Site-Specific Incorporation of Fluorescent Probes into Protein: Hexahistidine-Tag-Mediated Fluorescent Labeling with (Ni^{2+}:Nitrilotriacetic Acid)$_n$−Fluorochrome Conjugates

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Structural and mechanistic characterization of proteins by fluorescence resonance energy transfer (FRET)[1,2] requires the ability to incorporate fluorescent probes at specific, defined sites.[2] For proteins that do not contain cysteine residues, site-specific fluorescent labeling can be accomplished by use of site-directed mutagenesis to introduce a cysteine residue at the site of interest, followed by cysteine-specific chemical modification to incorporate the fluorescent probe.[2] However, for proteins that contain cysteine residues (most proteins with MW > 50 kDa), site-specific fluorescent labeling is difficult. Three strategies have been reported: (i) intein-mediated labeling ("expressed protein ligation"),[3] (ii) oxidation-mediated labeling,[4] and (iii) trivalent-arsenic-mediated labeling.[5] The first two strategies are limited to labeling of protein termini and do not permit in situ labeling (i.e., direct labeling of proteins in cuvettes, gels, blots, or biological samples—without the need for a subsequent purification step); the third strategy currently is limited to a single fluorochrome.

Here, we report a strategy that permits labeling of termini or internal sites, that permits in situ labeling, and that is compatible with a range of fluorochromes with different spectroscopic and photophysical properties. Our strategy involves use of the "hexahistidine tag"[6,7,8]— i.e., the amino acid sequence His_6— to target site-specific fluorescent labeling. The hexahistidine tag is known to interact tightly with transition-metal complexes, including Ni^{2+}:nitrilotriacetic acid (Ni^{2+}:NTA).[6−8] The hexahistidine tag can be introduced at protein termini or internal sites by using standard molecular-biology procedures[6] and is widely used in molecular-biology research for affinity-chromatography-based protein purification [with (Ni^{2+}:NTA)-agarose][6] and protein immobilization [with (Ni^{2+}:NTA)-coated surfaces].[6,7]

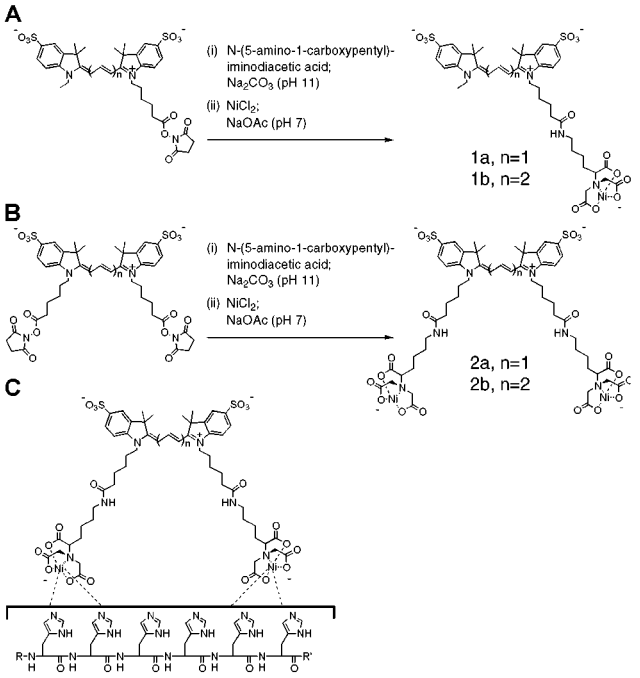

Figure 1. (Ni^{2+}:NTA)$_n$ derivatives of cyanine fluorochromes. (a) Synthesis of (Ni^{2+}-NTA)$_1$-Cy3 (**1a**) and (Ni^{2+}-NTA)$_1$-Cy5 (**1b**). (b) Synthesis of (Ni^{2+}-NTA)$_2$-Cy3 (**2a**) and (Ni^{2+}-NTA)$_2$-Cy5 (**2b**). (c) Schematic representation of the mode of interaction of **2a** or **2b** with the hexahistidine tag.

Table 1. Spectroscopic Properties of Fluorochrome Conjugates[a]

fluorochrome	$\lambda_{max,exc}$ (nm)	$\lambda_{max,em}$ (nm)	quantum yield (Q)
1a	550	562	0.05
1b	648	667	0.08
2a	552	565	0.04
2b	650	668	0.05

[a] Ni^{2+}-free analogues of **2a** and **2b** exhibit identical $\lambda_{max,exc}$ and $\lambda_{max,em}$ and 3.8-fold higher Q (with the higher Q presumably reflecting the unavailability of nonradiative decay pathways involving Ni^{2+} unoccupied d orbitals).

We hypothesized that the hexahistidine tag should interact tightly with (Ni^{2+}:NTA)$_n$-fluorochrome conjugates and thus should be able to mediate site-specific fluorescent labeling (Figure 1). We further hypothesized, based on molecular modeling, that the hexahistidine tag should be able to interact with up to two Ni^{2+}:NTA moieties without steric hindrance. To test these hypotheses, we prepared and analyzed (Ni^{2+}:NTA)$_1$-fluorochrome conjugates and (Ni^{2+}:NTA)$_2$-fluorochrome conjugates. We synthesized derivatives of the widely used cyanine fluorochromes Cy3 and Cy5[2,9] having one pendant Ni^{2+}:NTA− moiety [(Ni^{2+}:NTA)$_1$-Cy3 and (Ni^{2+}:NTA)$_1$-Cy5; **1a** and **1b** in Figure 1a] or two pendant Ni^{2+}:NTA− moieties [(Ni^{2+}:NTA)$_2$-Cy3 and (Ni^{2+}:NTA)$_2$-Cy5; **2a** and **2b** in Figure 1b] by reaction of mono- and bis-succinimidyl-ester derivatives of Cy3 and Cy5[9] with *N*-(5-amino-1-carboxypentyl)iminodiacetic acid,[10] followed by reaction with $NiCl_2$ (Figure 1a,b; Table 1).

Fluorescence anisotropy experiments[11,12] establish that **1a** and **1b** exhibit relatively low affinity for the hexahistidine tag ($K_D \geq 10\ \mu M$; Figure 2).[11]

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(1) Clegg, R. *Curr. Opin. Biotechnol.* **1995**, *6*, 103−110.
(2) Selvin, P. *Nat. Struct. Biol.* **2000**, *7*, 730−734.
(3) (a) Muir, T.; Sondhi, D.; Cole, P. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. (b) Ayers, B.; Blaschke, U.; Camarero, J.; Cotton, G.; Holford, M.; Muir, T. *Biopolymers* **2000**, *51*, 343−354. (c) Tolbert, T.; Wong, C.-H. *J. Am. Chem. Soc.* **2000**, *122*, 5421−5428. (d) Mukhopadhyay, J.; Kapanidis, A. N.; Mekler, V.; Kortkhonjia, E.; Ebright, Y. W.; Ebright, R. H. *Cell* **2001**, *106*, 453−463.
(4) Geoghegan, K.; Stroh, J. *Bioconjug. Chem.* **1992**, *3*, 138−146.
(5) (a) Griffin, B.; Adams, S.; Tsien, R. *Science* **1998**, *281*, 269−272. (b) Griffin, B.; Adams, S.; Jones, J.; Tsien, R. *Methods Enzymol.* **2000**, *327*, 565−578.
(6) (a) Hochuli, E.; Bannwarth, W.; Dobeli, H.; Gentz, R.; Stuber, D. *BioTechnol.* **1988**, *6*, 1321−1325. (b) Crowe, J.; Dobeli, H.; Gentz, R.; Hochuli, E.; Stuber, D.; Henco, K. *Methods Mol. Biol.* **1994**, *31*, 371−387.
(7) (a) O'Shannessy, D. J.; O'Donnell, K.; Martin, J.; Brigham-Burke, M. *Anal. Biochem.* **1995**, *229*, 119−124. (b) Gershon, P.; Khilko, S. *J. Immunol. Methods* **1995**, *183*, 65−76. (c) Nieba, L.; Nieba-Axmann, S.; Persson, A.; Hamalainen, M.; Edebratt, F.; Hansson, A.; Lidholm, J.; Magnusson, K.; Karlsson, A.; Plückthun, A. *Anal. Biochem.* **1997**, *252*, 217−228.
(8) (a) Kienberger, F.; Kada, G.; Gruber, H.; Pastushenko, V.; Riener, C.; Trieb, M.; Knaus, H.-G.; Schindler, H.; Hinterdorfer, P. *Single Mol.* **2000**, *1*, 59−65. (b) Schmitt, L.; Ludwig, M.; Gaub, H.; Tampe, R. *Biophys. J.* **2000**, *78*, 3275−3285.
(9) Mujumdar, R.; Ernst, L.; Mujumdar, S.; Lewis, C.; Waggoner, A. *Bioconjug. Chem.* **1993**, *4*, 105−111.
(10) Hochuli, E.; Dobeli, H.; Schacher, A. *J. Chromatogr.* **1987**, *411*, 177−184.

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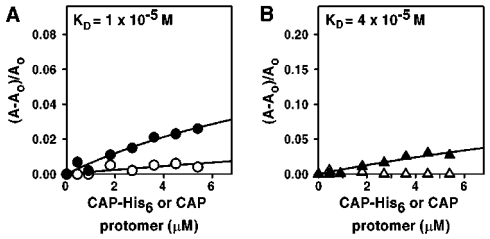

Figure 2. Specific interactions of **1a** and **1b** with hexahistidine-tagged protein: fluorescence anisotropy experiments.[11,12] (a) Titration of **1a** with CAP-His$_6$ (filled circles; $K_D = 10$ μM) and control titration with CAP (open circles; $K_D > 50$ μM). (b) Titration of **1b** with CAP-His$_6$ (filled triangles; $K_D = 40$ μM) and control titration with CAP (open triangles; $K_D > 100$ μM).

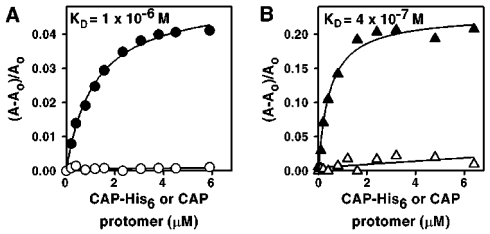

Figure 3. High-affinity, specific interactions of **2a** and **2b** with hexahistidine-tagged protein: fluorescence anisotropy experiments.[11,12] (a) Titration of **2a** with CAP-His$_6$ (filled circles; $K_D = 1.0$ μM) and control titration with CAP (open circles; $K_D > 50$ μM). (b) Titration of **2b** with CAP-His$_6$ (filled triangles; $K_D = 0.4$ μM) and control titration with CAP (open triangles; $K_D > 50$ μM).

Fluorescence anisotropy experiments[11,12] establish that, in contrast, **2a** and **2b** exhibit high affinity for the hexahistidine tag ($K_D = 1.0$ μM for **2a**; $K_D = 0.4$ μM for **2b**; Figure 3). Thus, titration of **2a** or **2b** with a protein having a hexahistidine tag (CAP-His$_6$; C-terminally hexahistidine-tagged derivative of the transcriptional activator CAP[13]) results in a large, saturable increase in fluorescence anisotropy (Figure 3, filled symbols). The interaction is specific (specificity >95%; Figure 3). Thus, titration of **2a** or **2b** with an otherwise-identical protein lacking a hexahistidine tag (CAP) results in little or no increase in fluorescence anisotropy (Figure 3, open symbols).

FRET experiments[1,2,14−16] confirm that **2a** and **2b** exhibit high affinity and high specificity for the hexahistidine tag ($K_D = 0.9$ μM for **2a**; $K_D = 0.3$ μM for **2b**; specificity >95%), establish that the stoichiometry of interaction of **2a** and **2b** with the hexahistidine tag is 1:1, and establish that **2a** and **2b** are suitable for use in FRET-based distance measurements (Figure 4). Thus, titration of a fluorescein-labeled, hexahistidine-tagged DNA-protein complex (DNA^F-CAP-His$_6$)[17,18]—but not an otherwise-identical fluorescein-labeled, nonhexahistidine-tagged DNA-protein complex (DNA^F-CAP)—with **2a** or **2b** results in large, saturable fluorescein→**2a** or fluorescein→**2b** FRET (Figure 4b,c). Titrations performed under stoichiometric-binding conditions (titrations with a concentration of DNA^F-CAP-His$_6$ substantially higher than K_D) indicate that saturation occurs at a 1.1(±0.2):1 mole ratio of **2a** or **2b** to hexahistidine [which corresponds to a 2.2(±0.4):1 mole ratio of Ni^{2+}:NTA− to hexahistidine; Figure 4d,e]. The measured efficiencies of FRET at saturation, together with the measured Förster parameters, yield an estimate of 56(±4) Å for the fluorescein-**2a** or fluorescein-**2b** distance in the complex (Figure 4b,c)[15,16]— an estimate in excellent agreement with the distance of ∼55 Å expected based on structural information (Figure 4a).[17,20,21]

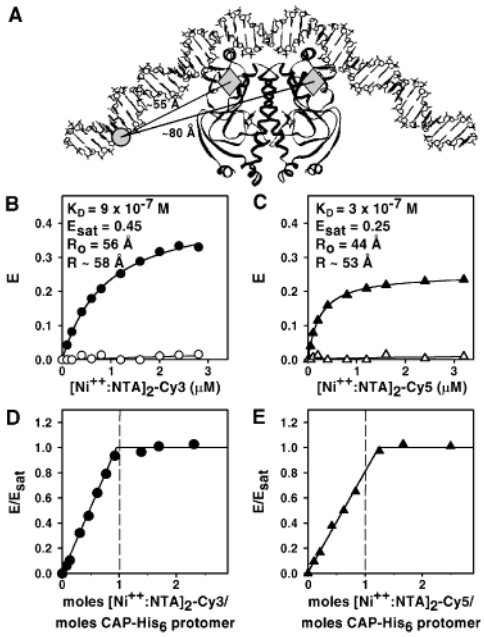

Figure 4. High-affinity, specific, stoichiometric interactions of **2a** and **2b** with hexahistidine-tagged protein: FRET experiments.[1,2,14−16] (a) Modeled structure of the DNA^F-CAP-His$_6$ complex,[17,20] showing the position of the fluorescein of DNA^F (circle), the position of the hexahistidine tag of each CAP-His$_6$ protomer (diamond), the distance between the fluorescein and the hexahistidine tag of the proximal CAP-His$_6$ protomer (∼55 Å), and the distance between fluorescein and the hexahistidine tag of the distal CAP-His$_6$ protomer (∼80 Å).[21] (b) Titration of 5 nM DNA^F-CAP-His$_6$ with **2a** (filled circles; $K_D = 0.9$ μM; $R = 58$ Å[21]) and control titration of 5 nM DNA^F-CAP with **2a** (open circles; $K_D > 50$ μM). (c) Titration of 5 nM DNA^F-CAP-His$_6$ with **2b** (filled triangles; $K_D = 0.3$ μM; $R = 53$ Å[21]) and control titration of 5 nM DNA^F-CAP with **2b** (open triangles; $K_D > 50$ μM). (d) Stoichiometric titration of 2.6 μM DNA^F-CAP-His$_6$ with **2a**. (e) Stoichiometric titration of 0.6 μM DNA^F-CAP-His$_6$ with **2b**.

FRET experiments assessing distances within RNA polymerase core enzyme (MW = 380 KDa) further confirm that **2a** and **2b** are suitable for use in FRET-based distance measurements (unpublished data).

We emphasize that the fluorescence anisotropy and FRET experiments in Figures 3 and 4 involved use of in situ labeling—i.e.,

(11) Fluorescence anisotropy experiments detect complex formation as an increase in fluorescence anisotropy, *A*, arising from the increase in molecular size and corresponding decrease in rotational dynamics.

(12) Jameson, D.; Sawyer, W. *Methods Enzymol.* **1995**, *246*, 283−300.

(13) Kolb, A.; Busby, S.; Buc, H.; Garges, S.; Adhya, S. *Annu. Rev. Biochem.* **1993**, *62*, 749−795.

(14) FRET experiments detect complex formation as an increase in the efficiency of FRET, *E*, arising from the increased proximity of fluorescent donor (fluorescein in this work) and fluorescent acceptor (**2a** or **2b** in this work).[1,2] FRET experiments also permit determination of the donor−acceptor distance, *R*.[1,2,15,16]

(15) $E = R_o^6/(R_o^6 + R^6)$, where *E* is the efficiency of FRET, R_o is the Förster parameter,[16] and *R* is the donor−acceptor distance.[1,2]

(16) R_o (in Å) $= (0.211 \times 10^{-5})(n^{-4}Q_D\kappa^2 J)^{1/6}$, where *n* is the refractive index of the medium, Q_D is the donor quantum yield in the absence of acceptor, κ^2 is the orientation factor relating the donor emission dipole and acceptor excitation dipole [approximated as $^2/_3$ in this work—justified by the low fluorescent anisotropy of the donor[17]], and *J* is the spectral overlap integral of the donor emission spectrum and the acceptor excitation spectrum.[1,2]

(17) Kapanidis, A. N.; Ebright, Y. W.; Ludescher, R. D.; Chan, S.; Ebright, R. H. *J. Mol. Biol.* **2001**, *312*, 453−468.

(18) DNA^F is a 52 bp fluorescein-labeled DNA fragment containing the consensus DNA site for CAP[19] (fluorescein incorporated at position −9 relative to the consensus DNA site for CAP). DNA^F is identical to DNA fragment ICAP52$^{F,-9}$ of ref 17.

(19) Ebright, R.; Ebright, Y.; Gunasekera, A. *Nucleic Acids Res.* **1989**, *17*, 10295−10305.

(20) (a) Schultz, S.; Shields, G.; Steitz, T. *Science* **1991**, *253*, 1001−1007. (b) Parkinson, G.; Wilson, C.; Gunasekera, A.; Ebright, Y.; Ebright, R.; Berman, H. *J. Mol. Biol.* **1996**, *260*, 395−408.

(21) The DNA^F-CAP-His$_6$ complex contains two CAP-His$_6$ protomers[13] and thus interacts with two molecules of **2a** or **2b** (Figure 4A). However, because of the inverse-sixth-power dependence of *E* on *R*,[1,2,15] and the high value of *R* for the fluorescein-distal molecule of **2a** or **2b** (∼80 Å; Figure 4A), the contribution to *E* of the fluorescein-distal molecule of **2a** or **2b** is expected to be negligible (∼10% the contribution of the fluorescein-proximal molecule of **2a** or **2b**).

direct labeling of the sample in the cuvette, without a subsequent purification step. Use of in situ labeling permitted rapid, "mix-and-read" assays. In addition, use of in situ labeling permitted the donor-only and donor-plus-acceptor measurements required for the FRET experiments to be performed on the same sample, in the same cuvette, thereby eliminating important sources of experimental error.

The strategy described here has important advantages, including the following: (i) compatibility with widely used hexahistidine-tag-based protein-purification and protein-immobilization systems, (ii) applicability to the large library of existing hexahistidine-tagged proteins, (iii) suitability for labeling of N-termini, C-termini, and internal sites, (iv) suitability for use in in situ labeling, and (v) suitability for use with cyanine fluorochromes having different spectroscopic and photophysical properties [Cy3 and Cy5 (Figures 1−4); probably also Cy3.5, Cy5.5, and Cy7[9]]. The size of the label is comparable to sizes of widely used labels.[2−5] Nevertheless, as with any labeling strategy,[2−5] it is essential to verify that incorporation of the label does not alter properties of the protein under study. We note that, in principle, the strategy described here also should be adaptable for detection of hexahistidine-tagged proteins in gels or blots, for staining of hexahistidine-tagged proteins in histological preparations, and for staining and FRET measurements in living cells.

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Supporting Information Available: Details of experimental procedures (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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