Double-Hexahistidine Tag with High-Affinity Binding for Protein Immobilization, Purification, and Detection on Ni–Nitrilotriacetic Acid Surfaces

Farid Khan, Mingyue He, and Michael J. Taussig*

Protein Technologies Laboratory, The Babraham Institute, Babraham Research Campus, Cambridge CB2 4AT, UK

There is a particular need in protein analysis and purification for specific, functional, and generic methods of protein immobilization on solid supports. Here we describe a double-hexahistidine (His6) tag sequence, comprising two hexahistidines separated by an 11-amino acid spacer, which shows at least 1 order of magnitude stronger binding to Ni–NTA-modified surfaces than a conventional single-His6 tag or two single-His6 tags at N- and C-termini. Using, as a model, tagged versions of green fluorescent protein (GFP), stable and tight binding of the double-His6 tag/Ni–NTA interaction was demonstrated by competitive elution from Ni–NTA agarose beads, surface plasmon resonance on a Ni–NTA chip, and ELISA in Ni–NTA microwell plates. Protein purification by Ni–NTA chromatography was improved by a 6–8-fold increase in imidazole concentration required for elution, while the dissociation rate of double-His6 GFP from Ni–NTA chips in SPR (BIAcore) was 10 times slower than for single-His6-tagged proteins. ELISA assays and protein microarrays constructed with double-His6 NTA demonstrated greater detection sensitivity with anti-His antibodies and Ni–NTA conjugates. Moreover, the double-His6 tag could serve simultaneously both for protein immobilization and for detection on surfaces. The double-His6 peptide has the potential to be a universal tag for protein immobilization and detection on arrays and single-step purification of proteins from crude mixtures.

Over the last several years, the binding of hexahistidine (His6) sequence tags to transition metal chelates of nitrilotriacetic acid (NTA) has been widely used as a powerful and universal means for affinity purification of recombinant proteins.1–3 Despite the development of other tags such as c-myc, glutathione-transferase, strep-tag, and maltose binding protein,4 the popularity of the His6 sequence lies in the rapid one-step purification of native and denatured proteins. Furthermore, it requires minimal addition of extra amino acids, can be fused at either the N- or C-termini or even within internal sites,5 and rarely alters protein folding and function.6,7 The His6 epitope is not particularly immunogenic and does not interfere with generation of antibodies against the protein of interest, while a number of high-affinity monoclonal antibodies specific to the epitope have been generated to detect fusion proteins.7,8

NTA forms a tetradentate chelate with the Ni2+ ion, although other transition metal ions with a coordination number of six can be used (e.g., Co2+, Cu2+, Zn2+). NTA occupies four of the six ligand binding sites in the coordination sphere of the metal ion, leaving two sites free to interact with the His6 sequence. Specifically, the electron donor groups on histidine imidazole rings readily form coordinate bonds with the Ni–NTA complex. The binding affinity (Kd) of single-His6 proteins for Ni–NTA-modified surfaces has been estimated as ~1 μM by surface plasmon resonance (SPR),8 while a solution-based assay using fluorescent Ni–NTA conjugates indicated that a single Ni–NTA binds to His6 with a Kd of 10 μM.9 The interaction has been exploited frequently for protein purification, e.g., using Ni–NTA coupled to agarose chromatography beads,10 the protein being eluted by an imidazole gradient (0–250 mM), decrease in pH, or chelation agents such as EDTA.

Although the His6/Ni–NTA interaction is well-suited to affinity purification, a major drawback for strong immobilization is its relatively low affinity, due to rapid dissociation, which reduces the yield of immobilized protein on Ni–NTA surfaces.9 In an effort to improve the binding characteristics of the His6 tag, we have designed model constructs in which green fluorescent protein (GFP) was fused with single-, double- or triple-His6 tags at either the N- or C-terminus or both. The double-His6 tag was originally introduced to immobilize antibody fragments and other proteins on Ni–NTA surfaces after in vitro protein synthesis.12 It consists

* Corresponding author. E-mail: mike.taussig@bbsrc.ac.uk. Tel.: +44 1223 496557. Fax: +44 1223 496045.
of two hexahistidines, separated by an 11-amino acid spacer and is fused to the protein by a flexible linker sequence. Here we report detailed binding characteristics of the double-His6 tag to Ni–NTA, demonstrating particularly tight binding as evidenced by affinity chromatography on Ni–NTA agarose beads, SPR on a Ni–NTA chip, and enzyme-linked immunosorbent assay (ELISA) in Ni–NTA microtiter wells. In addition, the double-His6 tag was detected with greater sensitivity than the single-His6 tag, using Ni–NTA conjugates and anti-His antibodies. We envisage that the double-His6 tag will find wide application for protein immobilization, single-step purification, and sensitive detection.

MATERIALS AND METHODS

Ni–NTA agarose, NTA-HRP, and Ni–NTA Hisorb 96-well microtiter plates were from Qiagen (Crawley, U.K.). Ni–NTA-coated microarray slides from Xenopore, and Nexterion amine reactive slides (slide-H) from Schott (Mainz, Germany). Imidazole, NiCl2, TMB solution, and all buffers were purchased from Sigma (Poole, UK). Biotinylated anti-GFP goat polyclonal antibody was from Abcam (Cambridge, UK). Anti-His–HRP monoclonal antibody conjugate and ProQ Saphire 532 oligohistidine gel stain (fluorescent Ni–NTA conjugate) were from Sigma and Invitrogen, respectively. Cy3-tyramide was from PerkinElmer (Boston, MA).

Instrumentation. The Akta explorer HPLC system (GE Healthcare) was used for analytical affinity purification and the Pharmacia FPLC for preparative purification of proteins. A Biacore 3000 was used for binding analysis with an NTA sensor chip. ELISA plates were read at A450 nm using a Multiskan EX microplate reader (Labsystems). Protein arrays were spotted using a eight-pin manual arrayer (Xenopore) and slides scanned using an Affymetrix 428 scanner.

Construction of GFP Expression Vectors and Tag Sequences. Throughout these studies, a variant of GFP, known as GFPuv (Clontech), was cloned into pENTR/D-TOPO Gateway vector (Invitrogen) as nontagged, C-terminal or N-terminal single-His6 and double-His6 constructs using PCR techniques. The gene encoding GFPuv was cloned into pENTR/D-TOPO Gateway vector (Invitrogen) as nontagged, C-terminal or N-terminal single-His6 and double-His6 constructs using PCR techniques. The resulting constructs were fully sequenced and are summarized in Table 1. In the double-His6 tag, the intervening 11-amino acid spacer was derived from a streptavidin-binding peptide and the flexible Gly-Ser sequence was from a linker sequence optimized for the stability of single-chain proteins.16

Expression and Purification of GFPs. Proteins were expressed in Escherichia coli using competent rosetta cells (Invitrogen). Single colonies of transformed E. coli harboring the GFP gene construct were picked from TYE ampicillin plates, inoculated into 5 mL of 2× TY media (containing 0.1 mg/mL ampicillin) and grown overnight on a shaker at 37 °C. The overnight culture was inoculated into 1× 2× TY media containing of 0.1 mg/mL ampicillin and grown overnight on a shaker at 37 °C. The overnight culture was inoculated into 1× 2× TY media containing of 0.1 mg/mL ampicillin and grown overnight on a shaker at 37 °C. The supernatant was loaded onto a 20-mL Ni–NTA agarose column until the green protein was bound visibly to the top of the column. After washing with six column volumes of buffer A, protein was eluted with buffer B (50 mM Tris, pH 8.0). Eluted fluorescent fractions were pooled, concentrated (~10 mL), and loaded onto a HiLoad 26/60 Superdex 75 column (Amersham Biosciences), pre-equilibrated in PBS, to remove any contaminants and imidazole. For analytical purposes, 2 mL of lysate was loaded directly on to a 1-mL Ni–NTA agarose column, washed with 10 mL of buffer A, and eluted with a gradient of buffer B (0–30 min), GFP concentration was determined using the extinction coefficient ε = 30 000 at A395 nm. Protein samples were concentrated, frozen at −80 °C, and analyzed on SDS–PAGE. All GFP proteins gave the characteristic GFPuv absorbance maximum at 395 nm and fluorescence maximum at 507 nm (excitation at 395 nm). In general, preparative purification of all GFP constructs gave protein yields of ~50 mg/L culture, except for the double-His6 GFP, which gave yields of 160 mg/L.

Biacore Binding Assay and Data Analysis. In each cycle on the NTA chip, 0.3 mM NiCl2 was injected at 15 μL/min for 1 min, followed by injection of protein with a constant flow of buffer A (containing 0.5 M NaCl) at 30 μL/min, with an injection time of 3 min and dissociation time of 30 min. The chip was regenerated with 1 M imidazole after each binding assay at 20 μL/min for 1 min before the next cycle. Sensorsgrams were run in duplicate.

Table 1. Summary of His6-Tagged GFP Constructs

<table>
<thead>
<tr>
<th>construct</th>
<th>no. of His6 tags</th>
<th>N or C terminus</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>nontagged</td>
<td>0</td>
<td></td>
<td>GFP (M1-K138)</td>
</tr>
<tr>
<td>GFP-single</td>
<td>1</td>
<td>C</td>
<td>GFP-HHHHHHHH</td>
</tr>
<tr>
<td>GFP-double</td>
<td>2</td>
<td></td>
<td>GFP-GGGGSGGGSGGTGGGSNGK RADAA</td>
</tr>
<tr>
<td>GFP-triple</td>
<td>3</td>
<td>N single His6 and C double His6</td>
<td>MHHHHHHHHH-GFP-GGGGSGGGSGGG</td>
</tr>
<tr>
<td>N + C-GFP</td>
<td>2</td>
<td>N single His6 and C single His6</td>
<td>TGGGSGGKGRRADAHHHHHHHESRA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>WHROPFGG HHHHH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MSYYHHHHHHHHLQSTSLLKKAGMGFP-HHHHH</td>
</tr>
</tbody>
</table>


B Analytical Chemistry
and in each data set, resonance units (RU) were averaged, normalized, and dissociation curves analyzed. Kinetic dissociation rate constants were obtained from a double-exponential fitting equation using the Kaleidagraph program (Synergy Software). Dissociation phases of the sensorgrams were fitted to the double-exponential function $Bt = A_1(1 - \exp(-k_1t)) + A_2(1 - \exp(-k_2t))$, where $Bt$ is the resonance signal in RU at time $t$ (s), and $A_1$ and $A_2$ are amplitudes of the resonance signal corresponding to dissociation constants $k_1$ and $k_2$ (s$^{-1}$), respectively. Fitted curves gave fitting correlation constant $R > 0.99$ and low residual errors of <0.1%.

**Enzyme-Linked Immunosorbent Assay.** For ELISA using Ni-NTA microtiter plates, nontagged, single-His$_6$- and double-His$_6$-GFP in buffer A were serially diluted (3 μg/mL−12 ng/mL) into a 96-well Hisorb plate (Qiagen) and incubated for 1 h at room temperature on a shaker. The plate was washed with PBS containing 0.1% Tween (PBS/Tween), and then wells were incubated for 1 h with 100 μL of biotinylated-anti GFP antibody (1/4000 dilution in blocking buffer consisting of 1% BSA in buffer A), washed ×3 with PBS/Tween, and incubated with 100 μL of streptavidin–HRP (1/5000 dilution in blocking buffer) for a further 30 min. After washing ×3 with PBS/Tween, 100 μL of TMB solution was added to each well and incubated until color developed, when the reaction was stopped with 100 μL of 1 M HCl. The plate OD was read at 450 nm. Alternatively, HRP-conjugated anti-His antibody was used instead of the biotin–anti-GFP/streptavidin–HRP, employing the same protocol.

For ELISA using Maxisorb (non-NTA) microtiter plates (Nunc), wells were coated with 100 μL of GFP or GFP–His fusions at (3 μg/mL) overnight at 4 °C followed by blocking with 1% BSA for 1 h at room temperature. For detection by anti-His antibody, the wells were incubated for 1 h with serial dilutions of HRP-linked anti-His antibody. Detection with anti-GFP was as above.

**Detection of Double-His$_6$ Tagged GFP on Microarrays.** Purified single- and double-His$_6$-tagged GFP were arrayed (100 ng/spot) onto Nexterion amine reactive slides (slide-H), incubated overnight in a humidified chamber, and reactive sites were blocked for 2 h with 50 mM ethanolamine in 100 mM Tris buffer, pH 9.0. The slides were rinsed with water and washed ×3 with PBS/0.1% Tween. One slide was incubated with Ni-NTA–HRP conjugate (1/1000 dilution in buffer A) for 1 h, washed ×3 with PBS/Tween, and incubated with Cy3-tyramide substrate solution for 10 min. Another was incubated for 2 h with fluorescent Ni-NTA conjugate solution (ProQ Sapphire 532) and washed ×3 with water. Slides were dried by centrifugation at 1000 rpm for 10 min and read using the Cy3 scanner channel. Nontagged GFP and single- and double-His$_6$-tagged GFP were similarly arrayed on a Ni-NTA slide (Xenopore), blocked with 1% BSA in PBS for 1 h, and developed with HRP-conjugated anti-His antibody and Cy3-tyramide.

**RESULTS**

**Ni-NTA Agarose Affinity Chromatography.** Analytical affinity purification from bacterial lysate of single-His$_6$-tagged GFP on Ni-NTA agarose gave a single elution peak at 0.1 M imidazole (Figure 1A). In contrast, double-His$_6$-tagged GFP eluted with two major peaks at 0.6 and 0.8 M imidazole, respectively, both of which were fluorescent and monomeric as determined by analytical gel filtration and of identical size by SDS–PAGE (Figure 1B) and MALDI mass spectrometry. When the two fractions were dialyzed and rerun separately on the column, each eluted at its previous imidazole concentration (data not shown). Using the same chromatography, the other His-tagged GFP constructs listed in Table 1 eluted as single peaks at ≤0.3 M imidazole.

![Figure 1](image-url)
Binding to NTA Chip Using SPR. (i) His<sub>6</sub>-Tagged GFP Constructs. Binding curves were obtained with a BIAcore NTA sensor chip. Figure 2A shows binding and dissociation curves of single-His<sub>6</sub> GFP at different concentrations. The levels of protein immobilized were directly proportional to the concentration of protein injected, and it is noteworthy that the protein was completely removed by a 30-min buffer wash. In contrast, Figure 2B shows binding curves of double-His<sub>6</sub> GFP, where the dissociation rate was slower and for most concentrations >50% of the protein remained bound after a 30-min wash.

Figure 3A compares the dissociation curves for nontagged, single-His<sub>6</sub> and double-His<sub>6</sub> GFP at 500 nM concentration, showing the unusually slow dissociation of double-His<sub>6</sub> GFP (1.6 × 10<sup>-4</sup> s<sup>-1</sup>), which remains ~40% bound after washing, compared to the fast dissociation of single-His<sub>6</sub> GFP (6.3 × 10<sup>-3</sup> s<sup>-1</sup>), which is almost completely removed from the Ni–NTA surface. Each curve was fitted to a double-exponential equation, and rate constants and the half-lives show the slower dissociation and tighter binding of the double-His<sub>6</sub> construct (Table 2).

Figure 3B shows the sensorgrams of the double-His<sub>6</sub> GFP compared with a construct in which single-His<sub>6</sub> tags were attached at both the N- and C-termini, (N + C)-His<sub>6</sub>-GFP. While the on-rate of both of these doubly tagged GFP were identical, the off-rate of double-His<sub>6</sub> GFP was clearly much slower than the N- + C-His-GFP. In Figure 3C, the double-His<sub>6</sub> GFP is compared with a triple-His<sub>6</sub> tagged (i.e., N-terminal His<sub>6</sub> plus C-terminal double-His<sub>6</sub>) from which it is seen that the triple-tagged protein had lower binding characteristics (i.e., lower on-rates and ~60% lower binding), whereas the dissociation rate was similar to that of the double-His<sub>6</sub> tag (Table 2).

ELISA on Ni–NTA Microtiter Plates. ELISA binding curves obtained on Ni–NTA microtiter plates also showed a ~10-fold
greater binding of the double-His<sub>6</sub> GFP compared to single-His<sub>6</sub> GFP, when developed with biotinylated anti-GFP antibody and streptavidin–HRP conjugate (Figure 4A). This assay reflects the strength of the His<sub>6</sub> interaction with the Ni–NTA-modified surface of the microwell plate. In a second ELISA format on Ni–NTA plates, anti-His antibody–HRP conjugate was used to probe accessibility of the immobilized His<sub>6</sub> tag itself on the Ni–NTA surface (Figure 4B) and showed that the Ni–NTA-bound single-His<sub>6</sub> sequence was unavailable to antibody except at high plate-coating concentrations, whereas the double-His<sub>6</sub> could be sensitively detected down to low plate-coating levels. Finally, in ELISA using conventional (non-NTA) microwells and also developed by anti-His antibody–HRP, immobilized double-His<sub>6</sub> GFP was detected ≥10 times more sensitively than an equivalent coating with single-His<sub>6</sub> GFP (Figure 4C, D).

**Detection of Double-His<sub>6</sub> GFP Arrayed on Protein Microarrays.** Protein arrays constructed on amine reactive slides showed a 3-fold increase in fluorescence spot intensity with the double-His<sub>6</sub> GFP compared to the single-His<sub>6</sub>-tagged construct when detected by HRP Ni–NTA conjugates and Cy3 tyramide (Figure 5A). The double-His<sub>6</sub> tag was also sensitively detected by fluorescent Ni–NTA (Figure 5B). As in the ELISA, the double-His<sub>6</sub> tag was detected by anti-His antibody even when used for immobilization on Ni–NTA slides (Figure 5C).

**DISCUSSION**

To date, binding studies of multiple-His-tagged proteins have focused on N- or C-terminal fusions to proteins, which are either monomers, dimers, or larger macromolecular complexes.

However, tagging a protein at both termini may affect protein folding, as some proteins are more sensitive to tag fusions at either terminus. We have used GFP<sub>svw</sub> as a model protein to compare different His<sub>6</sub> tags attached at the N- or C-terminus. GFP is known to tolerate fusions at either terminus, while the characteristic fluorescence serves as a useful probe for accurate quantitation and to follow protein elution (Figure 1A). All the designed N- and C-terminal GFP fusions (Table 1) were fluorescent and nontoxic, with yields of up to 160 mg/L culture medium.

Here we show that a double-His<sub>6</sub> tag sequence, in which two hexahistidines are separated by a short intervening peptide spacer, binds more strongly to Ni–NTA-modified surfaces than a single-His<sub>6</sub> tag, by at least 1 order of magnitude. This double-His<sub>6</sub> tag design was first introduced by ourselves for application in protein arrays, specifically to immobilize proteins synthesized by cell-free systems in situ on array surfaces, but its binding parameters have not been characterized previously. As well as the 11-amino acid sequence between the two hexahistidines, the tag carries a linker region, previously optimized for the stability of single-chain proteins, through which it is connected to the C-terminus of the protein.

Analytical affinity chromatography of the double-His<sub>6</sub> GFP on Ni–NTA agarose revealed two major protein peaks, eluting at 0.6 and 0.8 M imidazole, compared to the single peak obtained with the single-His<sub>6</sub> GFP eluting at 0.1 M (Figure 1A). Both double-His<sub>6</sub> GFP peaks exhibited green fluorescence, evidence for correctly folded tertiary structure, while MALDI mass spectrometry gave identical masses consistent for the full-length protein and SDS–PAGE also showed two similar molecular weight bands (Figure 1B).

In Figure 1B, it was noted that the two eluted species are likely to be independent conformations of the double-His<sub>6</sub> tag that allow differential spatial access for binding to Ni–NTA. Nevertheless, both species demonstrate tighter binding than the single-His<sub>6</sub> construct. In addition, elution characteristics after reapplication of each purified double-His<sub>6</sub> GFP peak were identical to those of the original peaks, suggesting that the putative structures are not in dynamic equilibrium but locked in either of two conformational states. It is also interesting to note from Figure 1A (peak area integration) that the two populations are approximately equal, indicating no preference in formation of either conformer.

The double-His<sub>6</sub> tag also serves as a useful tool for improved single-step protein purification. SDS–PAGE of the double-His<sub>6</sub> GFP eluted from Ni–NTA chromatography showed that the protein was purified to near-homogeneity from E. coli lysate, whereas the single-His<sub>6</sub> GFP coeluted with a number of contaminant bands (Figure 1B). A systematic comparison of a variety of affinity tags for purification has shown that a single-His<sub>6</sub>-tagged protein provided good yields of protein from E. coli but copurified with many contaminant proteins, and poor purification was obtained from yeast, Drosophila, and HeLa cell extracts. The higher purity obtained from elution of the double-His<sub>6</sub> GFP is made possible by its higher binding affinity (avidity), as proteins bound nonspecifically to the Ni–NTA agarose column will be eluted at considerably lower imidazole concentrations, as is the case for the single-His<sub>6</sub> GFP.

In the SPR sensorgrams for the single-His<sub>6</sub> GFP (Figure 2A), the dissociation curves were biphasic with similar (3-fold different) dissociation rates for the two phases, and the protein was completely removed after a 30-min buffer wash step. At 500 nM, the observed dissociation rate constants were $k_1 = 6.3 \times 10^{-3}$ s<sup>-1</sup> and $k_2 = 1.8 \times 10^{-3}$ s<sup>-1</sup> (Figure 3A, Table 2). The two phases are a consequence of two dissociation events, which is most likely due to two binding strengths of Ni–NTA to a single-His<sub>6</sub>-tagged protein through multivalent interactions.

Multiphasic behavior has also been observed in SPR analysis for other His-tagged proteins and such kinetic curves were difficult to fit to existing binding models for estimation of equilibrium binding constants. In contrast, the double-His<sub>6</sub>-tagged GFP (consisting of a mixture

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**Table 2. Summary of the Half-Life and Dissociation Rates Obtained with Different His<sub>6</sub>-Tagged GFPs at 500 nM**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$t_{1/2}$ (s)</th>
<th>$t_{1/2}$ (s)</th>
<th>$k_1$ (dissoc. s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>$k_2$ (dissoc. s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single His&lt;sub&gt;6&lt;/sub&gt;-GFP</td>
<td>110</td>
<td>385</td>
<td>$6.3 \times 10^{-3}$</td>
<td>$1.8 \times 10^{-3}$</td>
</tr>
<tr>
<td>Double His&lt;sub&gt;6&lt;/sub&gt;-GFP</td>
<td>173</td>
<td>4332</td>
<td>$4.0 \times 10^{-3}$</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>N + C His&lt;sub&gt;6&lt;/sub&gt;-GFP</td>
<td>141</td>
<td>330</td>
<td>$4.9 \times 10^{-3}$</td>
<td>$2.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>Triple His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>161</td>
<td>3465</td>
<td>$4.3 \times 10^{-3}$</td>
<td>$2.0 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

*The data were taken as an average of two sensorgrams, and each curve was reproducible (with SD < ± 0.4% RU) and residual errors for fitting curves were < 0.1%.*

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of the two apparent conformers) demonstrated much more stable binding in which a significant proportion of the protein (>50%) remained bound to the Ni–NTA chip after a 30-min buffer wash (Figure 2B). Analysis of the dissociation curves at 500 nM fitted a double-exponential equation with dissociation constants of $k_1 = 4.0 \times 10^{-3}$ and $k_2 = 1.6 \times 10^{-4}$ s$^{-1}$ (Figure 3A). This demonstrated the presence of a more weakly bound component, with a dissociation rate comparable to the single-His6 protein and a more strongly bound component dissociating 10-times more slowly (Table 2). An explanation for these observations is that they are a combined effect of the relatively weak binding of single-His6 to Ni–NTA and the subsequent stronger binding achieved through avidity of both His6 tags binding simultaneously to accessible Ni–NTA moieties.

A stoichiometric analysis of the single-His6 tag/NTA interaction in solution has demonstrated that three Ni–NTA moieties bind a single-His6 tag,$^{20}$ and the same interaction in SPR experiments yielded multiphasic dissociation curves,$^8$ an indication that the interaction is multivalent. The kinetics of binding become more complex with the double-His6 tag as the avidity to NTA increases with the second His6 sequence. Since there is little difference between the affinity for NTA of the two apparent conformers of the double-His6 GFP (as evidenced by competitive affinity chromatography elution, i.e., 0.6 and 0.8 M), it is likely that the tight binding is indeed due to cooperative effects through avidity of the double-His6 tag rather than conformational effects.

Our SPR binding study of GFP having single-His6 sequences at both the N- and C-termini (N + C) showed a dissociation that was remarkably faster than for the C-terminal double-His6 GFP (Figure 3B). The nature of the latter binding is thus dependent on the spatial proximity of the two His6 sequences, in which the cooperative effect (avidity) of a double-His6 with a short intervening spacer results in tight binding to Ni–NTA groups on surfaces. In contrast, the dissociation behavior of the (N + C)-His6 GFP was comparable to that of the C-terminus single-His6 GFP (Table 2; Figure 3A, B).

It is interesting to compare these observations with those of Nieba et al.,$^9$ who analyzed binding of several different proteins conjugated with different numbers of His6 tags for binding to a Ni–NTA chip surface, also measuring the interaction by BIAcore. Similarly, they found that single-His6-tagged monomeric proteins dissociated rapidly. Multimeric proteins carrying several His6 tags or those with dual-labeled (N + C)-His6 tags could show stable binding with very slow dissociation. Since GFP is a monomer,
than cooperatively. In contrast, our double-His 6 tag should be which could oblige (N- and C-) and dual (N- and C)-His6 tags may be determined by protein structure: where the N- and C-termini are both available for simultaneous binding to Ni-NTA, the enhanced avidity effect of two tags will be seen, but where binding of only one terminus can occur at a time, e.g., by reason of their relative locations on the protein surface, then there will be no advantage in (N + C) tagging. This is supported by the fact that the N- and C-termini of GFP are well separated (23 Å from the crystal structure), which could oblige (N + C) tags to behave independently rather than cooperatively. In contrast, our double-His6 tag should be universally applicable, avoiding the need to derivatize both N- and C-termini or reliance on oligomeric proteins.

It could be expected that increasing the number of His6 tags further might in turn increase the avidity for Ni-NTA even more. However, a triple-His6-tagged GFP (with an extra His on the N-terminus of the double-His6-GFP) gave 60% lower binding of total protein on the Ni-NTA chip in SPR and no advantage in immobilization over the double-His6 construct (Figure 3C). The triple-His6 construct had a strong tendency to aggregate visibly in solution, an effect that was exacerbated by an equivalent concentration of free Ni2+. This suggested that aggregates or multimers were formed by intermolecular Ni2+/His6 binding which were reversible by addition of imidazole. These results indicate that there is an upper limit on the number of His6 tags that can be introduced into a protein. His6 tag dependent protein dimerization has also been observed by others, e.g., in the DNA binding protein pi(30.5) protein of plasmid R6K.

ELISA data using anti-GFP detection showed that binding of the double-His6 GFP to Ni-NTA microtiter wells was ~10-fold greater than the single-His6 GFP (Figure 4A). This is agreement with the SPR data demonstrating 1 order of magnitude slower off-rate compared to the single-His6 construct. Sensitive detection by anti-His monoclonal antibody–HRP conjugate was observed with Ni-NTA immobilized double-His6 GFP, but not with the single-His6 GFP (Figure 4B). It appears that the binding of a single His6 to Ni-NTA masks the epitope recognized by anti-His antibody, which nonetheless remains available in the double-His6 tag. It is particularly advantageous that use of anti-His antibody is compatible with concurrent Ni-NTA binding to the double-His6 tag, enabling the latter to be used for both protein immobilization and detection on NTA surfaces in solid-phase assays such as ELISA and protein microarrays (Figure 5C). For protein immobilized conventionally onto non-NTA microtiter wells, ELISA detection of the double-His6 GFP by anti-His antibody was also considerably more sensitive than the single-His6 tag (Figure 4C, D), possibly due to increased avidity of the bivalent IgG binding to two adjacent His6 epitopes.

Another, nonantibody-based method for detection of His6 tags on surface-based assays is the use of fluorescent, biotin, or enzyme conjugates of Ni-NTA, which are versatile and relatively inexpensive. Once again, the double-His6 tag was more sensitively detected than single-His6. Thus, a Ni-NTA–HRP conjugate used as a detection reagent on covalently bound protein microarrays showed a 3-fold greater sensitivity of detection of the double-His6 tag compared to single-His6 (Figure 5A). Increased avidity of Ni-NTA conjugates for the double-His6 tag could be achieved by a greater conjugation ratio of NTA/protein. Fluorescent Ni-NTA has been used as a sensitive stain for detection of His6-tagged proteins in SDS–PAGE gels, but to our knowledge, the result in Figure 5B is the first reported application of fluorescent Ni-NTA in a microarray format. The ProQ Sapphire 532 conjugate has equivalent fluorescence spectral properties to Cy3, and therefore, this reagent can be used as a generic dye in dual fluorescence-based microarrays to detect protein–protein interactions, similar to methods established for DNA microarray technologies.

In conclusion, using GFP as a model, we have demonstrated high-affinity binding by a novel double-His6 tag sequence to Ni-NTA, leading to substantially improved binding on Ni-NTA surfaces, including chromatography media, Ni-NTA SPR chips, and ELISA wells, and greater detection with Ni-NTA-bound reagents and antibodies. In addition, we have demonstrated improved single-step protein purification. This immobilization method is particularly suited to biotechnological applications such as:

as solid-phase microarray assays for proteomics and diagnostics or enzyme immobilization on beads in biocatalysis.

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