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# Recombinant Antineuraminidase Single Chain Antibody: Expression, Characterization, and Crystallization in Complex With Antigen

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***ABSTRACT*** **The variable heavy (VH) and variable light (VL) genes of NC10, a monoclonal antibody with specificity toward N9 neuraminidase (NA), were cloned and sequenced. A single chain Fv (scFv) fragment of NC10, consisting of VH and VL domains joined by a peptide linker, was designed, constructed and expressed in the *E. coli* expression vector pPOW. The N-terminal secretion signal PelB directed the synthesized protein into the periplasm where it was associated with the insoluble membrane fraction. An octapeptide (FLAG) tail was fused to the C-terminus of the single chain Fv to aid in its detection and remained intact throughout the protein purification process. NC10 scFv was purified by solubilization of the *E. coli* membrane fraction with guanidinium hydrochloride followed by column chromatography. The purified NC10 scFv showed binding affinity for its antigen, NA, 2-fold lower than that of the parent Fab. The complex between NA and the scFv has been crystallized by the vapor diffusion method. The crystals are tetragonal, space group $P42_12$, with unit cell dimensions $a = b = 141$ Å, $c = 218$ Å.** © 1993 Wiley-Liss, Inc.

**Key words: X-ray crystallography, antibody domain, recombinant DNA, binding affinity, antigen–antibody complex**

## INTRODUCTION

Our understanding of the structural basis of the interactions between antibodies and protein antigens has been limited by the small number of X-ray crystal structures of these complexes. The structures of antilysozyme Fabs D1.3,[1], HyHEL5,[2] and HyHEL10,[3] which bind to different parts of the lysozyme surface, have been solved in complex with their antigen, and the structures of Fabs NC41[4,5] and NC10,[6,7] which recognize largely overlapping epitopes on NA from influenza virus, have been determined in complex with NA. The structure of NC41 Fab has been solved in complex with two related NAs which were isolated from tern (avian) and whale sources and are both of the subtype N9. The protein sequences of these variants differ at 14 residues, but these substitutions have no effect on the NA–Fab interface. The structure of NC10 Fab in complex with whale N9 NA has been solved and is in the process of final refinement.[8]

The nature of the interactions between antibodies and antigens may be probed by introducing a change in the structure or chemistry of the antibody paratope. Recently, the application of polymerase chain reaction (PCR) technology to cloning antibody genes has enabled the expression and rapid mutagenesis of selected antibody domains in *E. coli*.[9] It is now feasible to study a model antigen such as NA in complex with a wide range of recombinant antibody structures (initially a known structure such as NC10) which have been genetically manipulated.

The antibody Fv fragment, which consists of the associated VH and VL antibody domains, is the minimum size binding unit required to reproduce the antibody-combining site of the parent Fab. An Fv fragment may bind to the target antigen with an affinity approaching that of the parent Fab.[10] In the only published structure of a complex between a two-chain Fv and its protein antigen, namely D1.3 Fv and lysozyme, the authors reported that the modes of attachment of D1.3 Fab and Fv were very similar.[1] In addition, the smaller size of Fvs may

Abbreviations: $A_{600}$, absorbance at 600 nm; bp, base pairs; CDR, complementarity-determining region; *E. coli, Escherichia coli;* EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Fab, antigen-binding fragment of antibody; NA, neuraminidase; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline solution; PCR, polymerase chain reaction; PelB, pectate lyase secretion signal peptide from *Erwinia cartovora;* scFv, single chain Fv fragment; SDS, sodium dodecyl sulfate; VH and VL, variable domains of antibody heavy and light chains, respectively.

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allow higher resolution crystallographic studies than have been possible for Fabs and Fab complexes.[11] However, the usefulness of Fv fragments is limited by their apparent lack of stability at low protein concentration, including under physiological conditions.[12] Joining the VH and VL domains with a polypeptide linker to form a single chain Fv results in a more stable molecule without loss of affinity.[12] To date there is no published structure of a scFv in complex with a protein antigen, so the structural effect of introducing an additional protein strand between the two variable antibody domains is unknown. Here we report the cloning, expression and characterization of a scFv fragment of NC10 and the crystallization of a complex between NC10 scFv and tern N9 neuraminidase from influenza virus. We believe this is the first published report of crystals of a single chain antibody–antigen complex.

## METHODS

### Design of scFv Molecule

In the crystal structure of the whale N9 NA–NC10 Fab complex (space group *I*422; $a = b = 171.5$ Å, $c = 160.2$ Å) the constant domains of NC10 Fab are disordered, so it was anticipated that an NA–NC10 scFv complex could crystallize isomorphously with the parent complex. The atomic coordinates of the partially refined whale N9 NA–NC10 Fab complex[8] were displayed by the program FRODO[13] on an Evans and Sutherland PS300 graphics workstation, to design a scFv molecule which would cause minimal disruption to the antibody–antigen interface and the crystal contacts in the NA–NC10 Fab crystal structure. It did not appear that the order in which the VH and VL domains were linked would disrupt the antibody–antigen interface, however residues near the N-terminus of VH were involved in crystal contacts, so the linker was designed to extend from the C-terminus of VH to the N-terminus of VL. A peptide of fifteen residues' length was accommodated without geometric strain, so the linker $(GlyGlyGlyGlySer)_3$ was chosen as it had been used in the construction of several scFvs.[12,14] The hydrophilic octapeptide, AspTyrLysAspAspAspAspLys (FLAG),[15–17] was added to the C-terminus as an affinity label to aid in the detection of scFv. It was predicted that the presence of FLAG at the C-terminus of VL would not affect crystal contacts, since the antibody constant region is disordered and is therefore unlikely to be contributing to crystal growth.

### Cloning of VH and VL Genes

Total RNA was extracted from the hybridoma cell line secreting monoclonal antibody NC10[18] and mRNA was selected on poly(U)-Sepharose.[19] Synthesis and amplification of NC10 VH cDNA were based on the PCR method of Orlandi et al.[20] The VH cDNA fragment was obtained by reverse transcription of 1 μg of NC10 mRNA using VH1FOR[20] primer. The cDNA fragment was then amplified in a PCR reaction using VH1FOR and VH1BACK[20] primers, which contained *Pst*I and *Bst*EII restriction sites, respectively. The amplified DNA was purified by isolation as a single band from a 2% agarose submarine gel, digested with *Pst*I and *Bst*EII and inserted into the modified pUC19 vector pSW1.[9]

Since NC10 VL was not readily cloned by the PCR method described above, a cDNA library was constructed in bacteriophage λgt10 (Amersham) and screened with a light chain cDNA probe, B61K16.[21] Positive clones were subcloned into M13 and sequenced. NC10 VL cDNA was amplified by PCR using primers VK10FOR and VK10BACK (Fig. 1A). VK10FOR had been modified for exact 3′ sequence homology to the NC10 VL gene. NC10 VL cDNA was run on a 2% agarose gel and isolated as a single band, digested with *Sac*I and *Xho*I and ligated into pSW1.

### Construction of scFv Gene

The NC10 scFv-FLAG gene (Fig. 1B) was assembled in pSW1. The NC10 VH gene was inserted between the *Pst*I and *Bst*EII sites in pSW1, and the VL gene was inserted between the *Sac*I and *Xho*I restriction sites. A pair of complementary synthetic oligonucleotides were premixed in equimolar ratios to form the DNA duplex encoding the single chain linker (D1, Fig. 1A). This duplex was ligated into the *Bst*EII–*Sac*I restricted pSW1 plasmid. Similarly, a synthetic DNA duplex encoding the FLAG tail (D2, Fig. 1A) was inserted between the *Xho*I and *Eco*RI sites at the 3′ end of the VL gene.

### Expression of scFv Gene

The scFv-FLAG gene was inserted between the *Msc*I and *Eco*RI sites in the *E. coli* secretion vector pPOW[17] as a PCR fragment which had been amplified using primers P1[17] (5′ region consensus sequence for VH gene, containing a *Tha*I site) and FLAGBACK (reverse primer complementary to FLAG, containing an *Eco*RI site; Fig. 1A) and digested with *Tha*I and *Eco*RI. Prior to expression analyses, the construct was sequenced using the dideoxynucleotide method.[22] Expression was achieved in the cell line TG1 using the following protocol. A two litre batch fermenter (LH series 500, LH Engineering) was inoculated to an $A_{600}$ of 0.5 using an overnight culture grown at 28°C in fortified medium.[17] The initial incubation temperature of the fermentation was 28°C, the pH was controlled automatically at 7.0, and dissolved oxygen was maintained above 30% using air sparging and agitation. The medium used in the fermentation was identical to that used in preparing the inoculum. When the culture reached an $A_{600}$ of 6.0, expression of NC10 scFv was induced by raising the tempera-

**A**

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                                SacI
VK10FOR:   5'-TGT GAT ATC GAG CTC ACA CAG ACT-3'

                               XhoI
VK10BACK:  5'-CCG TCT TAT CTC GAG CTT GGT CCC-3'

                           EcoRI   stop
FLAGBACK:  5'-AT GAT GAA TTC TTA TTA TTT ATC-3'



D1:  5'-GTC ACC GTC TCC GGT GGT GGT GGT TCG GGT GGT GGT GGT TCG
             G CAG AGG CCA CCA CCA CCA AGC CCA CCA CCA CCA AGC

       GGT GGT GGT GGT TCG GAT ATC GAG CT-3'
       CCA CCA CCA CCA AGC CTA TAG C


D2:  5'-TC GAG ATA AGA GAC TAC AAA GAC GAT GAC GAT AAA TAA TAA G-3'
           C TAT TCT CTG ATG TTT CTG CTA CTG CTA TTT ATT ATT CTT AA
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**B**

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                                pelB signal sequence
 M  K  Y  L  L  P  T  A  A  A  G  L  L  L  L  A  A  Q  P  A
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCG

       H1    Pst1                      H10
 M  A  Q  V  Q  L  Q  Q  S  G  A  E  L  V  K  P  G  A  S  V
ATGGCGCAGGTGCAGCTGCAGCAGTCTGGGGCTGAACTGGTGAAGCCTGGGGCCTCAGTG

   H20                          H30
 R  M  S  C  K  A  S  G  Y  T  F  T  N  Y  N  M  Y  W  V  K
AGGATGTCCTGCAAGGCTTCTGGCTACACATTTACCAATTACAACATGTACTGGGTAAAA

   H40                                H50        H52A
 Q  S  P  G  Q  G  L  E  W  I  G  I  F  Y  P  G  N  G  D  T
CAGTCACCTGGACAGGGCCTGGAGTGGATTGGAATTTTTTATCCAGGAAATGGTGATACT

      H60                                H70
 S  Y  N  Q  K  F  K  D  K  A  T  L  T  A  D  K  S  S  N  T
TCCTACAATCAGAAGTTCAAAGACAAGGCCACATTGACTGCTGACAAATCCTCCAACACA

      H80      H82A  H82C                      H90
 A  Y  M  Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R
GCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGA

                H100                H100E                    BstE2
 S  G  G  S  Y  R  Y  D  G  G  F  D  Y  W  G  Q  G  T  T  V
TCGGGGGGCTCCTATAGATACGACGGAGGCTTTGACTACTGGGGCCAAGGGACCACGGTC

H110                          linker                        L1
 T  V  S  G  G  G  G  S  G  G  G  G  S  G  G  G  G  S  D  I
ACCGTCTCCGGTGGTGGTGGTTCGGGTGGTGGTGGTTCGGGTGGTGGTGGTTCGGATATC

 SacI                 L10                          L20
 E  L  T  Q  T  T  S  S  L  S  A  S  L  G  D  R  V  T  I  S
GAGCTCACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGT

                   L30                                    L40
 C  R  A  S  Q  D  I  S  N  Y  L  N  W  Y  Q  Q  N  P  D  G
TGCAGGGCAAGTCAGGACATTAGTAATTATTTAAACTGGTATCAACAGAATCCAGATGGA

                   L50                                    L60
 T  V  K  L  L  I  Y  Y  T  S  N  L  H  S  E  V  P  S  R  F
ACTGTTAAACTCCTGATCTACTACACATCAAATTTACACTCAGAAGTCCCATCACGGTTC

                L70                                    L80
 S  G  S  G  S  G  T  D  Y  S  L  T  I  S  N  L  E  Q  E  D
AGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGAACAAGAAGAT

                L90                                    L100
 I  A  T  Y  F  C  Q  Q  D  F  T  L  P  F  T  F  G  G  G  T
ATTGCCACTTACTTTTGCCAACAGGATTTTACGCTTCCGTTCACGTTCGGAGGGGGGACC

    Xho1                FLAG                      EcoR1
 K  L  E  I  R  D  Y  K  D  D  D  D  K  *  *
AAGCTCGAGATAAGAGACTACAAAGACGATGACGATAAATAATAAGAATTC
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Fig. 1. (**A**) VK10FOR and VK10BACK, forward and reverse PCR primers used to amplify VL DNA for insertion into pSW1; FLAGBACK, reverse PCR primer to amplify scFv–FLAG construct for insertion into pPOW. Below, DNA sequences of synthetic oligonucleotide duplexes encoding peptide linker between VH and VL (D1) and FLAG tail at C-terminus of VL (D2). The coding strand is indicated by underlining. (**B**) DNA sequence encoding the NC10 scFv–FLAG construct is given below the corresponding protein sequence. N-terminal PelB signal sequence is in italics, linker joining VH and VL is both in italics and underlined and C-terminal FLAG sequence is in italics. Restriction sites used for construction of gene are indicated by underlining. CDRs according to the definition of Chothia and Lesk[30] are indicated by boxes around the protein sequence. The numbering of the protein sequence is as defined by Kabat et al.[24] and insertions are labeled.

ture to 42°C for 15 min and then lowering it to 39°C for the remainder of the induction period. Samples were taken prior to induction and at hourly intervals during the induction period for calculation of cell density from measurements of $A_{600}$ and for analysis by SDS-PAGE and Western blot.

The cells were fractionated as follows: cells were harvested by centrifugation (Beckman JA14, 6000 rpm, 15 min) and the culture supernatant was removed. The cell pellet was resuspended in 10% of the original volume in 0.010M Tris-HCl pH 7.5/20% sucrose at 0°C and EDTA was added to a final concentration of 0.005 M. The cells were placed on ice for 10 min and then collected by centrifugation. Periplasmic proteins were removed by osmotic shock in water (0°C, 10 min) followed by centrifugation. The cell pellet was resuspended in phosphate-buffered saline (PBS) solution, lysed by sonication and centrifuged (Beckman JA14, 12000 rpm, 45 min) to remove soluble cytoplasmic proteins from the insoluble membrane fraction. The cell fractions were analyzed by 12% SDS-PAGE.

### Purification and Characterization of NC10 scFv

The bacterial membrane fraction containing NC10 scFv was extracted by resuspension in 6 M guanidium hydrochloride in 0.1 M Tris-HCl, pH 8.0, and stirred at 4°C for 16 hr, then centrifuged to remove insoluble matter. The soluble fraction was dialyzed extensively against 0.025 M Tris-HCl, 0.1 M NaCl, pH 8.0, at 4°C and centrifuged to remove proteins which precipitated on dialysis. The remaining soluble fraction, which contained approximately 50% of the extracted scFv, was concentrated by ultrafiltration and centrifuged again. The soluble concentrate was applied to a Sephadex G-100 column (60 × 2.5 cm) equilibrated with 0.025 M Tris-HCl, 0.1 M NaCl, pH 8.0. Eluted fractions containing scFv were pooled, concentrated and dialysed against 0.025 M Tris-HCl, pH 8.0, and chromatographed on a MonoQ column (Pharmacia-LKB) to yield a homogeneous preparation of scFv as judged by SDS-PAGE and N-terminal sequence analysis. The affinity constants for the tern N9 NA–NC10 scFv and tern N9 NA–NC10 Fab complexes were determined by sedimentation equilibrium as described by McInerney et al.[23]

### Crystallization and X-Ray Diffraction Analysis

Crystals of the complex between NC10 scFv and tern N9 NA were initially grown at room temperature in 24-well cell culture dishes sealed with greased coverslips, using the hanging drop method. NC10 scFv (5 mg/ml in PBS) and N9 NA (10 mg/ml in PBS) were mixed together (with scFv in slight molar excess) with an equal volume of potassium phosphate buffer (1.7 M, pH 6.6) in a 10 μl drop. The drop was equilibrated by vapor diffusion against phosphate buffer (1.3 M, pH 6.8). When this procedure was optimized it was scaled up to a 40 μl sitting drop experiment from which crystals of sufficient size for data collection were obtained. The crystals were characterized by precession and oscillation photography using Cu$K_\alpha$ radiation from an Elliot GX20 rotating-anode X-ray generator operated at 40 kV and 45 mA.

## RESULTS AND DISCUSSION

The NC10 VH gene comprises 366 bp, encoding 122 amino acids numbered from 1–113 in the scheme of Kabat et al.[24] According to this scheme there are nine insertions in the three complementarity determining regions (CDRs) (H52A, H82A-C, and H100A-E). The NC10 VL gene of 321 bp encodes a $V_\kappa$ domain of 107 amino acids, with no insertions as defined by Kabat et al.[24] (Fig. 1B).

The *E. coli* expression vector pPOW[17] was designed for synthesis of antibody domains for structural studies (X-ray crystallography, NMR spectroscopy) and other uses. Power et al.[17] reported the high-level expression of the NC41 VH gene in this system. Analysis of SDS-PAGE gels (Fig. 2) and Western blots which were probed with anti-FLAG M2 antibody (data not shown) revealed that, like NC41 VH, NC10 scFv was associated with the insoluble bacterial membranes in fractionated cells. There was no evidence of cytoplasmic "inclusion" bodies, and N-terminal protein sequencing of NC10 scFv demonstrated that the PelB signal sequence had been correctly cleaved, indicating that the protein had been secreted into the bacterial periplasm where it associated with the membrane. Approximately 50% of the expressed NC10 scFv was solubilized; the amount of pure soluble NC10 scFv obtained by the described procedure was 3 mg/liter of culture broth. The affinity constant of NC10 scFv for N9 NA was measured by sedimentation equilibrium ($K_a = 3 \times 10^7\ M^{-1}$ at 10°C) in a reaction with stoichiometry of one scFv molecule to one monomer of NA. This value was approximately 3-fold lower than the affinity of the parent Fab for tern N9 NA ($K_a = 7 \times 10^7\ M^{-1}$ at 10°C). The yield of pure protein and affinity for antigen of NC10 scFv was comparable to values reported for other scFvs; the yield of pure protein generally varied between 1 and 10 mg/liter of culture broth, and a slight reduction in affinity relative to the parent Fab has been observed for most scFvs.[25]

The tern N9 NA–NC10 scFv crystals are tetragonal prisms which grew to dimensions up to 500 × 300 × 300 μm over a period of 2–3 weeks (Fig. 3). The conditions under which these crystals grew were similar to those under which tern N9 NA and the N9 NA–Fab complexes have crystallized.[26,27] Crystals of diffraction quality were obtained from only 1 mg of protein using NA crystallization conditions as a starting point. The crystals were washed

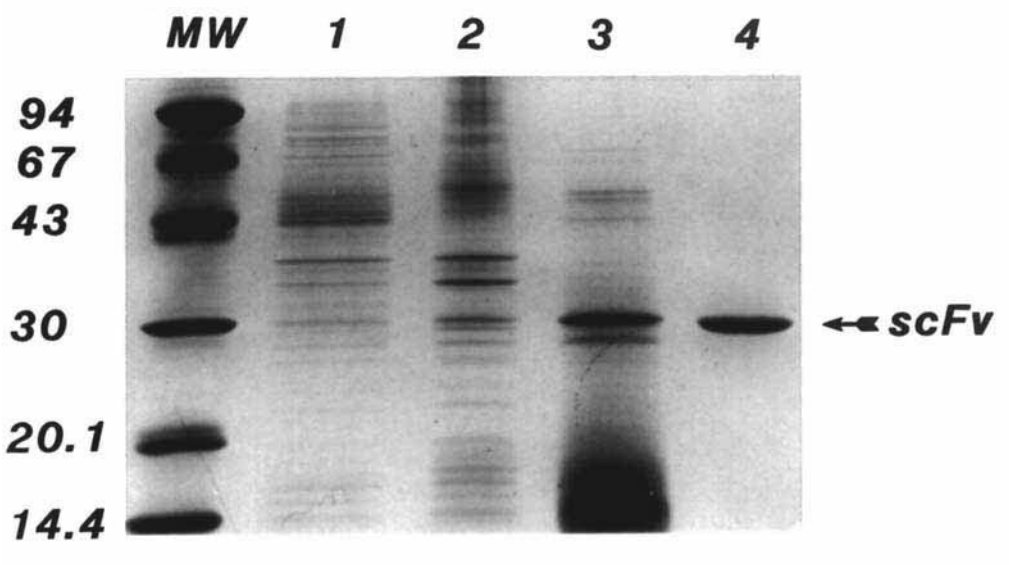


Fig. 2. Twelve percent SDS-PAGE stained with Coomassie blue, showing NC10 scFv at various stages of purification. **Lanes: MW,** molecular weight markers with mass/kDa indicated for individual bands; (**1**) whole cell sample 3 hr after induction of gene expression; (**2**) membrane fraction of cells; (**3**) soluble protein remaining after resuspension of membrane fraction in 6 M guanidium hydrochloride followed by dialysis; (**4**) purified NC10 scFv. The arrow indicates the location of the scFv band.

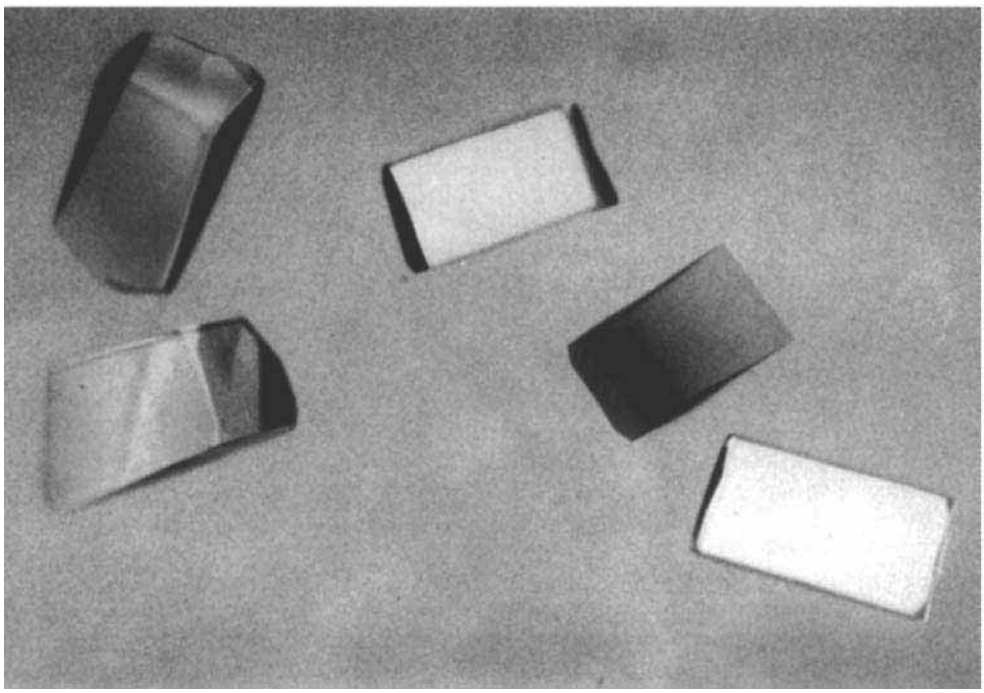

Fig. 3. Tern N9 NA–NC10 scFv complex crystals. Crystal dimensions were approximately 400 × 300 × 300 μm.

with phosphate buffer then dissolved and analyzed by 12% SDS-PAGE (Fig. 4). The presence of both NA and scFv protein bands in the dissolved crystal sample demonstrated that the crystals contain NA–scFv complex.

Although the scFv was designed to be compatible with the crystal lattice of the whale N9 NA–NC10 Fab complex, the scFv crystallized with tern N9 NA and a new crystal form was observed. The NA–NC10 scFv crystals belong to the space group $P42_12$ and

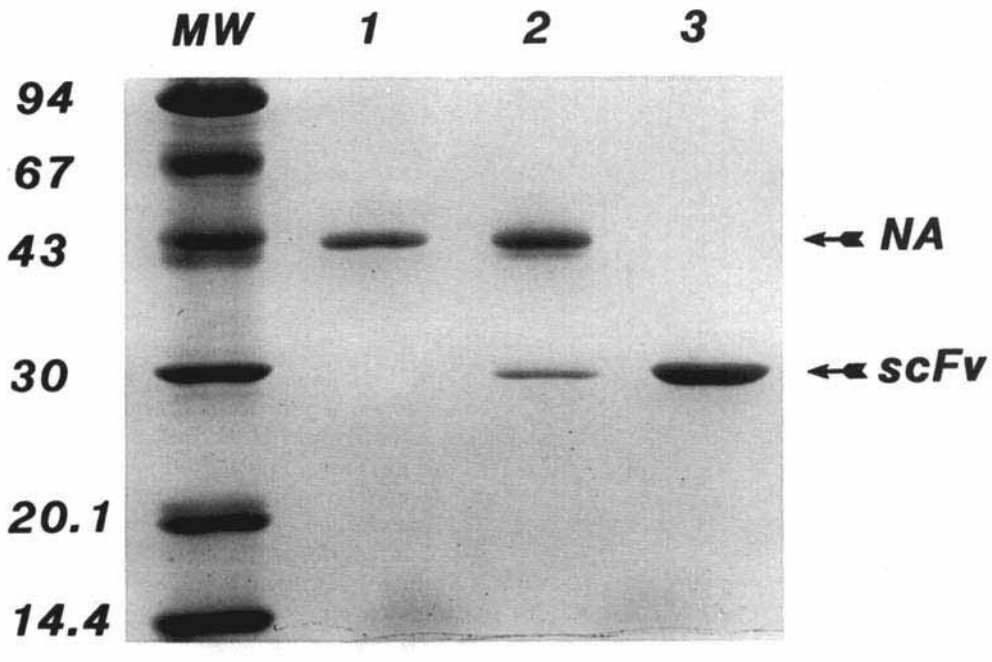


Fig. 4. Twelve percent SDS-PAGE stained with Coommassie blue, showing the two protein constituents of a dissolved N9 NA–NC10 scFv crystal. **Lanes: MW,** molecular weight markers with mass/kDa indicated for individual bands; **(1)** N9 NA; **(2)** dissolved N9 NA–NC10 scFv crystal; **(3)** NC10 scFv.

have unit cell lengths $a = b = 141$ Å and $c = 218$ Å; $\alpha = 90°$. Crystals containing tern N9 NA alone crystallize in the cubic space group $I432$ with $a = 185.2$ Å[26]; there was no evidence of NA crystals growing in any drops containing both scFv and NA. Assuming there are two NA monomer–scFv complexes per asymmetric unit, the crystal volume per unit of protein molecular weight, $V_M$, is 3.3 Å$^3$/Da, which corresponds to a solvent content of 63%.[28] These crystals diffract to at least 3.0 Å; collection of a high resolution data set is in progress. The structure of this complex should be amenable to solution by the molecular replacement technique[29] given the similarity of its component proteins to the whale N9 NA–NC10 Fab complex structure.

## CONCLUSION

We have established a system for the expression of single chain antibody domains with high affinity for influenza NA and we have crystallized an NA–scFv complex for structural analysis. Analysis of the NA–scFv structure will reveal the binding conformation of a scFv in complex with its target antigen and will provide the basis for rational protein design of scFv mutants with modified affinity and stability.

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