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Tandem Immobilized Metal-Ion Affinity Chromatography/ Immunoaffinity Purification of His-tagged Proteins— Evaluation of Two Anti-His-Tag Monoclonal Antibodies

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A tag comprising four to six histidines genetically fused to the protein of interest (His-tag) has been widely used to purify proteins by immobilized metal-ion affinity chromatography (IMAC). Here we report the utilization of the same tag twice in series, first for IMAC and subsequently for immunoaffinity purification. Both steps are based on completely different physical principles and can therefore remove different contaminants. Two anti-His-tag antibodies (3D5 and PentaHis) were characterized for their binding and elution properties using the BIAcore surface plasmon resonance biosensor. The dissociation constant of the PentaHis antibody was determined to be 1×10^{-8} M and for the 3D5 antibody 3.4×10^{-7} M at pH 7.4. Imidazole in the sample did interfere with binding, whereas chelating agents such as EDTA and high salt did not. The antibody 3D5 was coupled to a column matrix and used for a coupled two-step purification, in which the IMAC column is eluted with EDTA and the eluent is loaded directly on the immunoaffinity column. This method may constitute a very general procedure to purify proteins to near homogeneity without the need to tailor conditions individually, and it may thus be very attractive for high-throughput screening programs and for developing general protocols for clinical grade material.

Key Words: **His-tag; IMAC; immunoaffinity chromatography; BIAcore.**

Short peptide tags genetically fused to recombinant proteins have been widely used to facilitate detection or purification without the need to develop specific procedures. The His-tag, consisting of four to six consecutive histidine residues (1), has been in use for several years to purify proteins by immobilized metal-ion affinity chromatography (IMAC[2]) (2). The histidines coordinate metals such as Ni^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+}, which are chelated by nitrilotriacetic acid (NTA) (3) or iminodiacetic acid (IDA) (4) and thus immobilized on the gel matrix.

If the His-tagged protein is highly overexpressed in hosts such as *Escherichia coli*, is multimeric, or carries two His-tags, a one-step IMAC purification can result in sufficiently pure material for most applications. However, if the protein of interest is present only as a small fraction or a very high purity is needed, several contaminating proteins remain, which bind to the IMAC column under purification conditions and coelute. Certain proteins from *E. coli* have been identified, which might bind to IMAC columns depending on the conditions used, namely superoxide dismutase (SodA, 195 aa, 8 histidines), chloramphenicol acetyltransferase (Cat, 219 aa, 12 his), cAMP receptor protein (Crp, 210 aa, 6 his), heat-shock protein (HtpG, 624 aa, 14 his), host factor-1 protein (Hfq, 101 aa, 5 his) (H. Döbeli *et al.*, personal communication), and wondrous histidine-rich protein (Whp/SlyD, 196 aa, 18 his) (5).

Thus, there is a clear demand for a second purification step. For this purpose we employed anti-His-tag immunoaffinity chromatography, which keeps the procedure as simple and general as possible. In this strategy two different purification principles, which are based on unrelated physical interactions and thus give rise to very different contaminants, are applied sequentially, therefore removing most impurities, and a

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[2] Abbreviations used: CS, citrate synthase; DTPA, diethylenetriaminepentaacetic acid; IMAC, immobilized metal-ion affinity chromatography; mAb, monoclonal antibody; NTA, nitrilotriacetic acid, PBS, phosphate-buffered saline; RU, resonance units; scFv, single-chain Fv fragment of an antibody; IDA, iminodiacetic acid; FITC, fluorescein isothiocyanate.

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high degree of purity is obtained in a very general procedure.

Recently, several anti-His-tag monoclonal antibodies have been described, such as 3D5 (6), PentaHis (J. Ribbe, personal communication), 13/45/31 (7), and His-1/MRGs-His (8). The antibody 3D5 has also been obtained as recombinant scFv fragment and fusion protein to alkaline phosphatase as a one-step detection agent (6). We have now characterized two of them, 3D5 and PentaHis, regarding their suitability for immunoaffinity purification.

To assess the conditions for immunoaffinity purification, surface plasmon resonance measurements as implemented in the BIAcore instrument (9) were carried out, as they closely resemble the chromatographic procedure. The antibody is immobilized in a dextran layer of about 100 nm thickness, and the His-tagged protein is injected in a continuous flow. The advantages of the biosensor approach for working out chromatographic conditions, compared to sequential column runs, are the small amounts of material needed and the continuous and precise monitoring of the mass of protein binding in the dextran layer. The so-called regeneration of the surface equals the elution from the column. The mass in the dextran layer is given in resonance units (RU), which can be converted approximately according to 1 RU $\approx$ 1 pg/mm^2.

The characterization of the antibodies is important for determining their suitability for other applications as well, such as enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), and immunofluorescence. If a His-tagged protein is used in these techniques as the first reagent, e.g., in the form of an antibody fragment, a general detection scheme employing the characterized anti-His-tag antibodies may become possible. We have therefore studied the stability of the complexes for the correct application of the anti-His-tag antibodies.

MATERIALS AND METHODS

Proteins. The murine mAb 3D5, isotype IgG2b (6), was purified from hybridoma supernatant by protein A affinity chromatography (Hi-trap, Pharmacia). Lyophilized PentaHis antibody, isotype IgG1, was kindly provided by Dr. J. Ribbe, Qiagen GmbH. The antibody 3D5 is now available from Invitrogen, USA, and the PentaHis antibody from Qiagen, Germany.

Three His-tagged proteins were used as analytes for BIAcore measurements with immobilized anti-His-tag antibody. First, the single-chain Fv fragment FITC-E2-His (10) carrying a C-terminal His_5-tag; second, yeast citrate synthase (CS), which is a homodimeric protein with two N- terminal His_5-tags, named $(His\text{-}CS)_2$ and third, citrate synthase with N- and C-terminal His-tags, named $(His\text{-}CS\text{-}His)_2$ (11), were tested. In the scFv fragment FITC-E2 the His-tag is fused via a linker to the C-terminus resulting in the sequence FITC-E2-GSSGGAPHHHHH; in the citrate synthase, the His-tags are directly linked to the enzyme resulting in the sequence HHHHH-SSAS . . . Citrate Synthase . . . ESKN-HHHHH.

Immunoaffinity chromatography was carried out with two miniantibodies as the proteins to be purified, named M1dhlxh6 (12) and 425dhlxh6 (13), which are homodimerized single-chain Fv fragments. In both molecules, a His_6-tag is fused to the C- terminus via a linker resulting in the sequence scFv-GGSGGAPGHH-HHHH.

Biosensor measurements. Surface plasmon resonance (SPR) measurements were carried out with a BIAcore instrument (BIAcore, Sweden). Antibodies were immobilized in 10 mM Na-acetate at pH 4.5 on a CM5 sensor chip (BIAcore) using standard EDC [1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide-HCl] and NHS (*N*-hydroxysuccinimide) coupling chemistry and subsequent blocking with ethanolamine. Running buffer was 50 mM Na-phosphate, 150 mM NaCl, 0.005% Tween 20 at pH 7.4. Varying coating densities were obtained by varying the anti-His-tag antibody dilution as well as the injection time. For analysis typically a sample volume of 20 μl was injected at a flow rate of 20 μl/min.

Immobilized metal-ion affinity chromatography. Three different IMAC gel matrices were tested: Ni-NTA (nitrilotriacetic acid)–agarose (Qiagen, Hilden, Germany), Ni-NTA–Superflow (Qiagen), and Ni-IDA (iminodiacetic acid)–Fractogel–Chelate 650 (Merck, Darmstadt, Germany). Imidazole elution tests showed that His-tagged proteins bound strongest to the IDA–Chelate 650 gel and weakest to the NTA–agarose (data not shown). The intermediate NTA–Superflow material was chosen for further experiments. Running buffer was either TBS [50 mM Tris (tris-(hydroxymethyl)-aminomethane); 150 mM NaCl, 0.005% Tween, pH 7.4] or PBS (50 mM Na-phosphate, 150 mM NaCl, 0.005% Tween, pH 7.4). Three elution agents were tested: imidazole, EDTA (ethylenediaminetetraacetic acid), and DTPA (diethylenetriaminepentaacetic acid). Elution with pH was not tested, as it might have inactivated the protein. Cleared, 0.22-μm filtered cell lysates were applied in PBS with 10 mM imidazole, and washing steps of 15 column volumes each were performed at 10 and 25 mM imidazole. A concentration of 250 mM imidazole eluted all protein, whereas EDTA and DTPA had to be applied in concentrations of 400 mM to give focused peaks. Especially the NTA–Superflow material showed a tendency for peak tailing. EDTA and DTPA elutions were directly applied to immunoaffinity purification, whereas imidazole was first removed by dialysis. It is interesting to note that DTPA

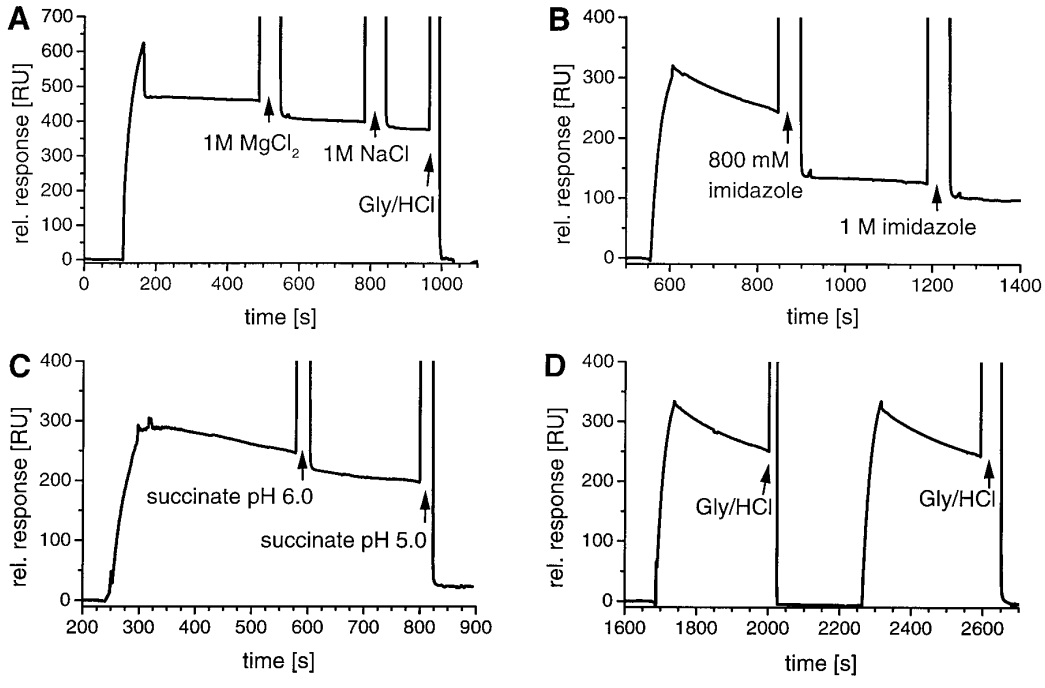

FIG. 1. Sensorgrams of $(His\text{-}CS)_2$ (A) and scFv FITC-E2-His (B–D) binding to immobilized PentaHis mAb coated to 2300 RU. Protein was injected in PBS buffer, pH 7.4, which was also the running buffer. The sensorgrams demonstrate the stability of the interaction after injection of 20 μl of various agents (shown by arrows). (A) 1 M $MgCl_2$, 1 M NaCl, and Gly/HCl injections; (B) 800 mM imidazole in PBS buffer and 1 M imidazole at pH 7.4; (C) 50 mM succinate, 500 mM NaCl at pH 6.0 and pH 5.0, respectively; (D) 100 mM glycine/HCl, 500 mM NaCl, pH 4.0.

was capable of resolving several peaks, when applied in a step gradient.

Immunoaffinity chromatography. About 3 mg of antibody 3D5 was purified by protein A affinity chromatography and crosslinked to Affi-Hz (Bio-Rad, USA) hydrazide gel via the carbohydrate moieties. After oxidation with $NaIO_4$ for 1 h, the desalted antibody was coupled overnight. The column was stored in PBS buffer with 0.02% NaN_3 at 4°C for up to 1 year, so far with no detectable loss of activity. All solutions for chromatography were sterilized by filtration through a 0.22-μm-pore-size filter.

RESULTS

While several buffers and methods have been described for IMAC and immunoaffinity purification, we restricted the vast choice of reagents and conditions to those which are likely to be of general utility. Using surface plasmon resonance measurements with the BIAcore instrument we determined under which conditions the antibody binds His-tagged proteins, how stable the interaction is, and under which conditions the protein can be eluted. As the immunoaffinity purification might most likely be used as a cleanup after IMAC, the typical elution conditions from IMAC are taken into account for sample application on the immunoaffinity column.

The standard running buffer was PBS, pH 7.4, in which all His-tagged proteins were bound. Two model proteins were tested: yeast citrate synthase, which is a homodimer carrying an N-terminal His-tag on both subunits, named $(His\text{-}CS)_2$, or carrying both N- and C-terminal His-tags resulting in a total of four His-tags, named $(His\text{-}CS\text{-}His)_2$; and a monomeric single-chain Fv fragment directed against fluorescein, named FITC-E2-His, which carries a single C-terminal His-tag.

The Antibody PentaHis

Conditions for association. The antibody PentaHis binds His-tags irrespective of whether they are fused to the N-terminus, as in the case of the citrate synthase $(His\text{-}CS)_2$ (Fig. 1 A), or to the C-terminus, as in the case of the antibody scFv fragment FITC-E2-His (Figs. 1B–1D). The effect of various agents on the association was analyzed by incubation of the His-tagged protein prior to injection and comparing the signals obtained at the end of association or at the start of dissociation of identical injections (Fig. 2, Table 1). Concentrations as high as 200 to 250 mM imidazole are frequently used for elution in IMAC. To test how imidazole added to the sample (which would be present, if the sample was directly taken from IMAC) affects binding, the same amount of the scFv FITC-E2-His was incubated with increasing amounts of imidazole (10, 20, 50, and 100

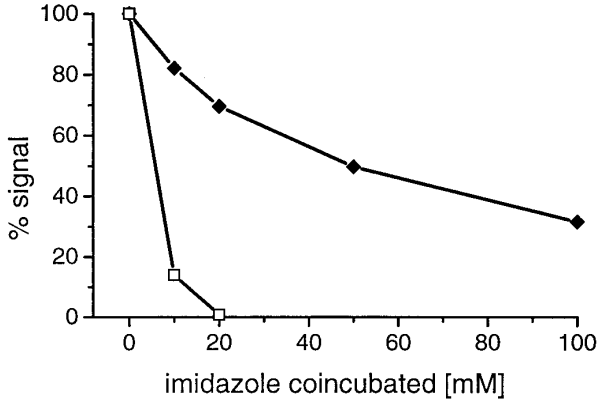

FIG. 2. Relative final signal of 20 μl 500 nM scFv FITC-E2-His binding to PentaHis mAb, coated to 2300 RU (filled diamonds) and 3D5 mAb, coated to 8400 RU (open squares), in the presence of increasing concentrations of imidazole (0, 10, 20, 50, and 100 mM). Prior to injection the protein was coincubated with imidazole for 5 min. Signals were measured at the start of dissociation for the PentaHis antibody and at the end of injection for the 3D5 antibody and were corrected for the bulk effect on a nonderivatized surface. Signals are given relative to the resonance units reached in the absence of imidazole.

mM). Figure 2 (closed diamonds) shows the relative amount of bound protein at the end of the association phase. Already at low concentrations of imidazole binding was effectively prevented. At 50 mM imidazole only half the amount, compared to the absence of imidazole, bound to the PentaHis antibody. The effect of NaCl, EDTA, DTPA, and higher pH was analyzed on a surface with 1600 RU PentaHis antibody coated, measuring the signal directly at the start of dissociation, since the huge bulk effect from the large refractive index change in the buffers prohibits analysis during association. To examine the effect with a tightly binding protein, $(His\text{-}CS)_2$ with two His-tags and therefore a very slow off-rate was used for coincubation (Table 1).

Thus, under typical conditions for elution from IMAC with a chelating agent such as EDTA or DTPA and high salt, binding of $(His\text{-}CS)_2$ to the anti-His-tag antibody is not affected. However, at slightly higher pH values, binding is weaker. If the flow rate is reduced from 20 to 5 μl/min, when the binding switches from an association-rate-limited to a mass-transport-limited interaction, the difference is less pronounced. At a flow rate of 5 μl/min, injection with PBS, pH 7.9, resulted in 86% of the signal compared to the same injection at pH 7.4. This indicates that both association and dissociation are slowed.

Stability of the interaction and elution conditions. Two His-tags in the analyte result in highly stable binding with nearly no detectable off-rate (Fig. 1A) on a "medium"-density anti-His-antibody surface, for which antibody was coated at a density of 2300 RU. As seen in this figure as well, the interaction is stable at high salt concentrations of 1 M NaCl or 1 M $MgCl_2$. This is also true for binding of the monomeric scFv FITC-E2-His (data not shown). Imidazole, which reduced association, was also tested as an eluent for protein bound to the PentaHis antibody (Fig. 1B). At a flow rate of 20 μl/min 40 μl of 800 mM imidazole in PBS or 1 M imidazole at pH 7.4 was injected. During the injection of 800 mM imidazole some limited elution of the $(CS\text{-}His)_2$ occurs. If small amounts of His-tagged protein are present, i.e., under conditions of rebinding, and bivalent binding to the PentaHis antibody is likely, imidazole is an ineffective elution agent. Another method for elution is to decrease the pH. Three pH values were tested: a succinate buffer (50 mM succinate, 500 mM NaCl) at pH 6.0 and pH 5.0 (Fig. 1C) as well as a glycine/HCl buffer (100 mM glycine, 500 mM NaCl) at pH 4.0 (Fig. 1D). Succinate at pH 6.0 has nearly no effect, whereas injection of succinate buffer at pH 5.0 results in nearly complete elution. Most effective is glycine/HCl, which results in a complete regeneration of the surface. As demonstrated in Fig. 1D, the surface can be reused shortly after such a regeneration without any loss in capacity. One surface could be reused at least 10 times without any change. However, it is noteworthy that performance of the antibody PentaHis immobilized on a sensor chip dropped significantly after drying and storage for several days (data not shown). The relatively slow dissociation from immobilized PentaHis antibody and its successful regeneration allow the use of this antibody as a general tool for directed immobilization of His-tagged proteins.

Determination of the kinetic parameters. Binding kinetics of the PentaHis antibody were determined with a series of concentrations (100, 200, 400, 800, and 1600 nM) of monomeric scFv FITC-E2-His injected at a high flow rate (30 μl/min) onto a surface with a low antibody density coated (1150 RU) to prevent rebinding (Fig. 3A). All sensorgrams obtained were globally fitted, including the association and dissociation phase at all concentrations at the same time, to an integrated first-order reaction scheme using BIAevaluation 3.0 software (BIAcore). The fit was not significantly im-

TABLE 1

Effect of Coincubated Chemicals on the Relative Signal of $(His\text{-}CS)_2$ Binding to the Immobilized Antibody PentaHis at the Start of the Dissociation Phase

Coincubated substance	Signal relative to PBS, pH 7.4 (%)
1 M NaCl	100
400 mM EDTA	100
200 mM DTPA, pH 7.6	91
BBS buffer, pH 8.0	53
PBS buffer, pH 7.9	71

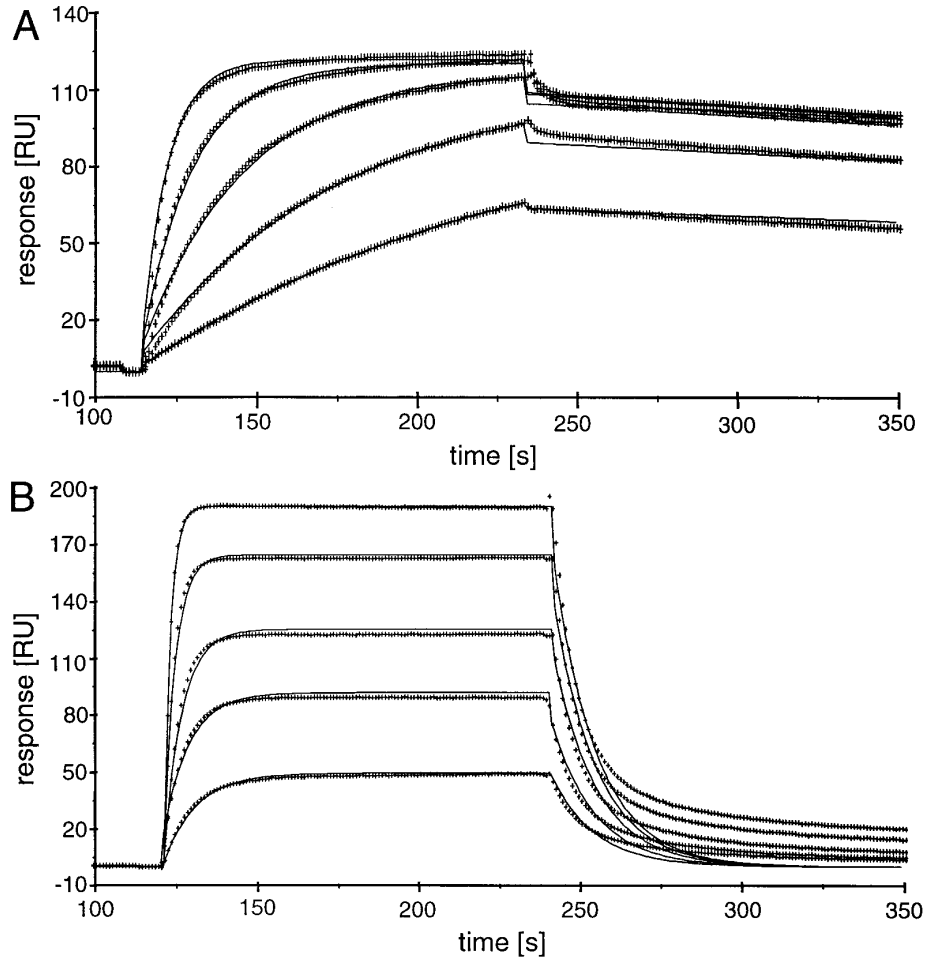

FIG. 3. Determination of binding kinetics of scFv FITC-E2-His to PentaHis mAb immobilized to 1150 RU (A) and mAb 3D5 immobilized to 1100 RU (B). A series of concentrations of 100 nM (bottom curve) and 200, 400, 800, and 1600 nM (top curve) of scFv FITC-E2-His was injected at a flow rate of 30 μl/min. Kinetics were evaluated using a global fit (BIAevaluation 3.0 software) with a simple first-order reaction. The measured data (+) and the fitted curve (—) are shown.

proved when rebinding was taken into account (data not shown). Rate constants of $k_a = 7.7 \times 10^4\ M^{-1}\ s^{-1}$ and $k_d = 7.2 \times 10^{-4}\ s^{-1}$ were obtained resulting in a dissociation constant of $K_d = 9.4 \times 10^{-9}$ M with a χ^2 of 2.33 for this protein at pH 7.4. For the presented curves part of the bulk effect has been corrected manually, as the BIAcore instrument used does not allow automatic reference surface correction.

The Antibody 3D5

It has been shown previously that the antibody 3D5 captures proteins with two His-tags effectively at pH 6.0 and 7.4, but not at pH 8.0, and that 1 M imidazole is an efficient elution agent. It had also been demonstrated that only C-terminal His-tags are recognized (6).

Conditions for association. The influence of various chemicals added to the His-tagged protein (His-CS-$His)_2$ during association was tested in the same way as for the PentaHis antibody. Imidazole added to the His-tagged protein prevented binding, as only 1% was bound in the presence of 20 mM imidazole (Fig. 2, open squares). Concentrations of 200 mM DTPA or 400 mM EDTA at pH 7.4 added to the sample had no effect on association as they led to the same final resonance units as pure PBS buffer and are therefore suitable eluents for IMAC.

Stability of the interaction and conditions for elution. To address the difference in binding between monovalent and bivalent His-tagged molecules, the monomeric scFv fragment FITC-E2-His (Fig. 4A) and the dimeric citrate synthase with C-terminal His-tag (Fig. 4B) were compared, both at a monomer concentration of 500 nM. To measure the influence of rebinding, measurements were performed on four surfaces with increasing amounts of immobilized 3D5 antibody (1100,

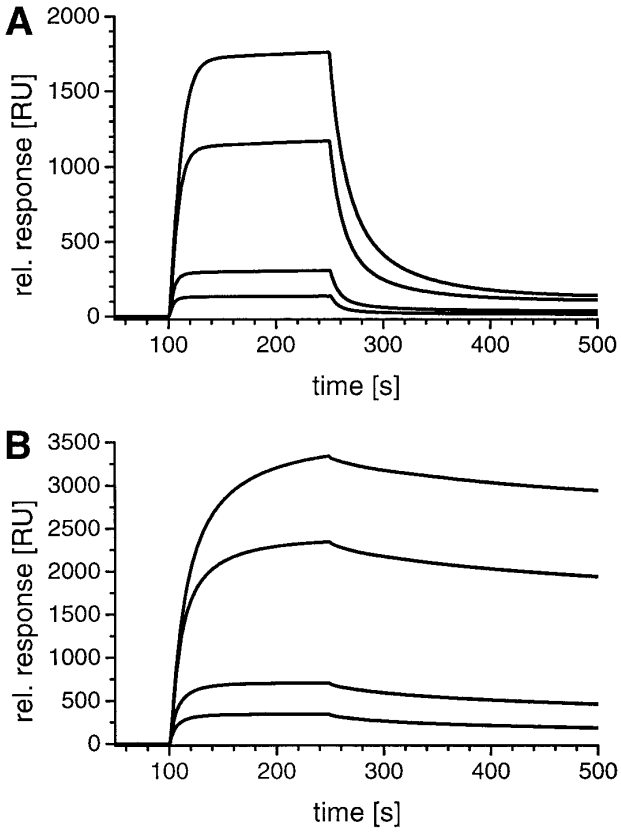

FIG. 4. Sensorgrams of identical concentrations of monomeric scFv FITC-E2-His (A) and dimeric (His-CS-His)$_2$ (B), both at a monomer concentration of 500 nM binding to 3D5 antibody immobilized at increasing surface densities (1100, 2400, 6770, and 10045 RU) (bottom to top).

2400, 6770, and 10045 RU). The difference in binding between monovalent and bivalent His-tagged molecules was dramatic and was growing more pronounced with increasing immobilized antibody density. For the monomeric protein at low coating densities, the off-rate was so high that no regeneration was required at pH 7.4. For the monomeric protein at the highest coating density and for dimeric protein at the lowest coating density, a 5-μl pulse of Gly/HCl, pH 4.0, was sufficient for regeneration, whereas at the highest coating density for the dimeric protein even a 20-μl pulse was not sufficient. For the regeneration Gly/HCl was compared with increasing imidazole concentrations, using citrate synthase with the C-terminal His-tag as a model protein and a surface coated with 6700 RU. The same procedure was used for all regeneration tests. Starting from a signal of 1400 RU, treatment with 20 μl Gly/HCl, pH 4.0, resulted in a remaining signal of 38 RU, while after 200 mM imidazole 48 RU remained, after 400 mM imidazole 9 RU remained, and after 1 M imidazole 0 RU remained.

Determination of the kinetic parameters. The rate constants for immobilized 3D5 binding to His-tagged proteins were determined at a low coating density of 1100 RU in the same way as with the PentaHis antibody (Fig. 3B). The second part of the dissociation phase did not fit to a first-order reaction, most likely due to rebinding and perhaps even due to some dimerized scFv molecules. Therefore, only the first 20 s of the dissociation phase was considered for the fit. Rate constants $k_a = 2.2 \times 10^5\ M^{-1}\ s^{-1}$ and $k_d = 0.075\ s^{-1}$ were obtained, resulting in an equilibrium constant of $K_d = 3.4 \times 10^{-7}$ M at pH 7.4. This is in good agreement with the value determined by immobilizing the His-tag and varying the antibody 3D5 as analyte (6).

Immunoaffinity Chromatography

About 3 mg of the antibody 3D5 was coupled via the carbohydrate moiety to a hydrazide-derivatized gel matrix and used for immunoaffinity chromatography. The goal was the purification of miniantibodies, which consist of a single-chain Fv fragment with a fused dimerization domain and a C-terminal His-tag and thus carry two His-tags (13, 14). These miniantibodies are functionally expressed in the periplasm of *E. coli*. Prior experiments had demonstrated that whole-cell disruption is most effective in releasing the recombinant protein, but results in a large amount of impurities. For well-expressed miniantibodies this is not a problem, as they carry two His-tags and can be purified to near homogeneity by a single IMAC step. However, to purify molecules which are poorly expressed to homogeneity from crude lysates, a two-column strategy is needed.

As the antibody 3D5 was shown to bind proteins with two His-tags quite well and as relatively mild elution conditions of 1 M imidazole are available, several combined IMAC–immunoaffinity purification schemes were tested. For IMAC the Ni-NTA–Superflow material was chosen (see Materials and Methods). For elution three agents were tested: (i) elution by competing the ligation of the histidines with imidazole or chelating the metal ion with (ii) EDTA or (iii) DTPA. A concentration of 250 mM imidazole was sufficient to elute all protein. EDTA and DTPA had to be applied in concentrations of 400 mM to give focused peaks, which was also required due to the tendency of the NTA–Superflow material for peak tailing. EDTA and DTPA elutions of the IMAC column could be directly applied to immunoaffinity purification, whereas imidazole first had to be removed by dialysis.

The IMAC elution was applied to the 3-ml immunoaffinity column at 0.8 ml/min. Washing was performed in the running buffer until the absorbance was close to baseline, typically after 3 column volumes. The protein was eluted with 1 M imidazole and collected in 1-ml fractions (Fig. 5A). The chromatography also worked well when operated manually with a syringe screwed on top of the column. After dialysis against the desired buffer,

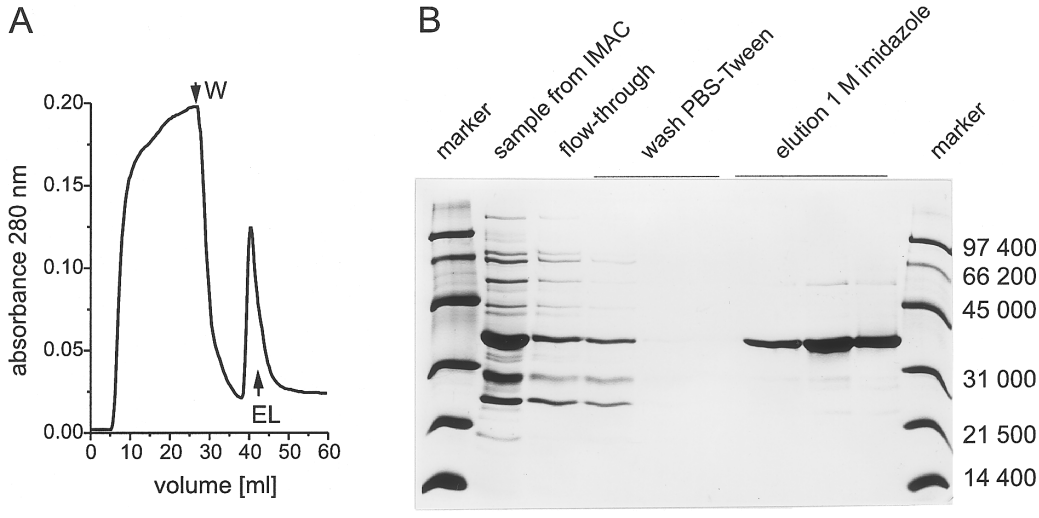

FIG. 5. Anti-His-tag immunoaffinity chromatography with immobilized antibody 3D5 subsequent to IMAC. (A) Chromatogram monitored at 280 nm of immunoaffinity chromatography of miniantibody M1dhlxh6. W denotes the start of washing buffer and EL the start of elution with 1 M imidazole. (B) Coomassie-stained SDS–PAGE of miniantibody 425dhlxh6 purified first by IMAC and subsequently by 3D5 immunoaffinity chromatography.

samples were analyzed on SDS–PAGE (Fig. 5B) and found to be efficiently purified. As determined by gel-scanning analysis, the purity after IMAC of 32% was increased to 95% after immunoaffinity chromatography.

DISCUSSION

The kinetic parameters of two anti-His-tag antibodies, named PentaHis (Ribbe *et al.*, unpublished data) and 3D5 (6), were determined as well as parameters affecting binding and elution of the His-tagged samples from the immobilized antibody. The antibody 3D5 was successfully employed for immunoaffinity chromatography.

An important question is which antibody to choose for immunoaffinity chromatography. If the protein of interest has only an N-terminal His-tag, there is no choice, as the antibody 3D5 recognizes only C-terminal His-tags. In BIAcore analysis proteins with an N-terminal His-tag generate only a transient response on the 3D5 surface, indicating at most a transient association. For proteins with a single His-tag the tight-binding speaks in favor of the PentaHis antibody, as with the 3D5 protein samples might bind too weakly at pH 7.4. On the other hand, the tight-binding necessitates harsher elution conditions, which is a clear disadvantage in the case of, e.g., pH-labile proteins. Thus, in the case of a protein with two His-tags, the weaker binding of the antibody 3D5 can be advantageous.

Unfortunately, monoclonal antibodies from commercial sources are in most cases only affordable on an analytical scale. In-house hybridoma production will thus usually be required for up-scaling of immunoaffinity columns, but in the near future recombinant antibody technology is likely to offer significantly better options. Recently, we have converted the antibody 3D5 to an scFv fragment which can be produced in *E. coli* (6). Meanwhile, analytical scale anti-His-tag immunoaffinity columns are also an interesting alternative to small IMAC columns for one-step purification due to their higher specificity.

Imidazole interferes with His-tag binding regardless of the antibody. Thus, if the immunoaffinity chromatography is performed directly after IMAC, the latter is preferably eluted with EDTA. Despite having a higher affinity for Ni^{2+}, DTPA shows no significant difference in IMAC elution, apart from a potentially higher selectivity. If the protein is pH-stable, a pH elution as acidic as possible might give the most focused peak, which, of course, needs to be titrated back to neutral before application to the antibody column. In the case of the PentaHis antibody it can be expected that prolonged washing even at high salt concentrations does not decrease the yield. In the case of the antibody 3D5, washing should not exceed a few column volumes due to the high off-rate.

In the case of the antibody 3D5 elution is best performed with an imidazole concentration higher than 800 mM. Imidazole concentrations as low as 200 mM will require additional changes in the buffer to be complete. Despite interfering with binding, imidazole even at a concentration of 1 M is not sufficient for elution from the PentaHis surface. This is most likely correlated with the slow off-rate, as dissociation is required before imidazole can interfere with binding. A glycine/

HCl buffer with 500 mM salt results in reproducible regenerations without affecting the performance of the PentaHis antibody. The high salt in the buffer has been shown to be useful for increasing effectivity in several cases (data not shown), most likely due to disruption of electrostatic interactions caused by the low pH.

The approach described in this paper presents a convenient way to evaluate affinity chromatography. The same His-tag, widely used for IMAC, was recruited a second time for a subsequent, different affinity chromatography, thus giving maximum specificity combined with highest generality and column lifetime. Since this second step can be directly used on the eluent of the first column, a very simple procedure results.

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