCT GGT ACT GAC
                                    220                                                     230                                                     240
Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Ile Tyr Phe Cys Ser Gln Thr Thr His Val Pro Pro Thr Phe Gly Gly
TTC ACC CTG AAG ATC TCT CGT GTC GAG GCC GAA GAC CTG GGT ATC TAC TTC TGC TCT CAG ACT ACT CAT GTA CCG CCG ACT TTT GGT GGT

Gly Thr Lys Leu Glu Ile Lys Arg *oc
GGC ACC AAG CTC GAG ATT AAA CGT TAA CTGCAG
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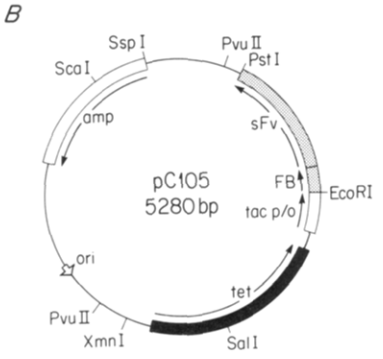

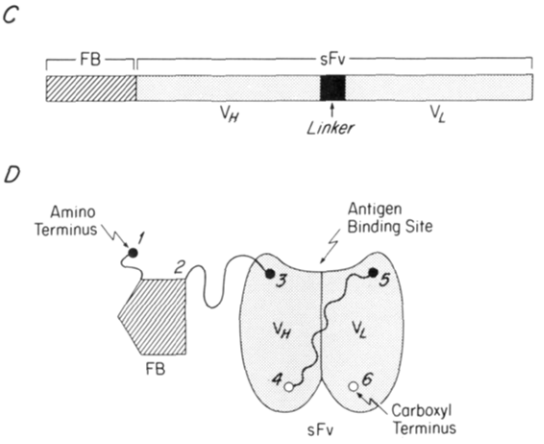

FIGURE 1: (A) Sequence of FB-sFv$^{26\text{-}10}$ fusion protein and the corresponding gene. The upper sequence is that of FB (FB-1 to FB-58) followed by -Ser-Asp-. The lower sequence is the 26-10 sFv (residues 1–248), in which the 15 residues bridging the V regions are underlined and complementarity-determining regions (CDRs) are shaded. The actual gene began with an *Eco*RI site followed by the initiation codon (ATG); the first amino acid (*N*-formylmethionine) of the translation product was removed in the bacterial host. (B) Circular map of the FB-sFv$^{26\text{-}10}$ expression plasmid. (C) Linear arrangement of protein domains and linker in the FB-sFv$^{26\text{-}10}$ polypeptide chain. (D) Schematic diagram of the folded bifunctional FB-sFv$^{26\text{-}10}$, drawn to emphasize the proximity of the sFv amino terminus to the antigen binding site, based on analogy with known structures (Huston et al., 1988a). Integers on the diagram indicate the following aspects: 1, amino terminus of the FB-sFv$^{26\text{-}10}$ polypeptide; 2, residue FB-47 corresponding to the end of the FB core region; 3, position of the amino terminus of the V_H and sFv, to which the FB sequence was fused; 4, carboxyl terminus of the V_H region, to which the 15-residue linker was fused (Huston et al., 1988a); 5, amino terminus of the V_L region, to which the linker was also fused (Huston et al., 1988a); 6, carboxyl terminus of the V_L and FB-sFv$^{26\text{-}10}$.

for 16 h at room temperature in the same redox buffer used for refolding FB-sFv$^{26\text{-}10}$ (3 M urea, 25 mM Tris, pH 8, 10 mM EDTA, 1 mM oxidized glutathione, and 0.1 mM reduced glutathione). At this juncture, the 26-10 sFv renaturation protocol diverged from that of the fusion protein, since their solution properties are quite different. The glutathione was removed by extensive dialysis in the cold against 3 M urea buffer lacking the redox couple. The protein was then dialyzed into 0.01 M sodium acetate and 0.25 M urea, pH 5.5, and this buffer was used in the affinity purification that was otherwise as described (Huston et al., 1988a). Final storage included the addition of 0.03% sodium azide.

Ultrafiltration Binding Assay of Digoxin Affinity. Affinity measurements were determined by an ultrafiltration assay (Sophianopoulos et al., 1978) in buffer solutions containing 1% horse serum (IgG-free horse serum, Gibco Laboratories, Grand Island, NY) or 0.1% gelatin with PBSA at pH 7.3 or with 0.01 M sodium acetate and 0.25 M urea at pH 5.5. The 26-10 binding-site species at 0.5 nM concentrations were incubated overnight at 4 °C with concentrations of [^{3}H]digoxin (NEN, Billerica, MA) varying from 10^{-10} to 10^{-7} M, in a total volume of 1.0 mL. Aliquots (0.9 mL) were placed in Centricon 10 microconcentrators having 10000 molecular weight cutoff membranes (Amicon Corp., Danvers, MA) and centrifuged at 1150*g* at 4 °C. Aliquots of the ultrafiltrate (0.3 mL) were collected and counted in 5.0 mL of scintillation fluid (Ultima Gold, Packard Instruments) on a Model 1500 Tri-Carb liquid scintillation analyzer (Packard Instruments, Downers Grove, IL). The $K_{a,app}$ values were calculated from the equilibrium binding data by using the LIGAND binding analysis program (Munson & Rodbard, 1980), as modified for calculation on microcomputers (McPherson, 1985). The values reported in

Table I: Digoxin Affinity Measurements of 26-10 Binding Sites

26-10 species	$K_{a,app} \times 10^{-9}$ (M^{-1}) pH 7.3	pH 5.5
sFv	2.4 ● 0.2	2.6 ● 0.3
FB-sFv	2.5 ± 0.3	2.6 ● 0.3
Fab	2.2 ± 0.3	2.4 ± 0.2
IgG	2.4 ± 0.5	2.5 ± 0.4

Table II: Specificity Analysis of 26-10 Digoxin Binding Sites[a]

cardiac glycoside	sFv	FB-sFv	Fab	IgG
digoxigenin	1.3	1.2	0.8	0.9
digitoxin	2.4	2.1	1.3	1.1
digitoxigenin	1.8	2.1	1.1	1.0
acetylstrophanthidin	2.8	1.7	2.6	1.1
gitoxin	12	13	11	8.9
ouabain	34	40	39	25

[a] Results are expressed as normalized concentration of inhibitor giving 50% inhibition of ^{125}I-digoxin binding.

Table I were obtained by analysis of data sets that combined results from three to seven experiments run in duplicate. The 26-10 IgG and Fab were prepared according to Mudgett-Hunter et al. (1982).

Antigen Binding Site Specificity Determination. The fine specificity of antidigoxin binding sites was assessed by comparative analysis of cardiac glycoside binding, using methods detailed previously (Hudson et al., 1987; Huston et al., 1988a). The interaction between each 26-10 digoxin binding species (bound to microtiter plates by affinity-purified GaMFab) and ^{125}I-digoxin (NEN, Billerica, MA) was inhibited by the addition of cardiac glycosides at 10^{-10}–10^{-4} M. For a particular 26-10 species, the relative affinity of each cardiac glycoside was calculated from its inhibition curve by dividing the concentration of each at 50% inhibition by the concentration of digoxin that gave 50% inhibition (Table II).

Determination of Fc Binding Affinity. The association between FB-sFv$^{26\text{-}10}$ and ^{125}I-hIgG (specific activity = 3.0 μCi/μg; NEN, Billerica, MA) was quantified in solution by using an immunoadsorbant to separate bound from free radiolabeled ligand. For this assay, ^{125}I-hIgG was diluted with unlabeled hIgG to give a final specific activity of 2.13 Ci/mmol. All reagents were diluted with 0.5% gelatin in PBSA at pH 7.3. The fusion protein (100 μL) at concentrations of (1–5) × 10^{-8} M was mixed with ^{125}I-IgG (100 μL) at concentrations ranging from 2.5 × 10^{-9} to 2 × 10^{-6} M and incubated overnight at 4 °C. Separation of bound and free ^{125}I-hIgG was effected by the addition of 100 μL of a 20% suspension of the digoxin–BSA–Sepharose immunoadsorbent (Mudgett-Hunter et al., 1982), with shaking during a 2-h incubation at room temperature. The ^{125}I-hIgG complexed with FB-sFv$^{26\text{-}10}$ was thus bound to the resin, which was separated from solution by filtration on no. 32 glass fiber filters (Schleicher and Schuell, Keene, NH) and rinsed with 6 mL of PBSA. Filters were counted on a 4/600 Micromedic automatic gamma counter (Micromedic Systems, Huntsville, AL), and apparent association constants, $K_{a,app}$, were calculated by using the program LIGAND, as described above. The $K_{a,app}$ for association between recombinant FB and rabbit Fc fragments (in 0.01 M sodium phosphate and 0.15 M NaCl at pH 7.4) was determined by a fluorescence method based on the observation that FB is devoid of tryptophan but is able to quench Fc tryptophan fluorescence upon binding (Lancet et al., 1978). Samples of recombinant FB were donated by Creative BioMolecules, and Fc was prepared from rabbit IgG by papain cleavage, followed by fractionation on Whatman CM 52, as originally described (Porter, 1959). The Fc fragment pool was repurified by affinity isolation on a column of protein A–Sepharose CL-4B equilibrated with 0.1 M borate, pH 8.5; Fc was eluted with 0.1 M glycine, pH 2.9, and renatured by dialysis against 0.01 M sodium acetate, pH 5.5. Homogeneity of the rabbit Fc was ensured by final fractionation on a Sephacryl S-200 size-exclusion column.

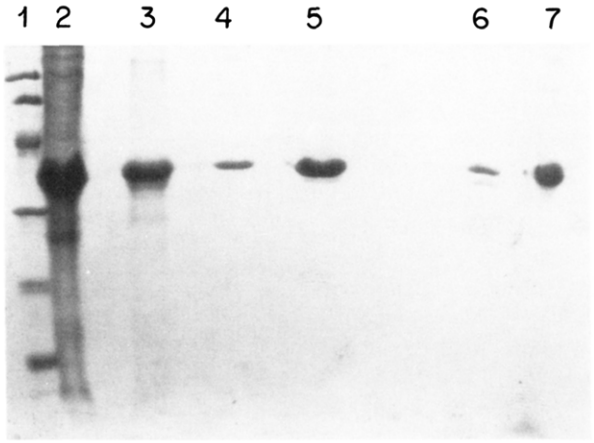

FIGURE 2: SDS–PAGE analysis of FB-sFv at progressive stages of purification. Samples in numbered lanes of the Coomassie blue stained 15% gel were the following: (1) standards (14.4, 20.1, 29, 43, 67, 94 kDa); (2) unpurified inclusion bodies; (3) DE 52 purified FB-sFv$^{26\text{-}10}$; (4) refolded FB-sFv$^{26\text{-}10}$ mixture (reduced); (5) affinity-purified FB-sFv$^{26\text{-}10}$ (reduced); (6) refolded FB-sFv$^{26\text{-}10}$ mixture (unreduced); (7) affinity-purified FB-sFv (unreduced). As discussed for 26-10 sFv (Huston et al., 1988a), the FB-sFv$^{26\text{-}10}$ migrated with a higher apparent molecular weight than its calculated molecular weight of 33 106; this is at least partially due to the low residue weight of glycine and serine in the linker sequence.

Fc Binding Site Specificity Determination. The Fc binding specificity of the FB domain was assessed with a method analogous to the digoxin specificity assay. FB-sFv$^{26\text{-}10}$ was anchored through its digoxin-combining site to digoxin–BSA adsorbed to the microtiter plate. Inhibition profiles were generated by measuring the concentration dependence of different inhibitory proteins on the association of FB-sFv$^{26\text{-}10}$ with ^{125}I-hIgG (Figure 3); the final results are the average of four data sets with standard deviations noted. Inhibitors for specificity measurements were unlabeled hIgG, rIgG, mIgG$_1$, and mIgG$_{2a}$; protein A and FB were competitive with FB-sFv$^{26\text{-}10}$ as inhibitors, and BSA was a control for nonspecific binding.

Ouchterlony Analysis. The Ouchterlony experiments were run on 1% agar plates that were prepared with PBSA buffer, which served as the diluent for fusion protein, nonspecific rIgG, and digoxin–BSA. The samples were allowed to diffuse for about 20 h at room temperature. To enhance precipitin lines, the plates were photographed with indirect light from below.

Results and Discussion

Protein Preparation. The directly expressed FB-sFv$^{26\text{-}10}$ formed protein inclusion bodies that represented the major intracellular protein. After cell lysis, FB-sFv$^{26\text{-}10}$ inclusion bodies were recovered by centrifugation, and protein purity was assessed by SDS–PAGE on a 15% polyacrylamide gel (Figure 2). Contaminating DNA and cellular proteins were eliminated by chromatography on a Whatman DE 52 column, and purified FB-sFv$^{26\text{-}10}$ was renatured in 3 M urea in the presence of a glutathione redox couple. Active FB-sFv$^{26\text{-}10}$ was purified from the pool of refolded protein by ouabain–Sepharose affinity chromatography with a recovery of up to 46% and yields of 110 mg/L of fermented cells (18.5-g cell paste). Automated Edman sequencing of active FB-sFv$^{26\text{-}10}$, before and after CNBr cleavage, confirmed that the fragments were

those expected from the fusion protein's sequence. The amino terminus of FB-sFv$^{26\text{-}10}$ began with alanine, indicating that the initial *N*-formylmethionine had been removed in vivo. Analysis by velocity sedimentation of the affinity-purified protein revealed some aggregated species that were eliminated by centrifugation at moderate speeds.[5] Therefore, samples were routinely spun in a preparative centrifuge for 1 h at 48000*g*, and the supernatant, accounting for at least 85% of the total protein, was removed for binding and specificity analysis.

Affinity for Digoxin. The digoxin-binding properties of FB-sFv$^{26\text{-}10}$ fusion protein were determined on dilutions of 1–3 mg/mL stock solutions stored in PBSA, conditions which irreversibly inactivate pure 26-10 sFv (Huston et al., 1988a). In the present study, we found that refolded 26-10 sFv could be assayed at pH 7.3 if the pH 5.5 stock solution (0.3 mg/mL in 0.01 M sodium acetate, 0.25 M urea, and 0.03% sodium azide) was diluted to nanomolar concentrations in 0.1% gelatin–PBSA or 1% horse serum–PBSA.

Affinity measurements relied on ultrafiltration to separate free digoxin from that bound to 26-10 species. The use of ultrafiltration permitted a rigorous determination of $K_{a,app}$, as it has been shown to be physicochemically equivalent to equilibrium dialysis for the measurement of association constants (Sophianopoulos et al., 1978), and its use eliminated the need for immunoprecipitation in radioimmunoassays. Affinity-purified FB-sFv$^{26\text{-}10}$ and 26-10 sFv exhibited $K_{a,app}$ values for digoxin binding that were essentially the same as those for 26-10 Fab and IgG (Table I). Thus, at pH 7.3 the apparent digoxin binding affinity of FB-sFv$^{26\text{-}10}$ was (2.5 ± 0.3) × 10^9 M^{-1}, compared with (2.4 ± 0.2) × 10^9 M^{-1} for 26-10 sFv, (2.2 ± 0.3) × 10^9 M^{-1} for 26-10 Fab, and (2.4 ± 0.5) × 10^9 M^{-1} for 26-10 IgG. On average, 79% of the FB-sFv$^{26\text{-}10}$ digoxin binding sites in solution were active at pH 7.3, and 76% were active at pH 5.5. These measures of activity were determined by comparing the molarity of active binding sites (calculated by means of the LIGAND program) to the total protein concentration (measured spectrophotometrically on the FB-sFv$^{26\text{-}10}$ stock solution).

Further experiments were conducted on the 26-10 sFv in order to relate our earlier data on its digoxin-combining site (Huston et al., 1989a) to the present analysis of FB-sFv$^{26\text{-}10}$. For this study, 26-10 sFv was refolded by a protocol that involved a redox buffer procedure at relatively high protein concentrations, in contrast to the dilution method used in our earlier work (Huston et al., 1988a). About 23% of the redox-refolded protein was recovered by affinity isolation versus 12.6% for the earlier method (Huston et al., 1988a). Simultaneous binding analysis of 26-10 sFv made by the two procedures demonstrated that the digoxin affinities of both proteins were comparable.[6] Therefore, affinity and specificity measurements (Tables I and II) were made on 26-10 sFv prepared by the redox refolding procedure, and its digoxin association constants were essentially identical at pH 7.3 [$K_{a,app}$ = (2.4 ± 0.2) × 10^9 M^{-1}] and pH 5.5 [$K_{a,app}$ = (2.6 ± 0.3) × 10^9 M^{-1}] and were indistinguishable from values measured for the fusion protein or parent 26-10 antibody. Previously, the digoxin affinity of 26-10 sFv and Fab had been determined by a radioimmunoassay that relied on double antibody precipitation at pH 5.5 (Huston et al., 1988a), where the primary precipitating antibody was GaMFab. We have found that this assay yields reduced values of $K_{a,app}$ under acidic conditions.[6] Furthermore, we observed in this investigation that all 26-10 species exhibited a reduced digoxin affinity at protein concentrations of 10 nM or more, regardless of the assay method used. Apparently, the 10–100 nM concentrations used in earlier 26-10 sFv measurements, together with the GaM-Fab-induced pH effect, gave lower than expected $K_{a,app}$ values at pH 5.5 for 26-10 sFv and Fab (Huston et al., 1988a).

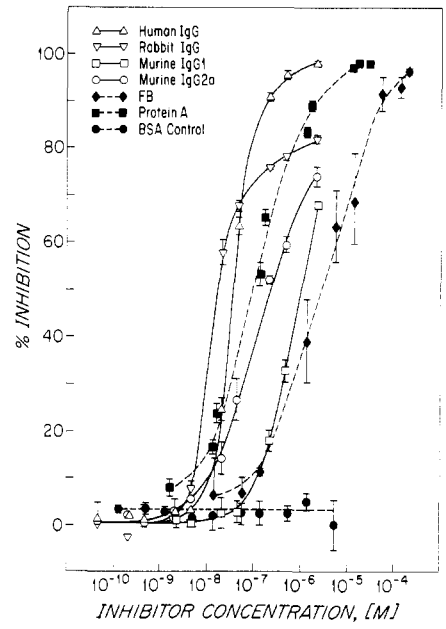

FIGURE 3: FB specificity profiles for FB-sFv$^{26\text{-}10}$. Association between ^{125}I-hIgG and FB-sFv$^{26\text{-}10}$ was inhibited by immunoglobulins, free FB, and protein A, with BSA as a nonspecific control. Standard deviations are noted as error bars.

Specificity for Digoxin Analogues. The binding of digoxin by FB-sFv$^{26\text{-}10}$ was highly specific, consistent with full recovery of the natural 26-10 binding site. The 26-10 sFv had been shown to retain common mouse V region determinants, which were recognized by GaMFab (Huston et al., 1988a). In the present study, FB-sFv$^{26\text{-}10}$ fusion protein was bound through these sFv epitopes to microtiter plates coated with GaMFab, and the association between FB-sFv$^{26\text{-}10}$ and ^{125}I-digoxin was inhibited by several related cardiac glycosides to obtain a specificity profile of the digoxin-combining site that was extremely close to those of 26-10 Fab and IgG (Table II). The 26-10 sFv, prepared by redox refolding, exhibits a specificity profile that is equivalent to that of the fusion protein and closer to that of the parent antibody than the profile observed for 26-10 sFv refolded by dilution (Huston et al., 1988a). GaM-Fab antibody did not induce detectable changes in the specificity profile of FB-sFv$^{26\text{-}10}$, since the values obtained with GaMFab (Table II) were within the experimental error of values obtained by substituting rabbit anti-FB antibody for GaMFab in the assay.[6]

Affinity and Specificity for Fc. Radioimmunoassay experiments indicated that the FB domain of FB-sFv$^{26\text{-}10}$ bound ^{125}I-hIgG with a $K_{a,app}$ of (3.2 ± 0.5) × 10^7 M^{-1}. This value was very close to a $K_{a,app}$ of 1.2 × 10^7 M^{-1} determined for the association of recombinant 58-residue FB with rabbit Fc fragments. By use of fluorescence quenching, association constants have also been determined for the interaction between rabbit Fc fragments and FB isolated by tryptic cleavage of protein A; Lancet et al. (1978) measured a $K_{a,app}$ of (3 ± 0.5) × 10^6 M^{-1}, whereas Wright et al. (1978) reported a range of values from (0.9–1.4) × 10^6 M^{-1}. The determination of $K_{a,app}$ seems to give higher values for our recombinant proteins than for naturally isolated FB protein, but the differences could also derive from inherent limitations in assay methods.

[5] Unpublished results of W. F. Stafford, III, and J. S. Huston.
[6] Data not shown.

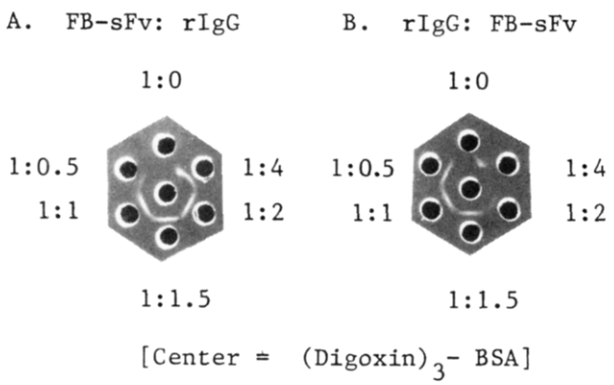

FIGURE 4: Double immunodiffusion analysis. Mixtures of FB-sFv$^{26\text{-}10}$ and nonspecific rabbit IgG in the outer wells diffused toward digoxin-labeled BSA placed in the center well. The concentration of FB-sFv$^{26\text{-}10}$ was 1.5 mg/mL and of rIgG was 3.3 mg/mL in the stock solutions, from which the indicated ratios were mixed and added to the outer wells. The center wells contained the digoxin–BSA conjugate at 0.35 mg/mL, which was a mixture of conjugates with a maximum of three glycosides per protein.

In specificity profiles presented in Figure 3, a measure of each relative dissociation constant can be derived from the protein concentration at 50% inhibition of ^{125}I-hIgG binding; comparing any two curves, the higher relative affinity corresponds to the lower midpoint concentration. Like protein A, the FB moiety of the fusion protein was able to distinguish between mouse isotypes, binding IgG_{2a} more strongly than IgG_1; these isotypes were similarly distinguished in specificity experiments[6] that used ^{125}I-rIgG instead of ^{125}I-hIgG. This feature of fusion protein specificity was significant, as the difference between the IgG_{2a} and IgG_1 curves at their 50% inhibition points was large even at the limits of the standard deviations shown for data points (Figure 3). Specificity profiles were also consistent with the fusion protein binding rIgG with a slightly greater affinity than hIgG (Figure 3), as noted for intact protein A (Langone, 1982). This difference was not large but appeared greater than experimental error.

Only the immunoglobulin profiles (solid lines, Figure 3) represent specificity curves for the FB moiety in the same sense as they were determined by the sFv region of FB-sFv$^{26\text{-}10}$ (Table II), in that the different IgG species bound directly to the FB site of the fusion protein to inhibit its binding to ^{125}I-hIgG. In separate experiments,[6] the 26-10 sFv at about 10^{-4} M was shown to exhibit no specific binding to human IgG that was immobilized as hIgG–Sepharose; this served as a control that confirmed IgG binding was mediated by the FB moiety of the fusion protein. BSA was a control for nonspecific binding and showed a negligible inhibitory effect over the full range of the IgG specificity profiles. In contrast to the immunoglobulins, recombinant FB and protein A bound directly to the ^{125}I-hIgG in competition with fusion protein, and both effected nearly complete inhibition of ^{125}I-hIgG binding to FB-sFv$^{26\text{-}10}$ on the plate. Thus, fragment B and protein A inhibition curves served as further controls which confirmed that FB-sFv$^{26\text{-}10}$ bound to ^{125}I-hIgG at the same sites as did the parent FB and protein A species. From the standpoint of competition assays, these data show the FB to have a much lower relative affinity for radiolabeled hIgG than protein A. This difference is expected from literature values for the IgG-binding affinity for protein A (Langone, 1982), reported to be 10–100 times higher than that for FB. Protein A has been found to form multimeric complexes with IgG (Hanson et al., 1984; Hanson & Schumaker, 1984), which can augment its apparent affinity for IgG over a single Fc binding site such as FB.

Dual Activity of Fusion Protein. Bifunctionality of FB-sFv$^{26\text{-}10}$ was demonstrated by its ability to simultaneously bind digoxin–BSA through its 26-10 sFv and ^{125}I-hIgG through its FB moiety in the microtiter plate assay. Ouchterlony analysis was also used to independently demonstrate dual activity in the fusion protein. The formation of precipitin lines in the double immunodiffusion experiments (Figure 4) presumably resulted from the formation of bivalent antidigoxin complexes [(FB-sFv)IgG(FB-sFv)] interacting with multivalent antigen [$(digoxin)_3$–BSA]. Typically, 98‰ of the ouabain–Sepharose-purified FB-sFv$^{26\text{-}10}$ subsequently bound to a hIgG–Sepharose column, thus providing another measure of the active bifunctional protein. The percentage of FB-sFv$^{26\text{-}10}$ that exhibited dual activity can be estimated to have been about 77%, calculated by multiplying the fraction of active FB sites in the fusion protein by the fraction of active sFv sites.

Conclusions. Our experiments demonstrate that an sFv fused at its amino terminus to an effector domain can refold into a native combining site. The FB effector domain can also recover intrinsic activity despite its attachment to the sFv. Given the proximity of the sFv amino terminus to its antigen binding site (Huston et al., 1988a), the fused FB might be expected to sterically hinder part of the combining site or to otherwise have an impact on binding (Panka et al., 1988). However, steric hindrance was not detectable, as FB-sFv$^{26\text{-}10}$ bound digoxin with the same affinity and specificity as the parent antibody. Access to the combining site may have been aided by flexibility of the carboxyl-terminal 11-residue segment of the FB that joins the FB helices to -Ser-Asp- at its junction with the sFv region.

In view of our results for FB fused to the amino terminus of 26-10 sFv and other data for a toxin fused to the carboxyl terminus of sFv (Chaudhary et al., 1989, 1990), bifunctionality appears attainable in sFv fusion proteins that have ancillary domains attached to either terminus of the sFv polypeptide chain. Thus, it should be possible to create multivalent proteins by connecting several sFv regions in succession on the same polypeptide chain. Multiples of the same or different binding sites might be linked in a single fusion protein, with effector proteins positioned on either side of sFv regions. The critical question of binding site fidelity appears to have been answered, in principle, by full recovery of the 26-10 combining site in the single-chain Fv and FB-sFv$^{26\text{-}10}$ fusion protein.

ACKNOWLEDGMENTS

We gratefully acknowledge the editorial advice of Thomas J. McVarish and the expert assistance of the following members of our laboratories: Marianne Wright, Julie Wong, Abbie White, Denny Maratea, Roberta Batorsky, Clare Corbett, Robert Juffras, and Christine Jost. We particularly thank Charles Cohen for his enthusiastic support and Professor Serge N. Timasheff for his scientific insights and encouragement.

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Contribution of Vitamin K_1 to the Electron Spin Polarization in Spinach Photosystem I†

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Received June 7, 1990; Revised Manuscript Received July 12, 1990

ABSTRACT: The electron spin polarized (ESP) electron paramagnetic resonance (EPR) signal observed in spinach photosystem I (PSI) particles was examined in preparations depleted of vitamin K_1 by solvent extraction and following biological reconstitution by the quinone. The ESP EPR signal was not detected in the solvent-extracted PSI sample but was restored upon reconstitution with either protonated or deuterated vitamin K_1 under conditions that also restored electron transfer to the terminal PSI acceptors. Reconstitution using deuterated vitamin K_1 resulted in a line narrowing of the ESP EPR signal, supporting the conclusion that the ESP EPR signals in the reconstituted samples arise from a radical pair consisting of the oxidized PSI primary donor, P_{700}^+, and reduced vitamin K_1.

A characteristic electron spin polarized (ESP)[1] electron paramagnetic resonance (EPR) signal was first observed in plant photosystem I (PSI) a number years ago (Thurnauer et al., 1979; McIntosh et al., 1979; McCracken et al., 1982; Gast et al., 1983). Yet, the identity of this signal is uncertain. Evidence suggests that the signal is due to $P_{700}^+A_1^-$, where P_{700}^+ is the oxidized primary chlorophyll donor of PSI and A_1^- is believed to be a PSI acceptor in the electron transport chain $P_{700}A_0A_1F_xF_aF_b$. Acceptor A_0 is probably a chlorophyll species, A_1 is believed to be a quinone-like molecule, and F_x, F_a, and F_b are iron–sulfur centers (Golbeck, 1987; Mathis &

† The work at Argonne National Laboratory was supported by the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Chemical Science, under Contract W-31-109-Eng-38. The work at Brown University was funded by the Competitive Research Grants Office of the USDA (88-37130-4135) and the National Science Foundation (DMB-86-03586).

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[1] Abbreviations: ESP, electron spin polarization; EPR, electron paramagnetic resonance; PSI, photosystem I; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; HPLC, high-performance liquid chromatography.

0006-2960/90/0429-8030$02.50/0 © 1990 American Chemical Society