Comparative Properties of the Single Chain Antibody and Fv Derivatives of mAb 4-4-20

RELATIONSHIP BETWEEN INTERDOMAIN INTERACTIONS AND THE HIGH AFFINITY FOR FLUORESCEIN LIGAND*

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Recombinant Fv derivative of the high affinity murine anti-fluorescein monoclonal antibody 4-4-20 was constructed and expressed in high yields, relative to the single chain antibody (SCA) derivative (2-3-fold), in Escherichia coli. Both variable heavy (VH) and variable light (VL) domains, that accumulated as insoluble inclusion bodies, were isolated, denatured, mixed, refolded, and affinity-purified to yield active Fv 4-4-20. Affinity-purified Fv 4-4-20 showed identical ligand binding properties compared with the SCA construct, both were slightly lower than the affinities expressed by Fab or IgG 4-4-20. Proper protein folding was shown to be domain-independent by in vitro mixing of individually refolded variable domains to yield functional Fv protein. In solid phase and solution phase assays, Fv 4-4-20 closely approximated the SCA derivative in terms of both idiotype and metatype, confirming identical active site structures and conformations. The equilibrium dissociation constant (Kd) for the VL/VH association (1.43 × 10−7 M), which was determined using the change in fluorescein spectral properties upon ligand binding, was relatively low considering the high affinity displayed by the Fv protein for fluorescein (Kd, 2.9 × 10−10 M). Thus, domain-domain stability in the Fv and SCA 4-4-20 proteins cannot be the sole cause of reduced affinity (2-3-fold) for fluorescein as compared with the Fab or IgG form of 4-4-20. With their identical ligand binding and structural properties, the decreased SCA or Fv affinity for fluorescein must be an ultimate consequence of deletion of the CH1 and C1 constant domains. Collectively, these results verify the importance of constant domain interactions in antibody variable domain structure-function analyses and future antibody engineering endeavors.

Antibody Fv fragments are composed of heavy chain (VH)1 and light chain (VL) variable domains. These two domains associate noncovalently to form the smallest functional antibody protein capable of antigen binding that most closely approximates the Ig molecule (1, 2). These proteins have been previously found to be less stable in terms of domain-domain association than Fab fragments due to the lack of covalent bonds between the two variable domains (3, 4). Single chain antibody (SCA) molecules have been produced to diminish this instability by the introduction of an interdomain linker peptide (5, 6). SCA proteins often mimic the parent antibody active site in terms of antigen binding and structural properties with usually some reduction in affinity for antigen (7-9). Recently, Fv proteins have been engineered to possess an interdomain disulfide linkage, effectively disallowing dissociation of the two domains (10). Due to their small size and amenability to genetic engineering, recombinant Fv proteins have been widely applied in the study of antibody active site structure-function (7, 11-13), idiotypy and metatypy (14-17), antibody bivalency and bispecificity (18-21), and in vivo immunodiagnostics and therapy (10, 22, 23).

Fv molecules have been efficacious proteins in the study of antibody active site structure-function and protein stability. Studies involving comparative analysis of Fv protein with other immunoglobulin constructs afford unique opportunities for determining domain-domain interactions and the effects these interactions exert upon the intrinsic conformational and antigen binding properties of the variable domains. Being covalently coupled by an interdomain linker, SCA proteins have been suggested to possess greater interdomain stability than their Fv counterparts due to the favorable entropic effect of domain coupling (6, 8). This would, in turn, suggest that in the appropriate Fv molecule (one with high affinity for antigen), interdomain associative properties would dictate the overall affinity displayed for antigen because only associated VL/VH proteins would bind antigen. In previous studies, dissociation constants for the VL/VH association in Fv molecules varied from 10−7 to >10−9 M (3, 24-26). These Fv molecules also displayed similar dissociation constants for their respective antigens (10−7 to >10−9 M), further supporting some correlation between interdomain and active site/antigen interactions. Further analysis of VL/VH association constants in relation to antigen affinity would allow identification of components necessary for the production of stable Fv molecules and novel variable domain proteins.

Fv molecules have been especially useful in the study of idiotypy and metatypy. Antibody idiotype and metatypy are immunologically resolved markers of active site structural and conformational determinants in the unliganded and liganded state, respectively (review in Refs. 27 and 28). Indeed, the transition between the idiotypic and metatypic states upon ligand binding emphasizes the dynamic properties of antibody
Comparative Analysis of SCA and Fv 4-4-20

Materials and Methods

Monoclonal Antibody 4-4-20—mAb 4-4-20 was generated by polyethylene glycol-mediated fusion of BALB/cJ immunoglobulin-secreting myeloma cells with nonsecreting Sp2/O-Ag14 myeloma cells as described previously (39). mAb 4-4-20 has been extensively characterized with an affinity for fluorescein of $1.7 \times 10^7 M^{-1}$ (34, 39–41). Fab fragments were prepared by papain ( Worthington) digestion of immunoglobulins as described by Oi and Herzenberg (42) and Weidner and Voss (14). Monoclonal anti-fluorescein activity, a solid phase ELISA assay similar to that in Strains, Plasmids, and Media—Escherichia coli strain GX7612 (F galK2 plsl $d$315) and plasmid pgX8773 were provided originally by the Genex Corp. (Inow Enzon, Inc.). Expression vector pgX8773 encodes the OmpA signal sequence and contains the variable domain 212 linker (GSTSSGSSKSEGKKG) (6, 45). The expression vector utilizes a hybrid $O_t$ promoter with protein expression initiated by temperature shift from 30°C to 42°C in E. coli strain GX7612 (46).

Fv 4-4-20 Construction—Polymerase chain reaction (PCR) methodology was used for construction of the $V_h$ and $V_l$ expression molecules from wild type SCA 4-4-20. Oligonucleotide primers were synthesized by the Genetic Engineering Facility at the University of Illinois (Urbana-Champaign) by the phosphoramidite method (47). Reaction conditions for amplification of DNA fragments with Vent were 10 mm Tris-HCl, pH 8.3, 2.5 mm MgCl$_2$, 50 mm KCl, 0.01% BSA (w/v), 0.1% Triton X-100, 4 mm each dNTP, 5.0 units of polymerase, 10 ng of template DNA, and 30 pmol of primer DNA. Reaction conditions for amplification with Taq were 20 mm Tris-HCl, pH 8.4, 2 mm MgCl$_2$, 50 mm KCl, 4 mm each of dNTP, 2.5 units of polymerase, 10 ng of template DNA, and 30 pmol of primer DNA. The reaction mixture was incubated in a thermal cycler (MJ Research) using the following program: 92°C for 5 min, 53–67°C (depending on primer sequence) for 5 min, 72°C for 1 min, followed by 30 cycles of 72°C for 1 min, 92°C for 1 min, and 53–67°C for 1 min. The $V_h$ and $V_l$ genes were amplified separately to contain the 5' OmpA signal sequence and the 3' terminator sequences necessary for expression from pgX8773.

Following amplification, $V_h$ and $V_l$ PCR products were purified by low melting temperature agarose (Seaplaque, FMC) and cloned into Smal digested pTZ18u (48). Correct clones were identified by restriction analysis and verified by dideoxy sequencing. To construct the $V_h$ gene, PCR was used to add the transcription stop codons by 3' primer overlap (Fig. 1). To incorporate the signal sequence in the $V_h$ gene, PCR was used to amplify the OmpA sequence with the addition of the 5' 5' sequence to the 3' end of OmpA. The resulting PCR product was then used as the 5' primer to incorporate the signal sequence to the $V_h$ gene (Fig. 1). Both amplified genes were cloned into pTZ18u to form pJ Wc2-2 and pJ Wc1-5, respectively. Following verification of proper primary sequence, the $V_h$ and $V_l$ genes were excised using ClaI-BamHI and cloned into pgX8773 for expression.

Sequence Determination—Following cloning, sequences of the PCR products were determined by the dye chain termination procedure using a double-stranded plasmid DNA template (49) and Sequenase® (U. S. Biochemical Corp.).

Large Scale Expression of Fv 4-4-20—Fv 4-4-20 was expressed in E. coli, denatured, and refolded using a modified version of the protocol used by Denzin et al. (45) and Rumbley et al. (14). The procedure was modified in two ways: 1) in scale, to accommodate 1 liter of bacterial culture instead of 12 liters, and 2) in molecular weight cutoff size, all chromatography and dialysis steps were performed using molecular weight cutoff of 3 kDa. Denatured $V_h$ and $V_l$ inclusions bodies were combined in a 1:1 mass ratio in the refolding solution to produce associated active Fv. Concentration of the diluted protein was accomplished using an Amicon Ultrafiltration Cell. Monomer $V_h$ and $V_l$ were produced by denaturation and renaturation in the absence of the other protein.

Purification of Fv 4-4-20—Refolded Fv 4-4-20 was dialyzed extensively against Tris-buffered saline (50 mm Tris-HCl, pH 8.0, 150 mm NaCl) followed by dialysis against phosphate-buffered saline (PBS; 50 mm PO$_4$, pH 8.0, 150 mm NaCl). Precipitates were removed by centrifugation. Active Fv 4-4-20 was purified by fluorescein-Sepharose affinity chromatography. After extensive washing, bound protein was eluted using 8.0 M urea/PBS followed by extensive dialysis against PBS. Individually refolded variable domain proteins were concentrated without purification. Purity of all proteins was evaluated by SDS-polyacrylamide gel electrophoresis using a 15% gel in the discontinuous SDS buffer system of Laemmli (50). Protein bands were visualized with Fast Stain (Zoion Research, Inc.). Anti-fluorescein Solid Phase ELISA Binding Assay—To demonstrate anti-fluorescein activity, a solid phase ELISA assay similar to that used in Denzin et al. (45) was performed. After addition of Fv 4-4-20 to fluorescein-BSA-coated wells and extensive washing, 50 $\mu$g of 10 $\mu$g/ml hamster anti-4-4-20 variable light domain-specific peptide antibody (3AS-1) (63) was added. Bound antibody was detected by horseradish...
peroxidase-labeled anti-hamster antibodies and 3,3',5,5'-tetramethyl-
benzidine (Pierce). Substrate was added and incubated at room tem-
perature for 30 min. Enzyme reactions were terminated with 2 N H2SO4
and optical densities determined using a Dynatech MR500 automatic
plate reader.

Anti-metatype Inhibition Solid Phase ELISA Binding Assay—For
comparison of the Fv metatypic state with SCA, a solid phase inhibition
assay was used (7). Inhibitors were preincubated with rabbit anti-
metatype γ-globulin (10 ng/ml) (15–16 h, 4°C) and added to polystyrene
wells preadsorbed with affinity-labeled 4-4-20 Fab fragments (see be-
low) (2 μg/ml).

Anti-idiotype Inhibition Solid Phase ELISA Binding Assay—For
comparative idiotype analysis between Fv and SCA 4-4-20, a solid
phase inhibition assay was performed (7). Inhibitors were preincubated
with horseradish peroxidase-labeled IgG 4-4-20 (10 ng/ml) and added to
polystyrene wells preadsorbed with polyclonal anti-idiotype antibodies
(1 μg/ml).

Dissociation Rate Kinetic Assay—Ligand dissociation rates of Fv
4-4-20 (90% liganded with fluorescein) were determined at 4°C as
described (51). Affinity constants were determined from the dissociation
rate using the previously determined association rate of 5 × 10^6 M^-1 s^-1
for anti-fluorescy1 antibodes to calculate intrinsic affinities (Ks = k1/k2)
(52, 53). Ligand dissociation rates were also performed as above, but liganded Fv 4-4-20 was preincubated with an active site molar excess of anti-metatype antibody active sites for 15–20 min at 4°C.

Fluorescein Fluorescence Quenching Assay—Fluorescein quenching
measurements of antibody-bound ligand were performed as described by
Watt and Voss (55). Fluorescein fluorescence quenching by affinity-
purified Fv and by equal optical density amount of preincubated Vκ and
Vλ protein was compared with SCA 4-4-20. Fluorescein protein samples were allowed to equilibrate for 4 min after each pressure change before spectra measure-
ments were taken.

VL/VH Interdomain Affinity Measurements—All fluorescence data
were collected on an ISS GREG PC photon counting spectrophotometer
(ISS, Champaign, IL) and all experiments were performed at room temperature in PBS. Anisotropy based domain-domain binding measurements were made using affinity-labeled Fv 4-4-20. Fluorescein was covalently coupled in the Fv 4-4-20 active site using the isothiocyanate derivative of fluorescein (FITC) (Sigma). A 1.1 molar excess of FITC was incubated with Fv 4-4-20 for 4–5 h with agitation at 37°C. Protein pressure samples were allowed to equilibrate against PBS to remove freeFITC. The concentration of fluorescein was determined on a Beckman DU-64 spectrophotometer using the absorbance at 492 nm and the extinction coefficient (ε_{492}) of 72,000 cm^-1 M^-1. The amount of affinity-labeled protein (R) was calcu-
lated as the ratio of fluorescein concentration and Fv protein concen-

![FIG. 1. Polynucleotide primers and amplification strategy used for con-
struction of the Vκ and Vλ gene products. Regions of complementarity with
4-4-20 are underlined. OmpA and terminator complementary sequences are in
italics, and vector complementary sequences are roman.](image)
trophoresis analysis on individually refolded domain proteins showed to be not shown). Additionally, the affinity-purified material was obtained using conventional cloning techniques (see “Materials and Methods”). The OmpA signal sequence was then used to amplify the entire VH gene, resulting in addition of the signal sequence. Protein yields from 1 liter of E. coli cultures were from 3 to 4 mg of affinity-purified Fv. This method of mutagenesis (59, 60). Briefly, the OmpA sequence was amplified from SCA 4-4-20 incorporating a portion of the signal sequence. Protein yields from 1 liter of E. coli cultures were from 3 to 4 mg of affinity-purified Fv. This represented a 2-3-fold increase of active anti-fluorescein protein as compared with expression yields of SCA 4-4-20. Protein concentrations were calculated from absorption spectra at 240-350 nm (61) using a Beckman DU-64 spectrophotometer. Extinction coefficients (ε_{max}) of 2.2, 2.1, 1.5 and 2.7 for SCA, Fv, VL, and VH proteins, respectively, were calculated from chromophore content (62).

Polycrylamide Gel Analysis—Fv 4-4-20 was purified by affinity chromatography using fluorescein-Sepharose as described (see “Materials and Methods”). SDS-polycrylamide gel electrophoresis analysis showed the purified Fv protein consisted of two detectable bands (14.0 and 12.5 kDa) corresponding to VL and VH proteins. Migration patterns indicated actual molecular weights for the two domains were in good agreement with their calculated values based on amino acid content (data not shown). Additionally, the affinity-purified material was shown to be >90% pure. Similar SDS-polycrylamide gel electrophoresis analysis on individually refolded domain proteins showed the VL and VH proteins were the major detectable bands found in their respective samples (data not shown).

Anti-fluorescein Activity of Fv 4-4-20—Fluorescein binding by purified Fv was examined using a solid phase direct binding assay, which compared SCA and mAb 4-4-20 to Fv. Proteins bound to fluorescein-BSA-coated wells were detected using hamster nonligand inhibitible anti-4-4-20 antibodies (16, 54, 63). Results showed that Fv 4-4-20 possessed similar levels of anti-fluorescein activity as compared with SCA 4-4-20 (Fig. 2A).

Anti-metatype Reactivity—To compare the degree of structural relatedness between the liganded states of SCA, Fab, and Fv 4-4-20, these proteins were used as polyclonal anti-metatype/liganded Fab 4-4-20-soluble inhibitors. All 4-4-20 proteins were affinity-labeled as described previously (Ref. 7; see “Materials and Methods”). Fig. 2B compares the inhibition titrations of affinity-labeled Fv with similarly labeled Fab and SCA 4-4-20. Unliganded Fab was also tested to determine the amount of anti-idiotypic and anti-constant domain activity present in the anti-metatype reagent. The anti-metatype reagent was not passed over an unliganded IgG 4-4-20 adsorbent to remove such activity prior to this experiment. Results indicated that Fv 4-4-20 possessed a similar anti-metatype inhibition profile as SCA 4-4-20, implying an overall structural similarity between their liganded states. Comparison of the unliganded and liganded Fab curves suggested the presence of anti-constant domain activity in the anti-metatype reagent, but confirmed specificity for the liganded state of the 4-4-20 active site.

Anti-idiotypic Reactivity—In terms of a polyclonal anti-idiotypic reagent, comparative inhibition studies revealed identical patterns of SCA and Fv anti-idiotypic recognition. Results suggested that SCA and Fv 4-4-20 were idiotypically identical (Fig. 2C). Previous idiotypic analysis of SCA with mAb 4-4-20 indicated that the two were idiotypically identical (7).

Spectral Properties of Fluorescein Bound to Fv 4-4-20—Anti-fluorescein antibodies have been characterized by their ability to quench (Q_{max}) the fluorescence of fluorescein (64). Q_{max} for Fv (87.5 ± 0.9%) compared well with SCA (85.9 ± 0.5%) (Table I). Identical Q_{max} values were found when affinity-purified Fv was compared with an equal optical density (278 nm) mixture of refolded VL and VH protein (Fig. 3). This suggested that each individually refolded domain protein had formed a dimerization competent structure in the absence of the other domain protein. Identical Q_{max} values confirmed the similar active site environments displayed by SCA and Fv 4-4-20.

Anti-fluorescein antibodies also produce a characteristic bathochromic shift of 10–20 nm in the ligands absorption maximum (λ_{max}) upon fluorescein binding (40, 64). The bathochromic shift in bound fluorescein absorption was identical for SCA and Fv 4-4-20 (504 nm) (Table I).

Affinity Measurements—Affinity-purified Fv 4-4-20 (liganded with fluorescein) was examined by dissociation rate fluorescence analysis. Fv 4-4-20 showed an affinity for fluorescein (3.5 × 10^6 M^{-1}) that was nearly identical to SCA (4.9 × 10^6 M^{-1}) within error limitation of the experiment (Table I). Similar affinity determinations were performed in the presence of excess polyclonal and monoclonal anti-metatype antibodies (reviewed in Refs. 28 and 54). These antibodies characteristically delay the fluorescein dissociation rate from the antibody active site against which they were raised. Both polyclonal and monoclonal anti-metatype reagents caused similar changes in the determined affinity values for fluorescein for SCA and Fv 4-4-20 (Table I). Cumulatively, binding data indicated that the Fv molecule effectively mimics the SCA, proving that the 212 linker peptide was not responsible for the original affinity decrease found in SCA as compared with mAb 4-4-20.

CD Spectra of Fv 4-4-20—Fig. 4 shows the CD spectra recovered for Fv and mAb 4-4-20. Results are expressed in terms of mean residue weight ellipticity ([θ]_222 nm (deg cm^2 dmol^{-1})). Analyses of CD spectra were carried out using previously computed CD spectra for poly-L-lysine containing varying amounts of α-helix, β-sheet, and random coil segments (65) as well as the previously determined CD spectra for SCA and mAb 4-4-20 (36). These analyses enabled estimation of the secondary structural characteristics of the SCA and Fab 4-4-20 anti-idiotype as well as the previously determined CD spectra for SCA (Fig. 4). The SCA protein also displayed the slight shift in extremum, as well as the pronounced negative value at 230 nm, that SCA did in comparison with mAb 4-4-20. Similar CD spectra were recorded for samples of refolded VL and VH proteins (data not shown).
Pressure-induced Dissociation of Fluorescein from Fv 4-4-20—Further structural comparison of Fv 4-4-20 with SCA was accomplished by measuring their hydrostatic-induced fluorescein dissociation parameters. Hydrostatic pressure has been shown to cause conformational changes (independent of protein tertiary structure) in proteins which can promote ligand dissociation (38, 66). Fig. 5 shows the effect of hydrostatic pressure on liganded SCA and Fv 4-4-20 while monitoring fluorescein fluorescence intensity. Increased fluorescein fluorescence intensity was correlated with structural changes in the active site resulting in alleviated quenching and the ultimate dissociation of fluorescein ligand. Fv and SCA displayed similar fluorescein dissociation profiles as pressure was increased from atmospheric to 2.4 kbar. Results further confirmed the overall similarity in structure between the Fv and SCA 4-4-20 molecule.

VL/VH Interdomain Affinity Analysis—The variable domain dissociation constant ($K_d$) was determined by diluting affinity-labeled Fv 4-4-20 and monitoring for increased fluorescein rotation by steady state anisotropy measurements. Being covalently coupled to the Fv binding pocket, changes in fluorescein anisotropy after dilution were due to domain-domain dissociation and not ligand dissociation. Fluorescence anisotropy-based binding curves were obtained by diluting affinity-labeled Fv protein and plotting degree dissociation values versus liganded protein concentration (see "Materials and Methods") (Fig. 6). Fluorescence anisotropy values of fluorescein.
ceindecreasedwithaffinityligandedFvconcentration,indicat-
ingdissociationofthetwodomains.Finalanisotropyvalues
\( r = 0.02 \) were higher than values for free fluorescein
\( r = 0.008 \) confirming the linkage of fluorescein to the
surface of an individual variable domain, not the static
interior of the binding pocket \( r = 0.320 \). In contrast to the
high affinity displayed \( (K_d, 2.9 \times 10^{-10} \text{ M}) \) by Fv 4-4-20
for fluorescein, the interdomain \( K_d \) value recovered from
Fig. 6 was relatively low \( (1.43 \times 10^{-7} \text{ M}) \). Similar
experiments performed with affinity-labeled SCA 4-4-20
showed no changes in fluorescein anisotropy over this
concentration range (data not shown).

\[ Q_{\text{max}}^a \]
\[ \lambda_{\text{max}}^b \]
\[ K_d \text{ for fluorescein}^c \]
\[ K_d \text{ (with polyclonal anti-metatype reagent)}^d \]
\[ K_d \text{ (with mAb 3A5–1 anti-metatype antibody)}^e \]

\[ \text{SCA 4–4–20} \]
\[ 85.9 \pm 0.5\% \]
\[ 504 \text{ nm} \]
\[ 4.9 \pm 0.4 \times 10^{10} \text{ M}^{-1} \]
\[ 2.0 \pm 1.0 \times 10^{9} \text{ M}^{-1} \]
\[ 9.1 \pm 1.0 \times 10^{8} \text{ M}^{-1} \]

\[ \text{Fv 4–4–20} \]
\[ 87.5 \pm 0.9\% \]
\[ 504 \text{ nm} \]
\[ 3.5 \pm 0.7 \times 10^{8} \text{ M}^{-1} \]
\[ 3.0 \pm 1.1 \times 10^{9} \text{ M}^{-1} \]
\[ 6.3 \pm 0.8 \times 10^{8} \text{ M}^{-1} \]

\( a \) Quenching of fluorescein fluorescence determined as
described in Mallender and Voss (1994). Briefly, antibody
protein was added sequentially to a fluorescein solution
and total fluorescence quenching monitored.

\( b \) Absorption maximum of fluorescein fluorescence
determined as described in Mallender and Voss (1994).
Briefly, the absorption maximum wavelength for fluorescein
was determined for a solution of 100% liganded fluorescein.

\( c \) Protein affinity for fluorescein determined as
described in Mallender and Voss (1994). Briefly, the
dissociation of fluorescein from the active site
was monitored after the addition of a large excess (>100-fold)
of fluorescein amine. Affinity constant was determined from
the dissociation rate using the previously determined association rate of
5 \times 10^{6} \text{ M}^{-1} s^{-1} for anti-fluorescyl antibodies.

\( d \) Affinity determination measurements were performed as above
(Footnote c) with the addition of a large (>100-fold molar active site)
excess of polyclonal anti-metatype antibodies.

\( e \) Affinity determination measurements were performed as above
(Footnote c) with the addition of a large (>10-fold molar active site)
excess of monoclonal anti-metatype antibody 3A5–1.

**DISCUSSION**

In terms of structure-function relationships, recombinant Fv proteins have been invaluable tools for experimental studies of immunoglobulins. More recent endeavors involving these recombinant proteins have included their engineering with specialized effector functions for in vitro and in vivo immunodiagnostic and therapeutic roles. A common characteristic upon production of these diminutive antibody proteins is that their affinity for antigen is often reduced (or abrogated) as compared with the parental IgG. The reduced affinity has been attributed to changes in the active site structure or variable domain associative properties upon removal of the constant domains (3). If the initial decrease in Fv affinity for antigen was due to decreased domain-domain interactions, the properties governing stable variable domain association in relation to antigen binding must be identified. As such antibody proteins continue to be modified and applied to different systems (reviewed in Refs. 67 and 68), the nature of this affinity decrease, including how \( V_L/V_H \) affinity correlates with antigen binding affinity,
must be defined and exploited. The well characterized 4-4-20/fluorescein system presented an ideal method to study this phenomenon, based on the fact that SCA 4-4-20 exhibits a slight decrease in affinity for antigen compared with IgG (7). This study addressed the question by production and characterization of the Fv analogue of the 4-4-20 active site. These studies were based on the premise that comparative analysis provided clarification of the correlation between antibody constant domains, variable domain stability, and affinity for antigen.

Using similar expression conditions for SCA, purified Fv 4-4-20 demonstrated nearly identical anti-fluorescein activity as SCA (Fig. 2A). Polyacrylamide gel analyses confirmed that the purified Fv protein contained only VL and VH domain proteins (data not shown). In terms of expression yield, E. coli cultures producing VL and VH protein consistently yielded 2–3-fold more active Fv protein than similar cultures producing SCA upon refolding and affinity purification. The fact that improper disulfide bonds could not form between variable domain proteins during expression and refolding was most likely responsible for this result (4). In terms of idiotypy and metatypy, Fv 4-4-20 showed properties identical to SCA 4-4-20 when examined with polyclonal 4-4-20 variable domain-specific antibodies (Fig. 2, B and C). These results suggested that despite the dependence on noncovalent interactions for association, Fv 4-4-20 closely approximated the SCA molecule in terms of unliganded and liganded state structure.

Ligand binding affinities and ligand-related spectral measurements were made to assess Fv homology to the SCA molecule (in terms of the initial decrease in affinity for antigen). Such spectral measurements involving fluorescein/anti-fluorescein antibodies are characteristic of the specific anti-fluorescein active site environment which are relatively independent of affinity (69). Fv 4-4-20 showed almost identical ligand-related spectral properties (Qmax and λmax) and affinity for antigen relative to SCA (Table I). Anti-metatype antibodies, both polyclonal and monoclonal, characteristically enhance the affinity for fluorescein displayed by the anti-fluorescein active site for which they are specific (15, 54). Fluorescein affinity measurements were repeated for Fv and SCA 4-4-20 in the presence of anti-metatype reagents to assess their relationship in terms of ligand binding kinetics and liganded state conformation. Fv and SCA showed similar (proportional) increases in affinity in the presence of anti-metatype antibodies, confirming that both active site structures possess the same conformational perturbations upon ligand binding (Table I).

In addition, CD analysis suggested identical overall secondary structures for Fv and SCA 4-4-20. Fv 4-4-20 showed the identical positive (204 nm) and negative extremum (217 and 230 nm) found characteristic in immunoglobulin CD spectra, are typical of proteins with β-sheet structure and a high aromatic content (70, 71) (i.e. SCA 4-4-20). Interestingly, the CD spectra of isolated variable domains consisted...
of negative extrema at 217 nm, indicative of β-sheet structure, but also showed negative values at 204 nm, possibly due to a higher degree of random structure (65) (data not shown). The shoulder at 230 nm in the CD spectra of both Vc and VH proteins was reduced compared with the Fv, suggesting a possible re-orientation of tryptophan and tyrosine side chains in their respective environments (37). This would indicate that isolated domain proteins undergo dynamic secondary structure rearrangement in order to dimerize and form active Fv protein.

To support this result, comparative fluorescence quenching studies were performed using affinity-purified Fv and associated Vc and VH proteins. Associated protein showed almost identical fluorescence quenching properties as compared with an equal optical density solution of Fv (Fig. 3). It was also demonstrated that the liganded Vc/VH dimers responded similarly to affinity-purified Fv when affinity measurements were determined in the presence of anti-metatype reagents (data not shown). Collectively, these results indicated that 1) Fv, Vc, and VH proteins consisted of mostly β-sheet structure and some random coil, 2) upon Vc and VH association some conformational changes are necessary for proper dimerization and active site formation, and 3) indistinguishably refolded domains maintain a dimerization competent form in the absence of constant domains which can form the proper active site environment for fluorescence binding and quenching.

As previously stated, hydrostatic pressure does not promote changes on the tertiary structure of proteins, but alters regions of secondary structure responsible for global protein conformation (38, 66). A comparison of the pressure induced dissociation of fluorescence profiles for Fv and SCA would be a definitive evaluation of their dynamic similarity. Identical fluorescence fluorescence profiles were recovered for the two proteins when exposed to increasing hydrostatic pressure (Fig. 5). This indicated that Fv 4-4-20 displayed the same standard volume change (ΔVsw = −50 ml/mol) upon fluorescence dissociation as SCA (38). Seeing that their structures were apparently identical, this suggested that the Fv 4-4-20 must have increased conformational dynamics relative to the IgG molecule (ΔVsw = −5 ml/mol) as originally postulated for SCA (15, 38, 41). This indicated that increased dynamics were responsible for the decreased affinity for antigen displayed by Fv and SCA. Determination of the Fv interdomain dissociation constant (1.43 × 10^{-7} M) showed that despite the relatively low associative affinity, the high affinity fluorescein interaction was unchanged relative to the SCA (Fig. 6). This excluded the possibility that the initial decrease in the affinity for fluorescein upon removal of the constant domains was due to decreased domain-domain stability. The large difference between Vc/VH and Fv/fluorescein Kd values (−400-fold) suggested that in terms of 4-4-20, there was little or no quantitative correlation between interdomain stability and antigen affinity. Seeing that individual variable domain proteins showed no affinity for antigen (data not shown), this confirmed that there was no coupling of fluorescein binding or domain association free energy in the formation of the Fv 4-4-20 (72). Thus, Fv structural characteristics responsible for interdomain association were independent of the structural features necessary for high affinity antigen binding. Collectively, results indicated that the absence of constant domains caused increased dynamic flexibility, not reduced variable domain associative affinity, in Fv and SCA 4-4-20 and resulted in decreased affinity for antigen.

Previous studies have demonstrated that heavy chain isotype (i.e., constant domain structure) influences antibody functional affinity against multivalent antigen (73, 74). The effect of constant domains reported in these studies, which depended on high multivalent antigen concentrations, suggested that the change in functional affinity was due to change in segmental flexibility of the IgG molecule. Antibody isotypes was, however, implicated in the expression of idiotopes on the variable domains of an anti-nitrophenyl antibody MOPC 315 (31). Idiotopes represent structural markers on the antibody active site which are sensitive to conformational fluctuations due to either ligand binding or natural protein dynamics (29, 30, 75). Such relationships would support the hypothesis that the interaction between the variable and first constant domains are necessary for proper variable domain conformational dynamics and not rigid structural features (Fig. 7). Results presented here support this hypothesis by demonstrating how the absence of constant domains influences active site/antigen interactions. In the case of 4-4-20, the binding of fluorescein can be considered a perturbation of the active site conformation which the constant domains can restrict to maintain the high affinity interaction. Removal of the constant domains from the SCA and Fv constructs resulted in the removal of this “dynamic buffering” effect. The ensuing increased domain dynamics translated into an increased dissociation rate of fluorescein from the active site. As studies progress on the re-engineering of antibody proteins, care must be taken to assess the importance of constant domain interactions for proper variable domain function. Methods which can both stabilize the active site structure and maintain wild type conformational dynamics may be necessary to ensure the success in producing recombinant Fv proteins which mimic parental IgG affinities.

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