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Histidine-tag-directed chromophores for tracer analyses in the analytical ultracentrifuge

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Abstract

Many recombinant proteins carry an oligohistidine (His_X)-tag that allows their purification by immobilized metal affinity chromatography (IMAC). This tag can be exploited for the site-specific attachment of chromophores and fluorophores, using the same metal ion–nitrilotriacetic acid (NTA) coordination chemistry that forms the basis of popular versions of IMAC. Labeling proteins in this way can allow their detection at wavelengths outside of the absorption envelopes of un-modified proteins and nucleic acids. Here we describe use of this technology in tracer sedimentation experiments that can be performed in a standard analytical ultracentrifuge equipped with absorbance or fluorescence optics. Examples include sedimentation velocity in the presence of low molecular weight chromophoric solutes, sedimentation equilibrium in the presence of high concentrations of background protein and selective labeling to simplify the assignment of species in a complex interacting mixture.

Keywords

Histidine tag; Nitrilotriacetic acid; Tracer sedimentation; Analytical ultracentrifugation; Fluorescence

1. Introduction

Many important physiological processes are mediated by macromolecular assemblies that contain multiple protein components. Examples range from transcription–initiation complexes [1,2] to cytoskeleton [3,4] and membrane-fusion machinery [5,6] to blood-clotting assemblies [7,8]. The determination of the compositions, structures and functions of these assemblies is a major theme of modern biomedical research and one in which analytical ultracentrifugation has made valuable contributions. In classical analytical ultracentrifugation, molecules are detected by absorbance at near-ultraviolet or visible wavelengths. One difficulty with this approach is that many biomolecules have overlapping spectra (for example most proteins have absorption maxima near 280 nm while nucleic acids absorb near 260 nm). This limits the number of molecular species that can be monitored in a single sample when one relies on natural chromophores.² The problem becomes more acute when the system of interest contains several protein molecules with nearly identical

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absorption spectra or when one solution component is present in such high concentration that its absorption masks the contributions of others.

Several powerful approaches have been developed for dealing with these challenges. Covalent labeling with exogenous chromophores by chemical methods (for example with fluorescein [9,10] or genetic methods (for example GFP or SNAP-tag labeling [11,12]) adds absorbance at wavelengths that are not masked by proteins or nucleic acids and offers the possibility to detect one protein in the presence of many others. Alternatively, fractionation of solutions brought to sedimentation equilibrium in small tubes carried in a preparative swinging bucket rotor allows a wide range of analytical techniques to be used to detect species of interest, including scintillation counting, enzyme activity assays and antibody binding [13,14]. This approach, which relies on the slow dissipation of concentration gradients after the centrifuge run, has been used in elegant analyses by Minton, Rivas and colleagues [15–17]. Finally, the development of analytical ultracentrifuges equipped with fluorescence optics [18,19] has stimulated interest in the sedimentation properties of macromolecules containing intrinsic and extrinsic fluorophores [20]. In the present generation of instruments, the light source is a laser emitting at 488 nm [21]; this is outside of the near-UV wavelength envelope characteristic of most proteins and nucleic acids.

Here we describe a strategy that complements those mentioned above. It involves labeling proteins with exogenous chromophores that are targeted to the oligo-histidine (His_X-tag) motifs widely used for protein purification [22,23]. The labeling reactions are gentle and reversible, and position the chromophores at unique, user-defined locations within the structures of the selected proteins. Available chromophores allow detection of the protein(s) of interest at wavelengths that are not obscured by the native absorbance(s) of the molecular system. Selective labeling of each protein component in a multi-protein system simplifies sedimentation velocity and equilibrium analyses. In addition, since many of the current generation of labels are fluorescent, this strategy widens the range of proteins that are immediately available for analysis by fluorescence-detected analytical ultracentrifugation. Finally, labeling with fluorophores allows performance of fluorescence analyses (such as FRET or anisotropy [24]) and sedimentation analyses on the same samples, increasing the number of properties that can be correlated. Examples that demonstrate these capabilities are discussed below.³

2. Materials and methods

2.1. Reagents and proteins

NiCl₂, CoCl₂, ZnCl₂ and the corresponding acetate salts were obtained from Aldrich. Ovalbumin was obtained from Sigma and was exhaustively dialyzed against 10 mM Tris (pH 7.6 at 4 °C), 75 mM KCl, 2 mM DTT. Plasmid pUC19 DNA was obtained from New England Biolabs. His₆-tagged *N*-ethylmaleimide-sensitive factor (NSF), O⁶-alkylguanine DNA alkyltransferase (AGT) and AdaC proteins were prepared as previously described [25–27]. DNAs containing full length open reading frames for yeast Gal3 and Gal80 proteins were cloned in the expression vector pET28c (Novagen). Plasmids were propagated, under kanamycin and chloramphenicol selection, in *Escherichia coli* Rossetta (DE3) (Novagen) and expression of cloned genes was induced with IPTG. His₆-tagged Gal3 and Gal80 proteins were purified to near homogeneity by immobilized metal chromatography and gel filtration (Tao and Hopper, unpublished result). Un-tagged Gal 80 protein was purified as a

²When the molecules of interest have different but overlapping spectra (e.g., protein and nucleic acid), multiwavelength analysis methods [59–62] can allow deconvolution of contributions from molecules of each type.

³Some results presented here have been described before [24] and are used with permission. These results (Figs. 2A, 6 and 7) are reproduced in order to allow us to discuss them in terms of their relevance to analytical ultracentrifugation.

complex with His₆-tagged Gal3 protein by immobilized metal chromatography in the presence of 2 mM ATP and 25 mM galactose, then eluted from the retained His₆-Gal3 protein with ATP- and galactose-free buffer. Further details of Gal3 and Gal80 preparations will be described elsewhere (Tao and Hopper, in preparation).

2.2. Synthesis of histidine-tag-directed dyes

The dyes that we have tested to date are bis-nitrilotriacetic acid (NTA)₂-containing derivatives of the widely used Cy3, Cy5 and Cy7 fluorochromes (Fig. 1). These are obtained by reaction of bis-succinimidyl-ester derivatives of the corresponding cyanine dyes (available from GE Healthcare) with *N*_α,*N*_α-bis(carboxymethyl)-L-lysine (Sigma), as originally described by Kapandis et al. [28]. Reaction products were purified by absorption on Sep-Pak C18 cartridges (Waters, Milford, MA, USA) and elution with 60% methanol and then by preparative thin-layer chromatography (silica gel G, Analtech, Newark) using NH₄OH/ethanol/water (33:21:6, v/v/v) mobile phase [24]. Representative properties of these dyes are given in Table 1. Transition metal complexes were formed at pH 7 using MCl₂ (where *M* is Ni²⁺, Co²⁺, Zn²⁺) as described [28]. Detected by fluorescence quenching, the order of metal affinities for (NTA)₂-CyX dyes is Zn²⁺ < Co²⁺ < Ni²⁺ (Zhao, unpublished result), consistent with the order found for unsubstituted NTA [29]. In our experience, the nickel complexes (Ni²⁺-NTA)₂-CyX, are stable for weeks-to-months in aqueous solution, frozen at -80 °C.

2.3. Protein labeling

Proteins must be expressed using a host-vector system that results in the incorporation of a His_X-tag or an HN-tag [30] into the target protein sequence. A longer (His)₁₀ motif gives higher dyebinding affinity than the His₆-tags in common use [31]. Other metal-binding motifs such as KGHK [32] or the (Asp)₄-tag [33] have yet to be evaluated.

Labeling is accomplished by direct addition of (Ni²⁺-NTA)₂-CyX to the protein solution, followed by gentle mixing. To date we have performed labeling reactions without difficulty in buffers containing Tris, HEPES, ATP (2 mM), 2-mercaptoethanol (2 mM), dithiothreitol (2 mM), glycerol (10% v/v), glucose (25 mM) and simple 1:1 salts at concentrations up to 300 mM. On the other hand, chelators such as EDTA and competitors for the His_X-tag, such as imidazole can cause dissociation of the (Ni²⁺-NTA)₂-CyX-protein complexes [24], so these reagents should be avoided. Under typical buffer conditions, in the absence of EDTA or imidazole, the dissociation constant for the His₆-(Ni²⁺-NTA)₂-Cy3 interaction is ~5 × 10⁻⁷ M [24,28]; for efficient labeling we use (Ni²⁺-NTA)₂-CyX concentrations well in excess of this value. If desired, the concentration of free dye can be transiently reduced by gel-filtration chromatography [24], although dye will be slowly released from the protein as the system relaxes (see below). Solutions of labeled proteins can be stored for days at 0-4 °C, or for longer periods (months) frozen at -80 °C. At this stage in technique development we are not aware of any systematic effects of (Ni²⁺-NTA)₂-CyX labeling on protein solubility.

2.4. Analytical ultracentrifugation

Sedimentation velocity measurements of proteins labeled with (Ni²⁺-NTA)₂-Cy3 were performed in a Beckman XL-A analytical ultracentrifuge (Beckman, Fullerton, CA), using an An-60 Ti rotor. The rotor speeds selected depended on sample properties (see figure captions for details). Radial absorbance distributions were recorded at 550 nm, corresponding to the absorbance maximum of Cy3-(Ni²⁺-NTA)₂. Velocity data were analyzed using numerical solutions of the Lamm equation implemented in the program SED-FIT [34,35], using estimates of protein partial specific volume and buffer viscosity that were calculated with the public-domain program SEDNTERP (developed by D.B. Hayes, T.

Laue and J. Philo; available from <http://www.rasmb.bbri.org/>). Buffer density was measured with a Mettler-Parr density meter.

Sedimentation equilibrium measurements were made at 4.0 ± 0.1 °C, at 3000, 4500 and 6000 rpm. After attainment of equilibrium at each speed, radial absorbance distributions were recorded at 750 nm, corresponding to the absorbance maximum of Cy7-(Ni²⁺-NTA)₂. At sedimentation equilibrium, the radial distribution of absorbance is given by Eq. (1), where the sum is over all macromolecular species, *s*.

$$A(r) = \sum_s \alpha_{s,0} \exp[\sigma_s(r^2 - r_0^2)] + \zeta \quad (1)$$

In this expression $A(r)$ is the absorbance at radial position r , $\alpha_{s,0}$ is the absorbance of species s at the reference position r_0 and ζ is a baseline offset that accounts for radial-position-independent differences in the absorbances of different cell assemblies. The reduced molecular weight of species s is given by $\sigma_s = M_s(1 - \bar{v}_s \rho)\omega^2/(2RT)$. Here M_s is the molecular weight of species s ; ρ is the solvent density, ω , the rotor angular velocity ($=\text{rpm} \cdot \pi/30$), R is the gas constant (8.314×10^7 erg mol⁻¹ K⁻¹) and T the temperature (Kelvin). Partial specific volumes of proteins were estimated using the program SEDNTERP, described above. The partial specific volumes of duplex NaDNAs at 0.1 M NaCl ($\bar{v}_{ds} = 0.55$ mL/g) was estimated by interpolation of the data of Cohen and Eisenberg [36]. The partial specific volumes of protein-DNA complexes were estimated as previously described [37]. Buffer densities were measured with a Mettler-Parr density meter.

2.5. Fluorescence anisotropy measurements

Fluorescence was measured with an LS 55 Luminescence Spectrofluorometer (PerkinElmer, Waltham, MA, USA) with excitation and emission slit widths set at 10 nm and excitation and emission wavelengths of 550 and 570 nm, respectively. Anisotropy was calculated from the ratio of polarized emission intensities (Eq. (2)).

$$A = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

Here I_{VV} represents the intensity observed with vertically polarized excitation and emission, I_{VH} the intensity observed with vertically polarized excitation and horizontally polarized emission, and $G = S_V/S_H$, the ratio of detector sensitivity to vertically and horizontally polarized light [38].

3. Results

3.1. Sedimentation velocity analyses in the presence of interfering, low molecular weight solutes

The *N*-ethylmaleimide-sensitive factor (NSF) is a homohexameric ATPase ($M_r \sim 550,000$) that plays critical roles in membrane fusion reactions essential to the functions of blood platelets and nerve synapses [39]. NSF undergoes a conformational change as it cycles between ATP- and ADP-binding states [24,40] however the corresponding hydrodynamic changes have been difficult to assess because the absorbance of ATP or ADP at 260 nm masks the native absorbance of protein at 280 nm (for example a solution containing 0.5 mM ADP has an $A_{260} \sim 7.7$ cm⁻¹). To circumvent this problem, sedimentation velocity

measurements were made using detection at 550 nm with $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ -labeled His₆-NSF, in buffer containing 50 mM HEPES (pH 7.4) 100 mM KCl, 1 mM MgCl₂, 2mM β -mercaptoethanol, 5% glycerol supplemented with 0.5 mM nucleotide (ADP or AMP-PNP) as appropriate (Fig. 2). The results show that nucleotide substitution is accompanied by a significant change in sedimentation coefficient ($\Delta s_{20,w} \sim 0.9$). Further analysis of this conformational change is now underway.

3.2. Sedimentation equilibrium in the presence of high concentrations of background polymers

Biological systems often contain high concentrations of macromolecules; for example the protein concentration in blood plasma is on the order of 80 mg/mL, while the total macromolecular concentration in *E. coli* cytoplasm has been estimated to be 340 mg/mL [41,42]. The high absorbance and refractive index values of such solutions represent a barrier to studies of macromolecular properties under native-like conditions, using standard spectrophotometric or interferometric methods. To overcome this problem, Minton and colleagues developed a post-centrifugation fractionation method that allows other properties, such as radioactivity or antibody binding to be used to detect the radial concentration distributions of the molecules of interest [13,14,17]. An alternate approach is to label the macromolecule of interest with a chromophore that absorbs outside of the spectral range of the background polymer(s) but still within the accessible range (190–800 nm) of the absorption optics of XL-A and XL-I centrifuges [9,10]. $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy}$ dyes provide a simple way to accomplish that labeling.

Shown in Fig. 3 is a sedimentation equilibrium experiment characterizing the interaction of a small His₆-tag-containing DNA-repair protein (O^6 -alkylguanine DNA alkyltransferase, AGT; $M_r = 21,519$ [43]) with negatively supercoiled pUC19 plasmid DNA (2686 bp, $M_r \sim 1,778,000$ [44]), in the presence of 160 mg/mL ovalbumin ($M_r = 42,780$ [45]). In the absence of ovalbumin, AGT binds this DNA to form complexes containing ~ 60 protein molecules ($M_r \sim 3,070,000$; M. Melikishvili, unpublished result). For the experiments shown in Fig. 3, the AGT protein had been labeled with $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy7}$ and absorbance data was collected at 750 nm. The data are noisier than is usual for absorbance data collected at near-UV wavelengths. We attribute this to the relatively low intensity of the light source at high wavelengths [46]. At 750 nm, the native absorbances of AGT protein and DNA are not detectable, so the data were fit with a two-species version of Eq. (1), with terms corresponding to free $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy7-AGT}$ and a $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy7-AGT-DNA}$ complex. This returned apparent molecular weights of $15,500 \pm 1080$ for the low molecular weight species and $698,000 \pm 19,300$ for the high molecular weight species. These values are considerably less than those observed for AGT and AGT-DNA complexes in the absence of ovalbumin. Although this is an expected effect of volume exclusion in these crowded solutions [16,47], it complicates assignment of species and interpretation of the experimental results. Two additional considerations lead us to a provisional identification of these species. First, the high molecular weight component was not observed when $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy7-AGT}$ was brought to sedimentation equilibrium in ovalbumin solutions in the absence of DNA and $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy7}$ was not found to co-sediment with ovalbumin in mixtures that contained only the dye and that protein (results not shown). Together these features suggest that the high molecular weight species is a complex containing AGT and DNA. Further analysis will be required to characterize the non-ideal sedimentation observed with this system and the effects of macromolecular crowding on its association equilibria.

3.3. Simplified observation and assignment of species in complex heteroassociation reactions

Protein oligomerization can greatly increase the difficulty of protein interaction analysis by analytical ultracentrifugation. For example, shown in Fig. 4 are $c(s)$ and $c(M)$ spectra for purified Gal3 and Gal80 gene-regulatory proteins from the yeast, *Saccharomyces cerevisiae*, obtained using the native absorbance of these proteins at 290 nm. According to a widely accepted model [48,49], these proteins interact in an ATP- and galactose-dependent manner to control the induction the *gal* gene system. On the basis of sequence [50], the monomer molecular weights of the Gal3 and Gal80 proteins are 58,127 g/mol and 48,321 g/mol, respectively. However, it is evident from the $c(M)$ distributions that both of these proteins form populations of multimers. If Gal3 protein binds Gal80, the large number of possible oligomeric species and the similarities in size of contributing proteins would make even a preliminary assignment of protein stoichiometries in these complexes challenging indeed. A further complication is that these interactions take place at $[ATP] \geq 1$ mM, masking the protein absorbance at 280 nm ($\epsilon_{260, \text{mM}} \sim 15.4 \text{ cm}^{-1}$). The data shown in Fig. 4 were acquired at 290 nm to minimize this interference, but poor detection sensitivity at this wavelength required use of relatively high concentrations of each protein (14 μM). This increases the mole fractions of protein oligomers in equilibrium with monomeric forms, adding to the complexity of the sedimentation pattern.

Labeling one of the proteins (in this case Gal3) with $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ and monitoring absorbance at 550 nm reduces these problems (Fig. 5). First, sensitivity is increased, allowing lower protein concentrations to be used. Mass action thus simplifies analysis by reducing the mole fractions of protein oligomers. Second, in solutions containing His₆-tagged Gal3 and un-tagged Gal80, the dominant contributions to absorbance are from species containing Gal3-His₆-($\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$. Small peaks in the $c(s)$ spectrum of a sample containing un-tagged Gal80 protein and $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ indicate that detectable dye binding does take place, but at these protein and dye concentrations, the absorbance of dye bound to un-tagged Gal80 protein is small in comparison to that bound to His₆-tagged Gal3. This labeling specificity allows provisional assignment of Gal3-Gal80 complexes by comparison of the $c(s)$ spectrum of Gal3-His₆-($\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ alone with that of Gal3-His₆-($\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ in the presence of Gal80 protein (peaks labeled with asterisks in Fig. 5). The apparent molecular weights of the complexes containing both Gal3 and Gal80 allow us to generate hypotheses about their stoichiometries that can be tested by standard methods [37,51,52]. A more complete analysis of these interactions will be reported elsewhere.

3.4. Equilibrium constants, complex lifetimes and use of $(\text{Ni}^{2+}\text{-NTA})_2\text{-CyX}$ dyes in tracer sedimentation

Several factors influence the utility of $(\text{Ni}^{2+}\text{-NTA})_2\text{-CyX}$ protein labels in sedimentation analyses. Perhaps the most important is the equilibrium stability of the dye complex with histidine tags. Dissociation constants on the order of 5×10^{-7} M have been found for $(\text{Ni}^{2+}\text{-NTA})_2\text{-CyX}$ interactions with His₆-tagged proteins in a range of buffers [24,28,31]; this value can be reduced by 5- to 10-fold if a His₁₀-tag is used [31]. Tri-NTA dye systems have been synthesized and may offer a further improvement in affinity [53,54], however at present, the modest affinity of $(\text{Ni}^{2+}\text{-NTA})_n\text{-dyes}$ for His_X-tags determines the concentrations of dyes and target proteins that give useful binding densities.

Specific binding compensates in part for the modest affinities described above. The simplest demonstration of specificity uses $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ as a stain for His₆-tagged proteins in an SDS gel (Fig. 6). This allows a large number of proteins to be screened in parallel for $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ binding; in this experiment, significant binding was only found for proteins that

carried His₆-tags (described in the figure caption). However, data from Ni²⁺-NTA-affinity chromatography [55] suggests that some proteins that lack an artificial His₆ moiety may bind the Ni²⁺-NTA structures of (Ni²⁺-NTA)₂-CyX dyes and it is possible that proteins with affinity for planar aromatic ligands may bind the cyanine groups as well. The weak-but-detectable binding of (Ni²⁺-NTA)₂-Cy3 to the untagged Gal80 protein (Fig. 5) is likely to result from one or both of these mechanisms.⁴

A second important feature for tracer sedimentation experiments is the typical lifetime of the His₆-(Ni²⁺-NTA)₂-CyX complex. Under standard buffer conditions (10 mM Tris (pH 7.5), 100 mM NaCl, 2 mM DTT) AGT-His₆-(Ni²⁺-NTA)₂-Cy7 binding densities were stable during sedimentation equilibrium experiments that lasted 8 days (L. Hellman, unpublished result), indicating that both protein and (Ni²⁺-NTA)₂-Cy3 are long-lived under these conditions, as required for sedimentation equilibrium experiments. On the other hand, reagents that destabilize the His₆-(Ni²⁺-NTA)₂ interaction, such as EDTA or imidazole, can induce net dissociation and depending on concentration, can reduce complex lifetimes to just a few minutes (Fig. 7A). Rapid dissociation/reassociation reactions may give rise to reaction boundaries during sedimentation velocity experiments [56,57]. Since one or both of these reagents may be present in proteins purified by immobilized metal chromatography, it is important to ensure that they are thoroughly removed before sedimentation velocity analyses of protein-His₆-(Ni²⁺-NTA)₂-CyX systems is undertaken. When these reagents are not present, estimates of the exchange rate of dye between His₆ moieties provides lower-limit estimates of the kinetic stability of protein-His₆-(Ni²⁺-NTA)₂-CyX complexes in the absence of exchange (described below).

Hexahistidine polypeptide (His₆) acts as a sink for free (Ni²⁺-NTA)₂-CyX dyes in measurements of the dissociation kinetics of protein-His₆-(Ni²⁺-NTA)₂-CyX complexes [24]. These measurements provide a basis for estimating the lifetime of dye-protein complexes during sedimentation experiments. Addition of His₆ to NSF-His₆-(Ni²⁺-NTA)₂-Cy3 resulted in a time-dependent decrease in fluorescence anisotropy, consistent with transfer of the dye to the lower molecular weight His₆-peptide or with its dissociation from the NSF protein into free solution (Fig. 7B). As peptide concentration increased from 1 to 200 μM (a 5- to 1000-fold molar excess over His₆-NSF), this sum of dissociation and exchange rates increased from $5.3 \pm 0.3 \times 10^{-6} \text{ s}^{-1}$ to $4.4 \pm 0.5 \times 10^{-5} \text{ s}^{-1}$; these rates correspond to characteristic lifetimes of ~50 h in a solution containing 1 μM His₆-peptide and ~6 h in a solution containing 200 μM His₆-peptide. The dependence of log (rate) on log [His₆-peptide] (Fig. 7C) shows that the kinetic order of the reaction in [His₆-peptide] is ~0.4. The simplest model consistent with these data is one in which ~40% of the dissociation is first order in His₆-peptide and ~60% is peptide-independent. Thus the dissociation rate measured at the lowest His₆ concentration rate provides an upper limit estimate for the rate of dissociation in the absence of exchange. It follows that the lifetime of the NSF-His₆-(Ni²⁺-NTA)₂-Cy3 in the absence of exchange must exceed 50 h. This interval is nearly a factor of 10 longer than a typical sedimentation velocity run; under these slow-exchange conditions the (Ni²⁺-NTA)₂-CyX should sediment with the protein(s) to which it is bound and not as a reaction boundary with properties intermediate between those of the dye-protein complex and the free dye.

4. Discussion

At present, we are at an early stage in the development of oligohistidine-tag-directed optical probes and much remains to be done to improve their affinities and specificities.

⁴These mechanisms are easy to distinguish: the binding of Ni²⁺-NTA to His₆-proteins is reversed by addition of a slight molar excess of EDTA, while in most cases binding via the dye moiety will not be sensitive to modest concentrations of this reagent.

Improvement is likely to result from increases in the number of NTA units per dye molecule [53,54], from use of longer His_X-tags [31] or other tags with elevated metal affinities [58], and from the use of fluorophores with reduced susceptibility to quenching by metal chelates (Table 1). However, even with these improvements, the easy reversibility that makes the metal–NTA–His_X-tag interaction useful in preparative biochemistry will continue to be a feature of its analytical roles. For this reason, experiments should be designed to tolerate low concentrations of free dye. The low molecular weights ($M_r \sim 1200$) of the (Ni²⁺–NTA)₂–CyX dyes make free and bound forms easy to distinguish in sedimentation experiments with most His_X-tagged proteins and large polypeptides, but the presence of sub-micromolar or micromolar concentrations of (Ni²⁺–NTA)₂–CyX raises the possibility of non-specific binding to the target protein or to other macromolecules in the sample mixture. Non-specific binding via the metal–NTA moiety may be detected in control experiments that use EDTA to extract the metal and to compete with NTA–protein interactions, while chromophore-binding may be detected by monitoring the fluorescence anisotropy or sedimentation of the parent cyanine dye added to the protein system.

A related consequence of weak, reversible dye binding is that quantitative labeling of His_X-tagged proteins is difficult to achieve. This does not preclude the use of (Ni²⁺–NTA)₂–CyX as a tracer in sedimentation experiments as long as the optical signal is proportional to the concentration of the His_X-tagged protein of interest. With the current generation of (Ni²⁺–NTA)₂–CyX probes, under typical buffer conditions, high binding densities that are not sensitive to dye- and His₆-tagged protein concentrations are attained when dye and protein concentrations are $>10 K_d$. For systems tested to date, $K_d \sim 5 \times 10^{-7}$ M [24,28,31], but this value may differ for other protein and buffer combinations and should be verified for each molecular system.

All tracer experiments suffer from the possibility that the probe might perturb the experimental system. Thus, it is important to verify that labeling with (Ni²⁺–NTA)₂–CyX does not modify ligand binding or enzymatic activities of the target protein or its sedimentation properties. The widely-successful use of immobilized metal chromatography suggests that most proteins will tolerate the conditions under which labeling is performed, while the ability to use immobilized metal chromatography to purify the His_X-tagged protein of interest should simplify comparison of relevant properties of (Ni²⁺–NTA)₂–CyX-labeled and unlabeled proteins.

Despite these caveats, His_X-tag directed optical probes are likely to become useful tools for the study of macromolecules and their interactions. For instance, (Ni²⁺–NTA)₂–CyX dyes offer an attractive route to labeling proteins for analysis by fluorescence-detected analytical ultracentrifugation. In addition, these dyes offer the possibility of combined characterizations in which fluorescence analyses using anisotropy or FRET are paired with hydrodynamic studies. The slow exchange of (Ni²⁺–NTA)₂–CyX between low concentrations of His₆-tagged proteins may allow more than one dye label to be used at a time; the possibility of monitoring non-overlapping optical signals from different components of a complex mixture calls for further development. Finally, the ability to shift the absorbance signal of a protein away from the near UV region makes it possible to perform analytical ultracentrifugation experiments in the presence of chromophores that would otherwise interfere with detection (see Figs. 2 and 3). This should simplify studies of macromolecular interactions in complex biological solutions and at concentrations that approach those found *in vivo*.

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Abbreviations

IMAC	immobilized metal affinity chromatography
NTA	nitrilotriacetic acid
GFP	green fluorescent protein
FRET	fluorescence resonance energy transfer
NSF	<i>N</i> -ethylmaleimide-sensitive factor
AGT	O ⁶ -alkylguanine DNA alkyltransferase
CyX	any of the Cy3, Cy5 or Cy7 cyanine dyes
His_X	oligohistidine
His₆	hexahistidine

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Fig. 1. Structure of $(\text{Ni}^{2+}\text{-NTA})_2\text{-CyX}$, shown schematically in complex with hexahistidine. For the Cy3 dye, $X = 1$, for the Cy5 dye, $X = 2$ and for the Cy7 dye, $X = 3$.

Fig. 2. Sedimentation velocity characterization of NSF in the presence of ADP and AMP-PNP. (A) Sedimentation of $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-labeled His}_6\text{-NSF}$, at 10 °C and 15,000 rpm. The sample buffer contained 50 mM HEPES (pH 7.4) 100 mM KCl, 1 mM MgCl_2 , 2mM β -mercaptoethanol, 5% glycerol supplemented with 0.5 mM ADP. Absorbance measurements were made at 550 nm. Scans were taken at 4 min. intervals. (B) $C(s)$ spectra for samples of NSF in buffer containing 0.5 mM ADP (●) or 0.5 mM AMP-PNP (■). (A) reprinted from [24], with permission.

Fig. 3.

Sedimentation equilibrium data for a solution containing AGT-His₆-(Ni²⁺-NTA)₂-Cy7 and pUC19 plasmid DNA in a concentrated ovalbumin solution. This sample contained labeled AGT (5.1×10^{-6} M), supercoiled pUC19 DNA (6.4×10^{-8} M) and ovalbumin (160 mg/mL) in a buffer consisting of 10 mM Tris (pH 7.6), 2 mM DTT, 75 mM KCl. Sedimentation was at 6000 rpm and 4 °C; the radial absorbance scans taken at 750 nm. The smooth curve is a fit to the two-species version of Eq. (1), with terms corresponding to free (Ni²⁺-NTA)₂-Cy7-AGT and a (Ni²⁺-NTA)₂-Cy7-AGT-DNA complex. The symmetrical distribution of residuals around zero indicates that this model is compatible with the data.

Fig. 4. Sedimentation velocity analyses for un-tagged yeast Gal3 and Gal80 proteins sedimented separately. Above: $c(s)$ distributions for Gal3 protein (14 μM , red line) and Gal80 protein (14 μM , black line) in buffer containing 1 mM ATP and 25 mM galactose. Absorbance scans made at 290 nm. Below: the corresponding $c(M)$ distribution showing apparent molecular weights. The numbers indicate provisional assignment of oligomeric state for Gal3 (red) or Gal80 (black), based on sequence molecular weight.

Fig. 5.

Sedimentation of $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-His}_6$ Gal3 proteins with and without unlabeled Gal80 protein. Above: $c(s)$ distributions for $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-His}_6$ Gal3 protein (5 μM , red line), $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-His}_6$ Gal3 protein plus un-tagged Gal80 protein (5 μM each, blue line), and un-tagged Gal80 protein plus 5 μM $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ dye (black dashed line). Asterisks indicate species that are not detected in solutions containing only the $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-His}_6$ Gal3 protein. Absorbance scans were made at 550 nm. Below: the corresponding $c(M)$ distributions showing apparent molecular weights.

Fig. 6. Specific interaction of $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ with His-tagged proteins. Staining of His₆ proteins after SDS-PAGE. Samples preparation and electrophoresis was as described [24]. Samples were His₆-NSF (0.06 nmol, 5 μg , lane 1), His₆- α -SNAP (0.23 nmol, 8 μg , lane 2), un-tagged ovalbumin (0.11 nmol, 5 μg , lane 3), and *E. coli* cell extract containing His₆-AdaC protein (total protein 50 μg , lane 4). Left panel: Fluorescence image of gel ($\lambda_{\text{ex}} = 532$ nm, $\lambda_{\text{em}} = 580$ nm) obtained with a Typhoon 9400 imaging system. Right panel: the same gel after staining with Coomassie Blue R-250, visualized with white light. Relative molecular weights (kDa) of protein standards are indicated in the left margin. Reprinted from [24], with permission.

Fig. 7.

Dissociation kinetics of the $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3-His}_6\text{-NSF}$ monitored by fluorescence anisotropy. Reactions were carried out with $0.2 \mu\text{M}$ $(\text{Ni}^{2+}\text{-NTA})_2\text{-Cy3}$ and $1 \mu\text{M}$ His₆-NSF in 50 mM Hepes/KOH (pH 7.4), 100 mM KCl, 1 mM MgCl_2 , 2mM β -mercaptoethanol, 5% glycerol, 0.5 mM ADP. (A) Reactions initiated by addition of the indicated concentrations of EDTA or imidazole (imid). (B) Mixed dissociation and His₆-exchange reactions. Reactions initiated by addition of His₆ polypeptide to give final concentrations of $0 \mu\text{M}$ ($\bullet$), $1 \mu\text{M}$ ($\blacksquare$), $10 \mu\text{M}$ ($\blacktriangle$), $20 \mu\text{M}$ ($\circ$), $100 \mu\text{M}$ ($\square$), and $200 \mu\text{M}$ ($\triangle$). The lines are linear fits to the data. (C) Dependence of $\log$ (initial rate) on $\log [\text{His}_6 \text{ peptide}]$ for dye exchange between His₆ groups. The line is a linear fit of the data from (B) with a slope of 0.40 ± 0.03 .

Table 1

Properties of (Ni²⁺-NTA)₂-CyX Dyes.

	<i>R</i> _f ^a	<i>M</i> _W	<i>λ</i> _{max,ex} (nm)	<i>λ</i> _{max,em} (nm)	Ni ²⁺ quenching ^b	Approx. extinction coefficient ^c (M ⁻¹ cm ⁻¹)
(Ni ²⁺ -NTA) ₂ -Cy3	0.24	1205	553	565	~2.2	<i>ε</i> ₅₅₀ = 150,000
(Ni ²⁺ -NTA) ₂ -Cy5	0.26	1231	650	668	~5.0	<i>ε</i> ₆₅₀ = 250,000
(Ni ²⁺ -NTA) ₂ -Cy7	0.32	1257	750	770	~3.2	<i>ε</i> ₇₅₀ = 250,000

^a Silica gel TLC developed in NH₄OH/ethanol/water (33:21:6, v/v/v).

^b Calculated as Em(apo)/Em(Ni²⁺).

^c Extinction coefficient of *N*-hydroxysuccinimidy ester [63].