Specific Detection of His-Tagged Proteins with Recombinant Anti-His Tag scFv-Phosphatase or scFv-Phage Fusions

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ABSTRACT

Using a cell-bound immunogen, we have generated a monoclonal antibody, 3D5, that recognizes carboxy-terminal oligo-histidine tags (His tags) on a wide variety of proteins. From this monoclonal antibody, we have generated a single-chain fragment of the variable domains (scFv), a dimeric scFv-alkaline phosphatase fusion and an oligovalent scFv-display phage. The antibody in its various formats is an effective tool used in fluorescence-activated cell sorting analysis, the BIAcore® method, Western blots and enzyme-linked immunosorbent assay (ELISA). Western blots and ELISAs can be developed directly by using crude extracts of E. coli *cells that produce the scFv-alkaline phosphatase fusion, thus providing an inexhaustable and convenient supply of detection reagent. Alternatively, oligovalent scFv-displaying phage can be used directly from culture supernatants for this purpose. The dissociation constants,* K_D*, of the peptide KGGHHHHH (*$K_D = 4 \times 10^{-7}$ *M) and of imidazole (*$K_D = 4 \times 10^{-4}$ *M) were determined. Molecular modeling of the Fv fragment suggests the occurrence of two salt bridges between the protonated histidine side chains of the peptide and the acidic groups in the antibody, explaining why the antibody or the substrate may be eluted under mildly basic conditions.*

INTRODUCTION

The use of tag sequences (22) has revolutionized protein science by rendering the purification and detection of recombinant proteins rational and predictable. One of the most widely used tagging systems consists of five or six consecutive histidines (1) ("His tag"). The adjacent imidazole side chains can bind to free coordination sites of metal ions such as Ni^{2+}, Co^{2+}, Zn^{2+} or Cu^{2+}, which are bound to a solid support by a chelator complex (e.g., nitrilotriacetic acid [NTA] or iminodiacetic acid [IDA]). This interaction is highly selective in the presence of high salt, which eliminates nonspecific ionic interactions, and many proteins can be purified to near homogeneity in a single step.

His tags have been used on both the N-terminus and the C-terminus of recombinant proteins and have been shown to be compatible with immobilized metal ion affinity chromatography (IMAC) purification of both native proteins and guanidinium hydrochloride-denatured proteins obtained from inclusion bodies (8,17). Proteins with intact His tags have been used as clinical-grade material (5,11), have been crystallized (17,35) and have had their nuclear magnetic resonance (NMR) structures determined (6).

To complement the inexpensive, scaleable purification of His-tagged proteins with an equally convenient and inexpensive detection, we have now prepared recombinant anti-His tag scFv reagents. They were derived from a monoclonal antibody (MAb) elicited against the C-terminal His tag by using a cell-bound immunogen. While recently other anti-His tagged monoclonal antibodies (24,34) and an alkaline phosphatase (AP)-NTA conjugate (3) have been described, the recombinant form used here can be used in an oligovalent phage display format (21) or as a bivalent scFv-AP fusion (4,33) (Figure 1), directly from crude *E. coli* extracts. Furthermore, the inexpensive reagent may be useful in generating a second purification principle based on the His tag, in order to have a two-step procedure using a single tag. We report here the construction and characterization of these recombinant proteins.

MATERIALS AND METHODS

Immunization and Fusion Protocol

A recombinant Fab fragment, directed against the murine Thy1.2 antigen on T cells, was generated from the hybridoma MmT1 (16,18) (Bauer et al., unpublished). Its expression in *E. coli* and IMAC purification by means of a $(His)_5$ tag on the C-terminus of its Fd chain were performed essentially as described earlier (7,17,23). Thymocytes (2×10^7 cells) from C57BL/6 mice were incubated with 5 µg of the purified Fab-MmT1-His fragment for 30 min at room temperature, washed with phosphate-buffered saline (PBS) and intraperitoneally injected into C57BL/6 mice four times in weekly intervals. After an additional 4-week interval, a final boost was given, and the mice were sacrificed three days later. The spleen cells were fused with the mouse myeloma cell line P3X63Ag8.653 (12) according to standard protocols (14). To screen for His tag-specific antibodies, enzyme-linked immunosorbent assay (ELISA) plates were coated with His-tagged citrate synthase, $(His)_5$-$CS(His)_5$ (17), at a concentration of 5 µg/mL. The plates were blocked with 1% milk powder in PBS and incubated with the hybridoma supernatants, followed by incubation with goat anti-mouse IgG or IgM conjugated to horse radish peroxidase (HRP) (Dianova, Hamburg, Germany). The color reaction was initiated by *o*-phenylenediamine (Sigma Chemical, St. Louis, MO, USA). The isotype was determined using biotinylated goat anti-mouse subclass-specific antibodies (Amersham, Arlington Heights, IL, USA) and avidin-peroxidase (Camon, Burlingame, CA, USA). Hybridomas were cloned twice by limiting dilution, and the secreted MAbs were purified by chromatography on a protein G

Sepharose® column (Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol.

Molecular Cloning of the Anti-His Tag Antibody

By using methods described elsewhere (23), the phagemid vector pAK100 (23) was chosen to clone the scFv fragment, assembled by polymerase chain reaction (PCR) in the orientation V_L-$(Gly_4Ser)_4$-V_H, as a fusion with the gene encoding the C-terminal part (amino acids 250–406) of phage coat protein 3 (g3p). The V_L domain carries a 3-amino acid N-terminal extension that is recognized by the Anti-FLAG™ antibody M1 (13). As the resulting construct, pAK100his2, contains an amber codon between the scFv of 3D5 and the g3 part, it was transformed into the suppressor strain XL1-Blue (Stratagene, La Jolla, CA, USA) for display of the scFv on the phage surface. To identify binding phages, the medium (2 mL 2× YT, 1% glucose and 25 µg/mL chloramphenicol) was inoculated with 12 single colonies and incubated at 37°C. At optical density $(OD)_{550}$ = 0.2, each culture was induced and infected by the addition of 1 mL 2× YT, 1% glucose, 25 µg/mL chloramphenicol, 1.5 mM isopropyl-β-D-thiogalactoside (IPTG) and 5×10^9 cfu (colony forming units) VCSM13 helper phage (Stratagene). The cultures were allowed to produce scFv-displaying phages during overnight incubation at 24°C. Phages from 1.6 mL culture supernatants were polyethylene glycol (PEG)-precipitated and dissolved in 400 µL PBS/1% milk. Duplicates of 100 µL per well were analyzed directly by ELISA to test specific binding to His-tagged citrate synthase by first preincubating the 100 µL with 1.7 mg/mL poly-histidine as competitor (Sigma Chemical). Coating and further treatment of the ELISA plates were carried out as described below. After identification of binding phages, the DNA sequence was determined.

Construction and Characterization of scFv and scFv-AP Fusions

The scFv, expressed in the non-suppressor *E. coli* host JM83, was affinity-purified directly from crude extracts by using the immobilized peptide KGGHHHHH (running buffer: 100 mM HEPES, 0.5 M NaCl, pH 7.0), which had been coupled to *N*-hydroxysuccinimide (NHS)-activated CM-Sepharose 4B. The protein was eluted with 700 mM imidazole in the same buffer. The recombinant scFv was found to be monomeric by gel filtration using a Sepharose 12 column on a SMART® system (Pharmacia Biotech) (data not shown).

The gene for AP was PCR-amplified from *E. coli* JM83, introducing an in-frame *Sfi*I site at the 5′ end and a *Hin*dIII site at the 3′ end of the gene, and cloned into the scFv-expression vector pAK100His2 (see above), replacing the g3 part. The resulting plasmid was called pAK100HP15I. To fuse the 3D5 scFv sequence to AP, the following amino acid sequence was chosen: ...TLVTVSAASGAEGGGSEGGGSGTPEMP.... The first underlined sequence is the C-terminal part of the scFv, followed by the linker based on the g3 protein domain linkers of fd-phage, and the next underlined sequence represents the N-terminal part of mature AP.

BIAcore® Experiments

The peptide KGGHHHHH was coupled to the sensor chip using an amine coupling kit (Pharmacia AB Biosensor, Uppsala, Sweden). The standard buffer was Pharmacia's HEPES-buffered saline (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl and 0.05% surfactant P20, pH 7.4). To measure the pH dependence of the binding of the anti-

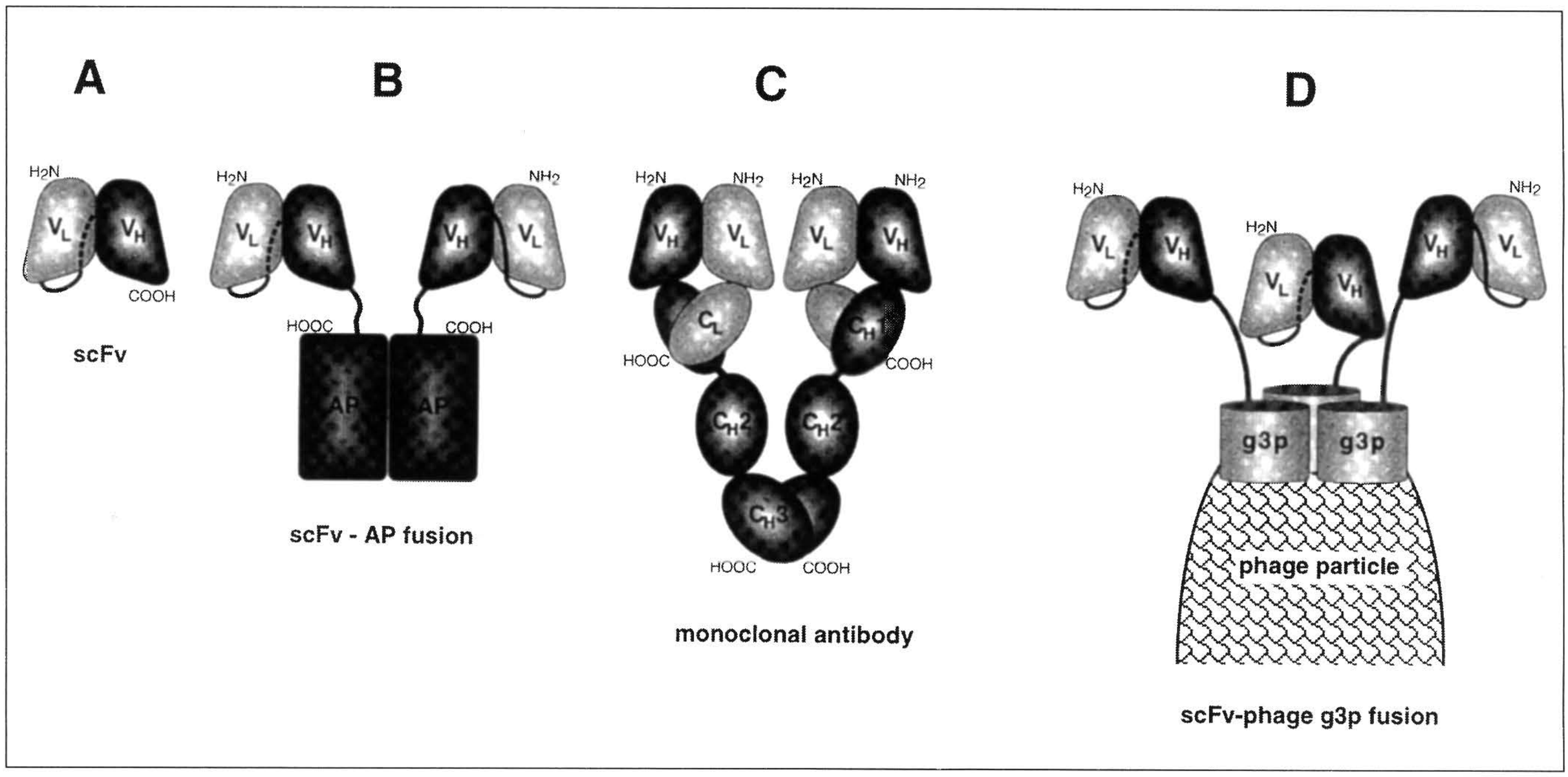

Figure 1. Schematic overview of anti-His tag formats. A) scFv fragment; B) scFv-AP fusion; C) whole antibody; D) phage with scFv-Δg3p fusion displayed. Note that the number of g3p on the phage is not precisely known, and the numbers three to five have been proposed.

body, acetate buffer (20 mM sodium acetate and 150 mM NaCl; pH 6.0), PBS (20 mM sodium phosphate and 150 mM NaCl; pH 7.0), HBS (20 mM HEPES and 150 mM NaCl; pH 7.4), borate-buffered saline (BBS: 20 mM boric acid and 150 mM NaCl, pH 8.2) and 50 nM MAb 3D5 were used. In the case of the immobilized peptide, the surface was regenerated by injection of 10 µL guanidinium hydrochloride (6 M, pH 6.0). Binding constants were determined by the competition BIAcore method (20), in which antibody and ligand are preincubated for 30 min at 10°C before injection. The binding constant was calculated from plots of k_{obs} vs. inhibitor concentration (20). In the experiments with immobilized 3D5, standard amine-coupling chemistry was used.

FACS Analyses

Binding studies were carried out in a FACScan™ cytometer (Becton Dickinson, San Jose, CA, USA). Thy-1.2-specific Fab fragments carrying a His tag were bound to T cells from C57BL/6 lymph nodes or onto EL-4 thymoma cells (29). The fragments were detected by fluorescein isothiocyanate (FITC)-coupled rat anti-mouse kappa (HB58; ATCC, Rockville, MD, USA) or unlabeled 3D5 which was detected by FITC-conjugated rat anti-mouse IgG2a/2b (HB90; ATCC) or FITC-coupled 3D5. B cells were counterstained by phycoerythrin-conjugated anti-B220 (Medac, Hamburg, Germany).

Protein Gels and Western Blotting

To test the specificity of the antibody constructs, we used crude extracts of *E. coli* cells expressing C-terminally His-tagged forms of the tryptophan synthase β-subunit, the *E. coli* co-chaperone GroES, a scFv of an anti-β-lactam antibody, the two N-terminal domains of phage M13 gene3 protein, mature yeast citrate synthase carrying an N-terminal $(His)_5$ tag [$(His)_5$-CS] or the same enzyme carrying $(His)_5$ tags on either end [$(His)_5$-CS-$(His)_5$]. Variable equivalents of *E. coli* extract, depending on the expression level of the individual proteins, were loaded onto sodium dodecyl sulfate (SDS) polyacrylamide gels and blotted (32) onto a polyvinylidene fluoride (PVDF) membrane (Immobilon™-P; Millipore, Bedford, MA, USA). The blotting membrane was blocked with 5% milk powder (low fat) in a slightly modified Tris-buffered saline ("high TBST": 50 mM Tris-HCl, 500 mM NaCl and 0.2% Tween® 20, pH 7.0) for 30 min, washed 3 times with high TBST and incubated with the anti-His tag antibody in its various formats (see below) in high TBST with 0.5% milk powder for 90 min at room temperature.

The bound anti-His MAb 3D5 was detected by a 45-min incubation of goat $F(ab')_2$ anti-mouse IgG, coupled to HRP (Pierce Chemical, Rockford, IL, USA), in high TBST containing 0.5% milk powder. Luminescence was detected with the ECL™ kit (Amersham, Arlington Heights, IL, USA), or color was developed with BM blue peroxidase precipitating substrate (Boehringer Mannheim, Mannheim, Germany).

For the oligovalent scFv-displaying phage, *E. coli* XL1-Blue cells containing the appropriate DNA were grown overnight at 30°C (2× YT-medium, 1% glycerol, 15 µg/mL tetracycline, 25 µg/mL chloramphenicol), leading to approximately 10^{11} phages/mL. Five milliliters of the phage supernatant were diluted 1:10 into high TBST/0.5% milk powder and were applied directly to the blot membrane. Detection of the phage particles was carried out using an HRP/Anti-M13 antibody conjugate (Pharmacia Biotech).

For the anti-His tag-scFv-AP fusion, an *E. coli* culture harboring the expression plasmid pAK100HP151 was induced overnight with IPTG, and the cell pellet was resuspended in 0.005 vol of TBS buffer, pH 7.0, and homogenized twice with a French Press (Aminco, Urbana, IL, USA). The supernatant was

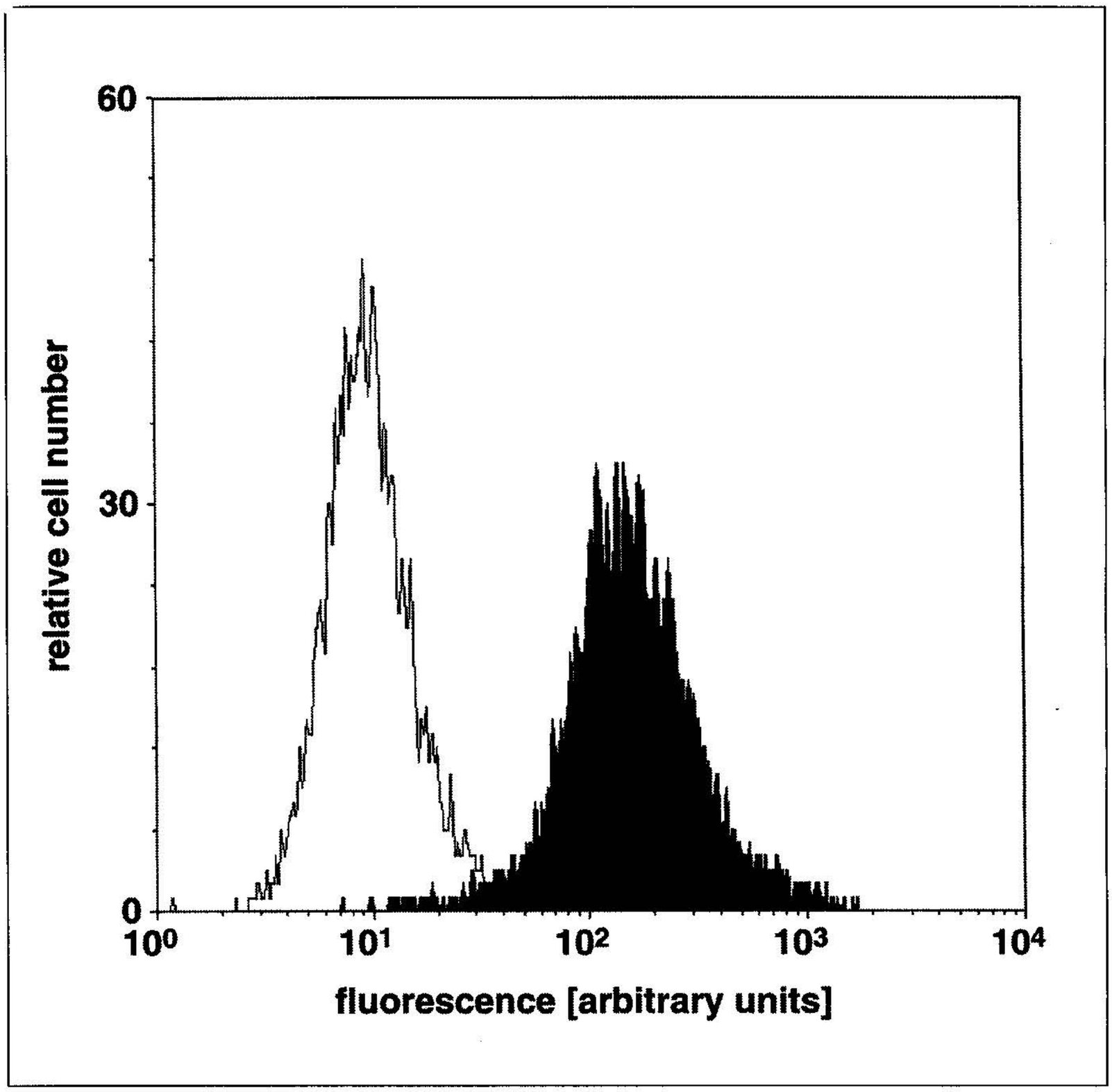

Figure 2. Detection of a cell-bound His-tagged antibody fragment by FACS. EL-4 thymoma cells were coated with Fab-MmT1-His, which was detected with anti-His MAb 3D5 and FITC-conjugated rat anti-mouse IgGFc. For the control (open histogram), cells were incubated only with 3D5 and anti-mouse-IgGFc-FITC.

aliquoted and stored at -20°C. Upon use, the extract was diluted 1:500 into high TBST buffer, pH 7.0, with 0.5% milk powder and incubated on the blot for 90 min using approximately 0.3 mL TBST solution per cm^2 blotting membrane. For luminescence detection, a mixture of 170 µL CSPD® (substrate for AP) and 1 mL amplifier (Tropix, Bedford, MA, USA) in 10 mL assay buffer (100 mM ethanolamine and 1 mM $MgCl_2$, pH 10.0) was added, while colored bands were generated with the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) according to standard protocols (2,27).

ELISA

The ELISA plate wells were coated with 10 µg of purified $(His)_5$-CS or CS-$(His)_5$ in 100 µL PBS buffer and blocked with 5% milk powder in PBS. After washing, 100 µL of the different detection reagents were added and incubated for 1 h at RT. After four washing steps, we added to MAb 3D5 (hybridoma supernatant diluted 1:250 to 1:50 000) 100 µL of a 1:2000 dilution of goat F(ab′) anti-mouse IgG conjugated to HRP (Pierce Chemical). In the case of phages, an *E. coli* culture supernatant with approximately 10^{11} oligovalent anti-His tag scFv displaying phages per mL (diluted 1:10 to 1:5000) was detected with 100 µL of a 1:5000 dilution of an anti-M13-HRP conjugate (Pharmacia Biotech). For the *E. coli* extract containing the anti-His tag scFv-AP fusion (diluted 1:10 to 1:10 000), the AP activity of bound scFv-AP fusion was detected directly by adding 100 µL/well of substrate solution (20 mg di-sodium 4-nitrophenyl phosphate hexahydrate in 10 mL 0.1 M glycine, 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, pH 10.4). After five washing steps, bound HRP-labeled antibodies were detected with 100 µL BM-blue substrate, soluble (Boehringer Mannheim). To minimize background, all dilutions and incubation steps were done in PBS containing 2% milk powder.

Molecular Modeling

A model of the anti-His tag Fv fragment was generated by homology modeling using the InsightII (Version 2.3.5) Software Package from Biosym/MSI (San Diego, CA, USA). For energy minimization, the Discover Software (V.94.0) in conjunction with the CFF91 force field was used (Biosym/MSI). The sequence of the V_L domain of the anti-His tag antibody was found to be very similar (92% sequence identity, no insertions or deletions) to that of the V_L domain of the anti-cholera toxin antibody TE33 (1TET) (30), which was used as a modeling template. The V_H domain model was predominantly based on the structure of the anti-digoxigenin antibody 26-10 (1IGI) (9), to which it showed 75% sequence identity. However, the length of the V_H CDR III loop of the anti-His tag antibody 3D5 was only three amino acids, compared to eight in the CDR III of the antibody 26-10, and the former showed no significant sequence homology to any CDR III of similar length in the Brookhaven Protein Databank (PDB). Therefore, the CDR III loop was modeled by a conformational search. The best initial conformation found was close to that of the CDR III of the unliganded form of the anti-HIV-peptide antibody 50.1 (1GGB) (26). Upon further examination of the initial model, it became obvious that changing the conformation of this loop to the conformation of the liganded form of antibody 50.1 would lead to the formation of a deep binding pocket that would be ideally suited to bind a protonated histidine side chain. The pentahistidine was built into this structure.

RESULTS AND DISCUSSION

The generation of MAbs against the His tag may be hampered by the fact that a His peptide is not always sufficiently immunogenic when delivered as a part of a soluble fusion protein (19) (E. Kremmer, unpublished results). For example, a hexahistidine-bearing malaria vaccine peptide was reported to elicit no immune responses against the His moiety in animal models (31). Therefore, we constructed a His-tagged antibody Fab fragment with a cell-binding specificity. The recombinant Fab fragment was cloned (18) from the MAb MmT1 (16), which binds the murine pan T-cell antigen Thy-1.2. It was expressed in *E. coli*, purified on an IMAC column and its T-cell binding activity was verified by FACS analyses (not shown). For immunization, the fragment was coated onto C57BL/6 thymocytes and injected into C57BL/6 mice. Even though the Fab fragment was of murine origin, it may elicit anti-idiotypic responses; and, to search for specificities that recognize the His tag independent of the protein context, hybridoma supernatants were primarily screened by ELISA for binding to His-tagged yeast citrate synthase (17). Positive ELISA signals were confirmed by testing binding to immobilized His-tagged scFv or Fab of the unrelated murine antibody McPC603 and the Fab MmT1-$(His)_5$, which had been used for immunization. The hybridoma 3D5 (isotype IgG2b) was selected for further analysis.

The specificity and utility of the anti-His tag antibody 3D5 were clearly demonstrated by FACS analysis, where murine T cells (which carry the Thy-1.2 surface antigen) (25) were incubated with the recombinant His-tagged anti-Thy-1.2 Fab fragment and subsequently with the anti-His antibody. Detection of the latter was carried out using a fluorescein-labeled anti-Fc antibody. A specific signal was obtained only in the presence of the His-tagged Fab fragment (Figure 2). Direct FITC labeling of the 3D5 antibody was also found to lead to a specific signal in the FACS analysis (data not shown).

We then tested binding of the monoclonal antibody with the BIAcore instrument, using a chip derivatized with the peptide KGGHHHHH coupled at the amino groups of the N-terminal lysine; its C-terminus was free. The maximal response was 1000 RU (resonance units). To obtain quantitative affinity data and to eliminate any artifacts due to rebinding or bivalency, we measured the competition for antibody binding between immobilized and injected ligand (10,20). We determined a monomeric dissociation constant of 4×10^{-7} M. The functional affinity (avidity) can be greatly increased by multimerizing the scFv (see below and Figure 1), which is particularly useful in surface binding assays. We found that imidazole can be used as a convenient eluent for regeneration of the sensor chip surface (as well as for affinity chromatog-

raphy), and we determined a dissociation constant for imidazole of 4×10^{-4} M. Interestingly, we observed no binding of histidine. The binding of the peptide was then measured at different pH values (pH 6.0, 7.0, 7.4 and 8.2). No significant difference between pH 6.0 and 7.4 was visible, but at pH 8.2 only very weak binding could be detected (Figure 3A). Thus, increasing the pH provides another mild and convenient method of elution. This observation is also consistent with our molecular model, which indicates protonated histidines interacting with glutamate H93 and aspartate H50 in the antibody (see below and Figure 4).

By coupling the bivalent antibody 3D5 to the chip surface by means of its NH_2-groups, His-tagged proteins can then be bound in an oriented fashion (Figure 3, B and C). If the tagged protein is itself dimeric or multimeric, the interaction is very stable—leading to an almost flat response curve—and the binding of another protein or substrate to the tagged partner can be detected. Our results show that, after binding of $(His)_5$-CS-$(His)_5$ to the immobilized antibody 3D5, nearly no decrease of RU during the dissociation phase was visible, and even after 2000 s, only weak dissociation was detectable (Figure 3B). In Figure 3C, *E. coli* extracts containing different citrate synthase constructs were injected onto the matrix. Signals were detected from CS-$(His)_5$ and $(His)_5$-CS-$(His)_5$, whereas no binding was obtained from untagged citrate synthase and $(His)_5$-CS. The two His tags in the homodimeric CS-$(His)_5$ are already sufficient for a very slow off-rate, and the two C-terminal ones are primarily recognized in $(His)_5$-CS-$(His)_5$. These experiments show that no prior purification of the His-tagged protein is necessary for BIAcore applications when the 3D5 antibody is immobilized as a capture reagent. Furthermore, its specificity for C-terminal His tags is apparent from this experiment.

To exploit the advantages of recombinant antibody technology, the antibody 3D5 was cloned as a scFv-fragment. After sequence determination, a structural model of this anti-His tag Fv fragment was generated by homology modeling (Figure 4). In our model, the deprotonated side chain of glutamic acid H93 at the bottom of the pocket can form a hydrogen bond with the protonated imidazole side chain of the peptide. The amide proton of this histidine residue could hydrogen bond to

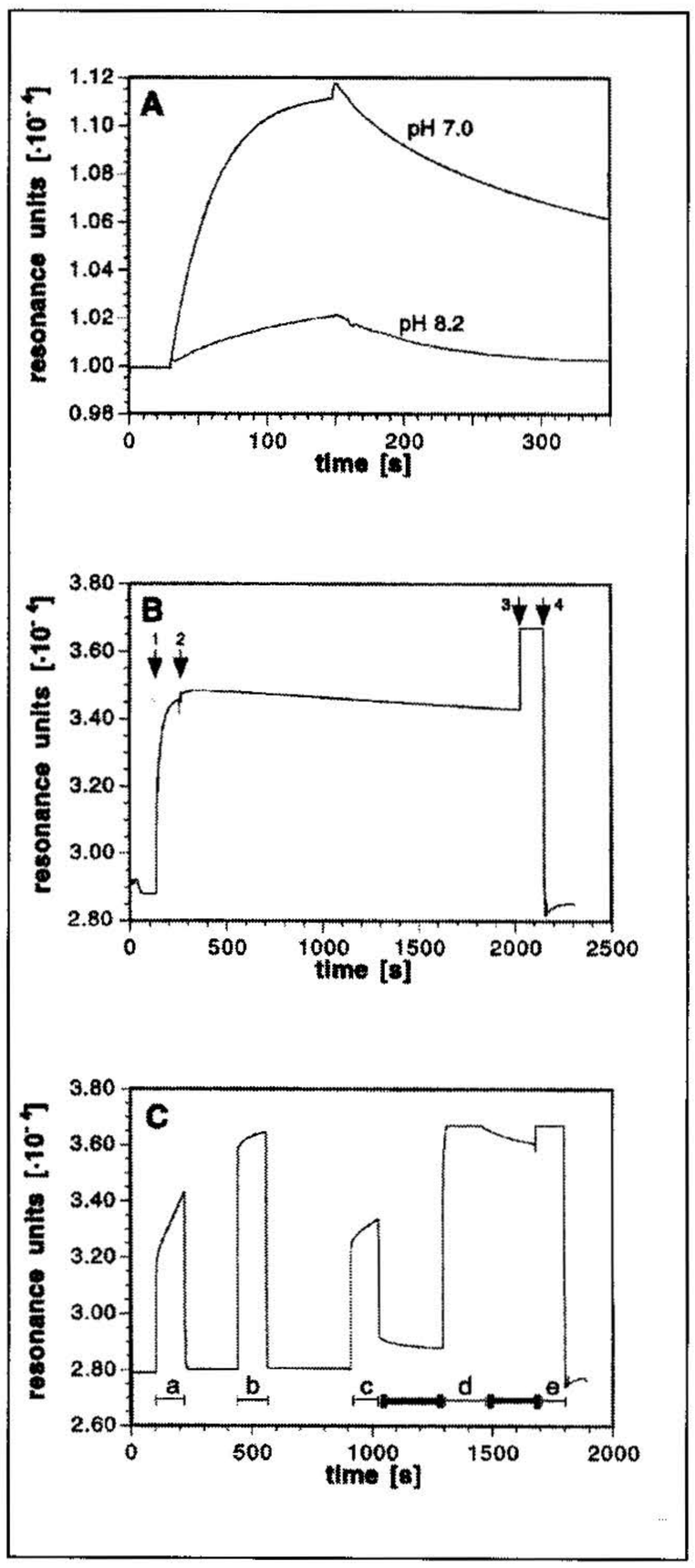

Figure 3. Binding behavior of the MAb 3D5 in BIAcore experiments. The peptide KGGHHHHH (A) or the monoclonal anti-His tag antibody (B,C) was coupled to the dextran matrix. A) pH dependence of the binding of the MAb 3D5. B) Purified $(His)_5$-CS-$(His)_5$ (50 nM) was injected (arrow 1). Almost no dissociation is detectable (arrow 2 to arrow 3) during 2000 s. Regeneration was carried out with 1 M imidazole (arrow 3 to arrow 4). C) Crude extracts of different citrate synthase constructs were injected into the sensor cell containing immobilized antibody: Phase a: $(His)_5$-CS (N-terminal His tag): no binding detectable, baseline immediately returned to zero after end of association phase (indicated by thin bar); phase b: CS (no His tag): no binding detectable, baseline immediately returned to zero after end of association; phase c: CS-$(His)_5$ (C-terminal His tag): significant binding detectable (bold bar indicates the dissociation phase); phase d: $(His)_5$-CS-$(His)_5$ (both N-terminal and C-terminal His tag): high signal was detectable (bold bar indicates the dissociation phase); phase e: regeneration was carried out with 1 M imidazole.

the glutamic acid L34 side chain. Starting from this core complex, the histidine peptide was model-built into the putative antigen binding pocket of the 3D5 Fv fragment (Figure 4). An additional histidine residue, attached to the C-terminal side of this core histidine, could be placed such that its carboxyl group forms a salt bridge with lysine L50, explaining the preference for C-terminal His tags shown by our antibody 3D5. Two additional histidine residues could be added to the N-terminal side of the core histidine in such a way that the resulting peptide is well embedded in the binding cleft of the antibody. They interact with the antibody both through main-chain and through side-chain hydrogen bonds. The side chain of the N-terminal of one of those histidine residues may form a salt bridge with the side chain of aspartate H50. A further, fifth histidine residue, attached to the N-terminus of the tetrapeptide (not shown in Figure 4), would probably start to emerge from the binding cleft but may still contribute to binding. The multiple, well-buried charge interactions between the histidine side chains of the antigen and the side-chain carboxylate groups of the antibody are consistent with the observed pH dependence of the interactions, which indicates that the His tag has to be protonated for optimal binding affinity.

The most frequently used applications for the detection of His-tagged protein lie in its use in the detection of recombinant proteins using Western blots and ELISA. To simplify and speed up those techniques, we constructed a dimeric scFv-AP fusion protein (4,33) and oligovalent scFv displaying phages (21), and compared their properties with the monoclonal anti-His tag antibody 3D5. We used crude extracts of *E. coli* cells, expressing a variety of unrelated recombinant proteins, to test the specificity of the antibody in its various forms. All proteins with C-terminal His tags of 5 or 6 consecutive histidines were specifically detected in both assays, whereas N-terminal His tags alone were not recognized (Figures 5 and 6, and data not shown). No cross-reactivity against *E. coli* proteins was seen, as no band was detected in an *E. coli* JM83 crude extract (Figure 5, lane 1). Therefore, we

conclude that recognition of His tags is specific, and that the lower bands, which are visible in lanes 2 and 4 of Figure 5, correspond to C-terminal products of citrate synthase degradation.

We found that the scFv-AP fusion protein, in particular, leads to very strong and specific signals in both detection assays (Figures 5B and 6B). Moreover, there is no need for any purification of this molecule, but rather *E. coli* lysates can be used directly for detection of His-tagged proteins. In fact, in conjunction with conventional blocking agents, the *E. coli* lysate itself blocks nonspecific binding when carrying out a Western blot of proteins expressed in *E. coli*. In addition, a one-step procedure is much faster than the traditional sandwich assay, since only a single reagent is needed. Furthermore, the reagent is stable when frozen as crude extract, or it can be stored as plasmid DNA for extended periods.

We have compared the scFv-AP approach with another recombinant strategy, namely, the use of phages that display the scFv fragment of the antibody 3D5 directly from culture supernatants (Figures 5C and 6C). Since initial investigations with a monovalent phagemid/helper phage system gave relatively weak signals (data not shown), we cloned the 3D5 scFv gene into a chloramphenicol-resistant derivative of fd-phage (15), where the wild-type gene3 was replaced by the scFv-g3 fusion. This makes fusion proteins of all copies of g3p, thus leading to oligovalent display (Figure 1) of scFv and subsequent much stronger signals. This underscores again the advantage offered by multivalency in the detection of a His-tagged protein bound to a solid phase. However, when compared to the use of the direct AP fusions in Western blots (Figure 5B), the phages were somewhat less specific and appeared to bind to the blocked membrane, resulting in an irregular background (Figure 5C), probably because the phage coat might bind by means other than by the cognate interaction. Furthermore, in this strategy a "classical" antibody-enzyme conjugate still has to be used as a detection module. Therefore, the use of scFv-displaying phages is not superior to the other two formats we have used. However, since the bound phage contains DNA, this allows for the possibility of amplifying signals by PCR techniques (28).

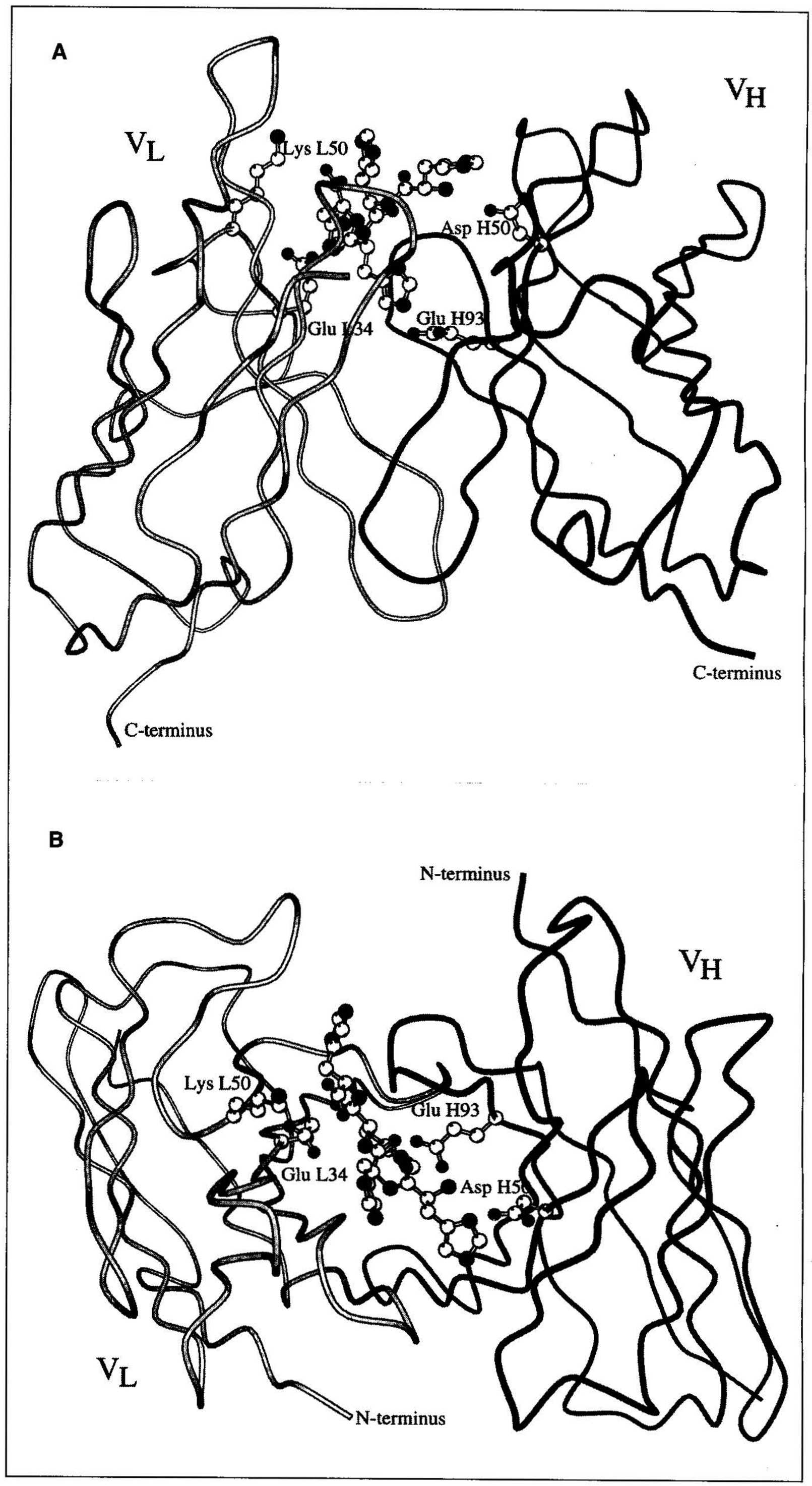

Figure 4. Model of the anti-His variable domain complexed with a $(His)_4$ peptide. The Fv structure was obtained by homology modeling based on the known structures of antibodies TE33 and 26-10. The peptide ligand was manually built into the combining site, starting with the deeply buried side chain of the histidine residue at the penultimate position. A) side view; B) top view on antigen binding site.

CONCLUSIONS AND PERSPECTIVES

The scFv-AP conjugate and the scFv-phage reagents have been shown to be highly specific and versatile tools for binding C-terminal His tags. Since these are recombinant reagents, the antibody's binding properties can be improved and altered by different methodologies using rational and random approaches.

Crude extracts of *E. coli* cells producing the recombinant protein can be used directly as the detection agent on the blot membrane or in ELISA. The anti-His tag antibody 3D5 has been shown to be sensitive and specific in various applications including FACS analysis, BIAcore and Western blots. Now that the 3D5 antibody has been produced in the form of a scFv in bacteria, it will be possible to use this scFv in the preparation of inexpensive immunoaffinity columns from which elution may be achieved under mild conditions using imidazole or pH 8.5. While IMAC is a very general and powerful technique, it does not always allow homogeneous protein to be obtained in a single step. By using the same tag with two different purification principles, proteins might be purified in a rational and predictable way independent of their nature. It should then be possible to work out a generally applicable method to produce clinical-grade material of His-tagged recombinant pharmaceutical proteins.

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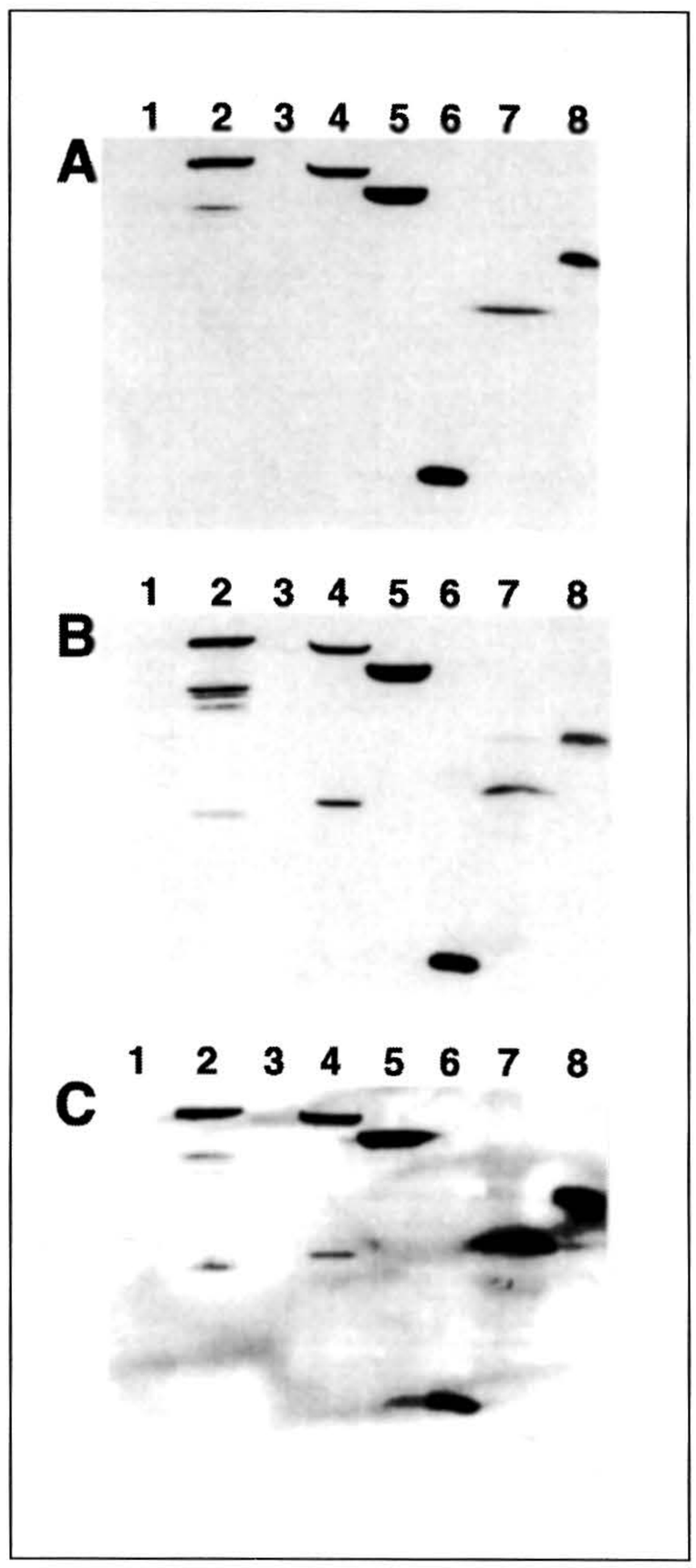

Figure 5. Western blot analysis of several His-tagged proteins expressed in *E. coli*. Variable amounts of crude extract (soluble fraction) were loaded for each protein using the same amount for each individual sample in all experiments. Lane 1, *E. coli* JM83 without plasmid; lane 2, *E. coli* W620/$(His)_5$-CS-$(His)_5$; lane 3, strain W620/$(His)_5$-CS; lane 4, strain W620/ CS-$(His)_5$; lane 5, strain W620/ tryptophan synthase-β-subunit-$(His)_6$; lane 6, *E. coli* W3110/ GroES-$(His)_6$; lane 7, *E. coli* RV308/ aL2-scFv-$(His)_6$; lane 8, *E. coli* W620/ g3p (amino acid 1-183)-$(His)_6$. A) Detection with whole anti-His antibody 3D5, from culture supernatant. B) Detection with 3D5 scFv fused to phoA-gene via 15-mer linker (V_L-V_H-AP). C) Detection with phages displaying a 3D5 scFv fused to gene3 protein in all copies. The uneven background appeared whenever phage supernatant was used as detection agent.

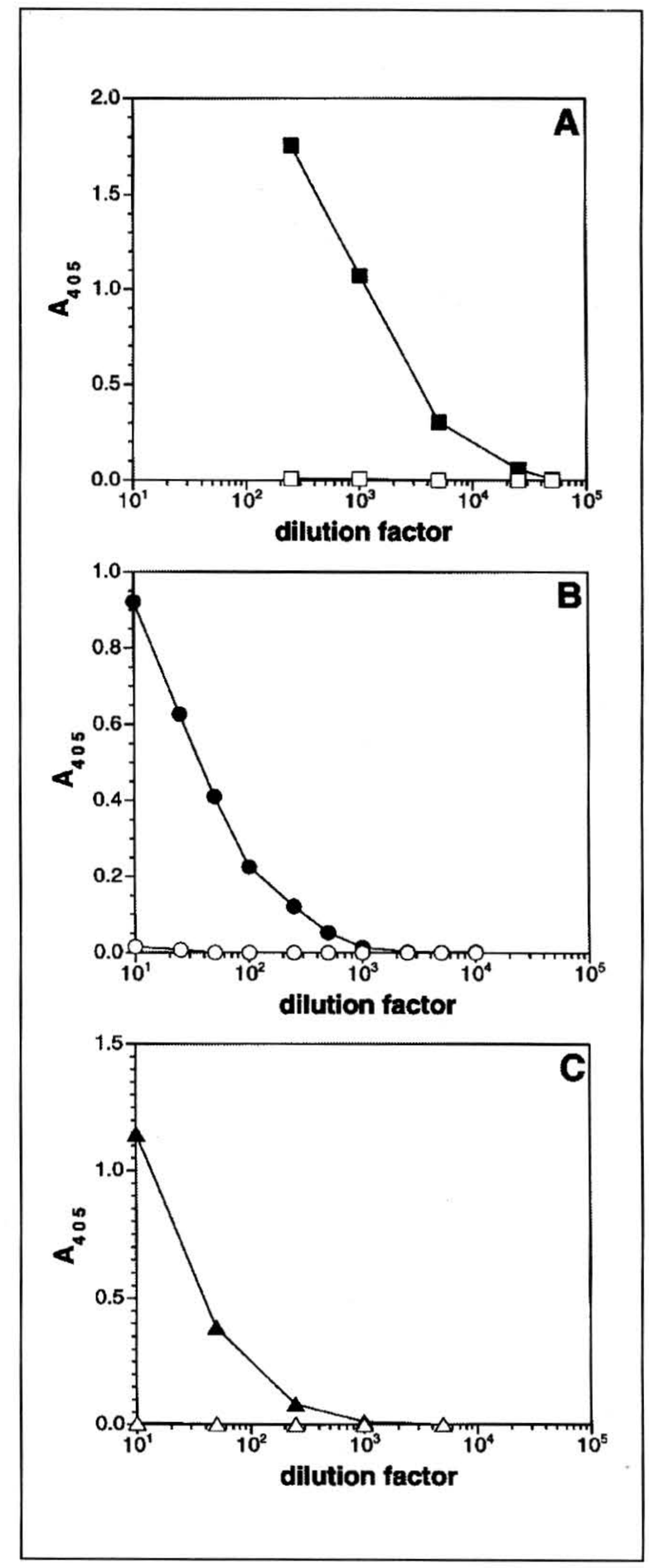

Figure 6. ELISA detection of His-tagged citrate synthase. Detection was performed by a dilution series of (A) hybridoma supernatant of monoclonal anti-His tag antibody (squares), (B) French Press extract of *E. coli* cells expressing anti-His tag scFv genetically fused to AP (circles) and (C) culture supernatant with oligovalent anti-His tag scFv displaying phages (triangles). Open symbols indicate that the His tag is fused to the N-terminus ($(His)_5$-CS), while filled symbols indicate C-terminal fusion (CS-$(His)_5$) to citrate synthase (CS). Detection was carried out (A) with a second antibody (anti-mouse IgG) conjugated to HRP, (B) directly by the fused AP activity and (C) by anti-M13-HRP conjugate.

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