Immunological and Structural Characterization of a High Affinity Anti-fluorescein Single-chain Antibody*

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Single-chain antibody of the (NH2)-VL-linker-VH (COOH) design, was constructed based on prototype high affinity anti-fluorescein monoclonal antibody (mAb) 4–4–20. Purified single-chain antibody (SCA) 4–4–20/212 was studied relative to Ig mAb 4–4–20 in terms of ligand binding, kinetics, idiotypic, metatypic, and stability in denaturing agents. Ligand-binding data correlated with metatypic relatedness of the liganded site. Anti-metatypic reagents reacted preferentially with the liganded conformer of the 4–4–20 antibody active site and were unreactive with free ligand and the non-liganded (idiotypic) state. All results were consistent with the conclusion that SCA 4–4–20/212, with a 14-amino acid linker folded into a native conformational state that closely simulated the prototypical mAb. Furthermore, GdnHCl unfolding and refolding studies demonstrated H and L chain variable domain intrinsic stability between SCA 4–4–20/212 and a 50 kDa antigen-binding fragment were nearly identical. This suggested Cα1 and Cα1 domain interactions may be more prevalent in V region molecular dynamics than structure.

The synthesis and expression of variable region immunoglobulin genes producing functional single-chain antibodies (SCA)* has proven an important innovation with significant potential in clinical and basic research areas (Huston et al., 1988; Bird et al., 1988; Chaudhary et al., 1989; Glockshuber et al., 1990). Single-chain antibodies represent an alternative to both Fab and Fv fragments and have already proven valuable potential in clinical and basic research areas (Huston et al., 1988). Single-chain antibody constructs involved de novo bacterial synthesis of a single gene encoding mAb V regions (VH and VL). Modeled linkers (14–25 amino acids) covalently bridged the H and L chain V domains similar in design to insertion of short loop sequences between domains in de novo design of proteins (Mutter, 1988; Regan and De Grado, 1988). SCA proteins have been synthesized as products of single-chain gene constructs with either VH or VL in the amino-terminal orientation (Bird et al., 1988; Huston et al., 1988). In principle, linkers must allow VH and VL domains to fold independently with the final antigen-binding site in proper conformation (i.e. native immunoglobulin fold) and without interfering with access to the active site or the molecular dynamics of either domain. However, the role or effect of the linker (if any) has not been fully assessed. Escherichia coli-expressed SCA protein has been purified in the approximate native folded state as evidenced by significant binding of homologous ligand (Bird et al., 1988). Although ligand binding properties of folded single-chain Ig molecules have not perfectly simulated parental mAb binding, approximate prototypical ligand reactivity has been obtained (Huston et al., 1988; Bird et al., 1988; Glockshuber et al., 1990). Thus, the SCA model represents a useful reagent to study V domain and ligand interactions independent of constant regions as in Fab and Ig molecules.

SCA has been synthesized based on high affinity anti-fluorescein mAb 4–4–20 (Bird et al., 1988). mAb 4–4–20 was a suitable prototype since the V region primary structures have been determined (Bedzyk et al., 1989; Bedzyk et al., 1990) as well as the three-dimensional liganded structure (Herron et al., 1989). Previous experiments revealed an SCA analogue of mAb 4–4–20 (4–4–20/202') exhibited an affinity.

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1 The abbreviations used are: SCA, single-chain antibody; AB, antibody molecule; Fab, 50 Da antigen-binding fragment derived from papain digestion of immunoglobulin molecules; Fv, VH and VL domain fragment; Fl, fluorescein; H, immunoglobulin heavy chain; Id, idiotypic; L, immunoglobulin light chain; mAb, monoclonal antibody; Met, metatypic; Qmax, maximum Fl fluorescence quenching expressed as percent; VH, H chain variable region; VL, L chain variable region; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay
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Qass and bathochromic shift approximating that of the parent Ig molecule (Bird et al., 1988). These parameters suggested that refolded SCA 4-4-20/212 had approximated the original mAb active site naive microenvironment.

To more closely examine fidelity of refolding in the presence of a linker idiotypic and metatypic (Voss et al., 1988b; Voss et al., 1989) comparative properties between mAb 4-4-20 and SCA 4-4-20 were examined. Idiotype and metatypic markers reflected topological features of the antibody active site in two different conformations. Metatypic was based on elicitation of antibodies which preferentially bound the liganded conformer of an antibody active site. Anti-metatypic reagents reacted inefficiently with the homologous antibody non-ligated (idiotype) state were unreactive with free hapten and did not react with the liganded form of idiotypically unrelated murine monoclonal antibodies of similar specificity and affinity (Voss et al., 1988b; Voss et al., 1989). We demonstrate here that SCA 4-4-20/212 closely simulated the mAb 4-4-20 active site based on idiotype, metatypy, ligand binding, and stability in denaturing agents. In addition, GndHCl-induced denaturation results suggested minimal CH1 and CL domain contributions to V region structure.

MATERIALS AND METHODS

Monoclonal Antibodies—mAb 4-4-20 was generated through chemically mediated cell fusion of splenocytes obtained from an adult BALB/c mouse hyperimmunized with Fl (T-KLH emulsified in Freund's complete adjuvant (Difco Laboratories). Mab 4-4-20 (IgG, s) possessed an affinity of 1.7 x 1015 M-1 (Kranz and Voss, 1981; Bates et al., 1985) for the fluorescyl ligand.

Single-chain Antibody—The 4-4-20 single-chain gene construct containing linker 212 was generated, expressed, and the protein purified as described (Bird et al., 1988).

Nucleotide Sequence Determination—The single-chain antibody 4-4-20/212 gene construct was subcloned into pTZ19U (Mead et al., 1985) for the fluorescyl ligand. Linker 212 was generated, expressed, and the protein purified by successive passages over Fl-Sepharose to remove any anti-Fl antibody activity caused by conjugation of fluorescein isothiocyanate to reactive sites external to the 4-4-20 active site and 4-4-20-Sepharose to remove anti-4-4-20 Id activity. The adsorbed γ-globulin fraction was tested for anti-metatype activity in an ELISA (see below). After the quaternary immunization (8 months post-primary), a strong positive anti-Met reaction was observed with anti-Met antibodies specific for ligand 4-4-20 Fab fragments. Finally, the anti-met reagent was not specific for Fl when tested in a solid-phase ELISA for reactivity with Fl BSA and did not quench Fl in a fluorescence quenching assay (Voss et al., 1988).

Solid-phase Metatypic Binding Assay—Polyacrylamide wells (Dynatech Immulon II plates) coated with the appropriate Fl (12 µg/ml) in 50 µl of 50 mM phosphate buffer, pH 8.0, were incubated at 37 °C for 2 h. Coated wells were washed three times with TBS and masked with TEBT and bound radioactivity quantitated in a Searle model 210 gamma counter (TM Analytic).

SCA 4-4-20 Dissociation Rate Analysis—Purified liganded SCA 4-4-20/212 was analyzed using off-rate fluorescence analysis as previously described (Bird, 1984). SCA 4-4-20/212 possessed an affinity of 5 x 108 M-1 based on the dissociation rate analyses at 2 °C (Fig. 1A). This compared with 1 x 109 M-1 for mAb 4-4-20 (Fig. 1B).


4 K. M. Weidner and E. W. Voss, Jr., unpublished experiments.
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Anti-Id Reactivity—To compare the degree of idiotypic relatedness between SCA 4-4-20/212 and mAb 4-4-20, Ig molecules were used as anti-4-4-20 Id/125I-4-4-20 soluble inhibitors. Fig. 2 compares inhibition titrations with SCA 4-4-20 and mAb 4-4-20 normalized to nanomoles of inhibitor active sites. Both inhibition curves represented titrations over a 10^6 mol active sites range and resulted in nearly identical inhibition curves. Maximum inhibition (90%) by mAb 4-4-20 and SCA 4-4-20/212 was achieved at 0.13 and 0.38 pmol of active sites while 50% inhibition was at 0.02 and 0.04 pmol of active sites, respectively. In control studies, polyclonal xenogenic anti-idiotypic reagent was further characterized in terms of Fl inhibition. Fig. 3 shows anti-Id interaction inhibition with either 125I-4-4-20 or 125I-SCA 4-4-20/212 inhibited by Fl-BSA or Fl ligand. In both cases, multiple Fl groups attached to the BSA macromolecule inhibited more efficiently, which was consistent with previous studies (Kranz and Voss, 1981). Ligand inhabitable anti-Id specificity has been a distinguishing factor relative to xenogenic anti-metatype reagent (described below). Protein inhibition results (Figs. 2 and 3) suggested, within limits of error inherent in the solid-phase assay, that SCA 4-4-20/212 and mAb 4-4-20 were idiotypically indistinguishable.

Anti-metatype Reactivity—In terms of the xenogenic polyclonal anti-Met reagent, comparative binding studies revealed only slight differences between liganded 4-4-20 Fab fragments and SCA 4-4-20/212 recognition. Fig. 4 compares inhibition titrations of liganded 4-4-20 Fab fragments and liganded SCA 4-4-20/212 normalized to nanomoles of inhibitor active sites. Maximum inhibition was 88.0 and 82.5% while 50% inhibition was achieved at 0.20 and 0.45 pmol, respectively. In addition, neither non-liganded 4-4-20 nor SCA 4-4-20/212 inhibited the anti-Met reaction with affin-
ity-labeled 4-4-20 Fab fragments.

Stability Studies—As an additional parameter to study SCA 4-4-20/212, GdnHCl-induced denaturation and subsequent renaturation studies were conducted. Fig. 5A represents an unfolding and reversible folding curve for SCA 4-4-20/212 obtained from triplicate trials. The transition curve (solid line) was normalized assuming the fluorescence for folded and unfolded proteins could be extrapolated linearly to the transition zone and was the theoretical curve expected for a two-state (N ⇔ D) unfolding model. From this curve, the midpoint (Cm) for unfolding was 0.98 M GdnHCl. In addition, determination of ΔGΔ for each transition region point (Fig. 5A) and plotted at the corresponding GdnHCl concentration resulted in Fig. 5B. The unfolding free energy change in the absence of GdnHCl (ΔGΔH2O), obtained from linear extrapolation to zero M GdnHCl, was 2.85 ± 0.51 kcal/mol. Finally, the slope (m = -d(ΔGΔ)/d[GdnHCl]) of the linear denaturation plot (Fig. 5B) was determined to be -2.99 ± 0.35 kcal/ (mol M).

**DISCUSSION**

Independent of potential therapeutic applications, single-chain antibodies represent a useful macromolecular analogue to resolve immunoglobulin structure-function relationships. As indicated previously, active site comparisons of Fab and Ig molecules led to definitive conclusions regarding identity; however, Fv fragment simulation with the parental site represents different antibody active site conformational states or topological features (Voss et al., 1989b, 1989), they were important parameters to verify prototypic active site reproduction in structures such as SCA or Fv fragments. In terms of idiotypic and metatypic topological markers, inhibition studies showed that SCA 4-4-20/212 closely resembled 4-4-20 Ig and 4-4-20 Fab fragments (Figs. 2-4). In addition, spectral properties (Qm and λmax) of SCA 4-4-20/212-bound fluorescent ligand were identical to mAb 4-4-20 Ig and Fab fragments (data not shown). Therefore, based on these parameters and since SCA 4-4-20/212 possessed nearly an identical affinity with the monoclonal Ig parental molecule, the SCA 4-4-20/212 active site closely approximated the prototype. Furthermore from these results, the 14-amino acid linker not only allowed appropriate VH and VL domain folding, but also did not cover any accessible surface area contacted by the anti-Met or anti-Id antibodies.

Statements assessing SCA homology gained validity through critical evaluation of each measurement. Ligand-related spectral measurements (Qm and λmax) have been found to be unique and characteristic of each anti-fluorescein monoclonal antibody. Both spectral measurements were environment sensitive but relatively independent of antibody affinity (Voss, 1989). Idiotypic relatedness (Figs. 2 and 3) was a relatively sensitive measurement which monitored non-liganded active site topology but deviations in antibody structure which affect idiotypic remain undetermined. Studies dealing with the for-
mulation of idioype families (Bates et al., 1985) and comparative primary structures of Ig members comprising idioype families (Bedzyk et al., 1990) in general suggested that only Ig molecules of closely related primary structures share idioype epitoeps. In terms of structure-function correlates, metatypic relationships also remain ill-defined (Voss et al., 1989).

In the case of a high affinity mAb (such as 4-4-20), it has been shown that upon stoichometric additions of ligand, there was a concomitant conversion from idioype to metatype based on xenogenic anti-Met reagent reactivity. Differences in sensitivity between idioype and metatypic assays can be attributed, in part, to variations in the xenogenic antibody reagents. However, differences were more likely to reside in the fact that idioype probably represented a series of conformational states (Voss et al., 1988a) not recognized equivalently by the anti-Id reagent. Conversely, the ligand-induced metatypic more likely represented a single conformational state or a tighter distribution of closely related states (especially in high affinity sites) all reactive with the anti-Met reagent.

Important questions regarding appropriate folding and possible linker effects must be assessed in terms of the assays employed. It was imperative to consider that the antibody-dependent results reported in these studies may be misleading and conclusions regarding variable domain folding and the linker role must be appropriately tempered. Conceivably, SCA 4-4-20/212 folding reached only a partially complete state and various xenogenic secondary reagents (i.e. anti-Id and anti-Met) or bound ligand selected a subset of SCA protein conformers in near native conformational states. The two anti-topological assays approached this question in different ways. The anti-Id assay was independent of ligand, but it was not possible to assess the role of the anti-Id reagent to force a partially folded SCA into the final conformer. Two factors were important in this assessment. First, there was the question of degree of recognition by the anti-Id reagent of a partially "correct" structure. Second, there was the importance of the relative affinity of the anti-Id reagent. In contrast, the anti-Met reaction depended on both bound ligand and reactivity with the anti-Met reagent. Both factors may have contributed synergistically toward finalizing the native conformer.

Finally, relative SCA 4-4-20/212 stability in GndHCl (Fig. 5) was determined to elucidate the $V_h$ and $V_l$ domain interaction contribution to active site stability. Since the transition curve, derived from increased GndHCl concentrations (Fig. 5A, solid line), was representative of a two-state unfolding model, $V_h$ and $V_l$ domains unfolded at similar conditions with near identical unfolding free energies. This was expected since $V_h$ and $V_l$ domain tertiary structures were homologous. In the absence of ligand, therefore, linker 212 did not make unfolding more complicated. Similar results were observed with other linkers as well. Like SCA 4-4-20/212, previous studies (Rowe and Tanford, 1973; Rowe, 1976) have shown another Fab fragment to have an unfolding transition near 1.0 M GndHCl. This revealed that SCA 4-4-20/212 gained as much $V_h$ and $V_l$ domain stability from the linker as $C_{H1}$ and $C_{L}$ domains contributed to a Fab fragment $V$ domains. Linkers were designed to minimally interact with $V$ domains and thus indicated that $C_{H1}$ and $C_{L}$ domains did not significantly add to intrinsic $V$ domain stability. Fig. 6 shows predicted SCA 4-4-20/212 $V_h$ and $V_l$ domain interactions, which were also present in Fab fragments, that resulted in this similar $V$ domain stability.

In summary, these studies confirmed that SCA possessing an appropriate linker refolded into an antibody active site that closely simulated the parental site. A multi-parameter approach determined that all measurements (individual and collectively) confirmed a close simulation between the mAb and SCA active sites. Therefore, recombinant Fv molecules can be designed and produced with prototypical properties. In addition, intrinsic $V$ domain stability of a SCA was shown to mimic a Fab fragment. This did not suggest $C_{H1}$ and $C_{L}$ domain interactions were not important; however, their function may be more involved in molecular dynamics than structure.

REFERENCES


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Rowe, E. S. (1976) *Biochemistry* **15**, 905-916


Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody.
W D Bedzyk, K M Weidner, L K Denzin, L S Johnson, K D Hardman, M W Pantoliano, E D Asel and E W Voss, Jr


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