The biologically and clinically important membrane transporters are challenging proteins to study because of their low level of expression, multidomain structure, and complex molecular dynamics that underlies their activity. ATP7B is a copper transporter that traffics between the intracellular compartments in response to copper elevation. The N-terminal domain of ATP7B (N-ATP7B) is involved in binding copper, but the role of this domain in trafficking is controversial. To clarify the role of N-ATP7B, we generated nanobodies that interact with binding domains 1–3. Modulation of these interactions by nanobodies in the absence of elevated copper provides direct evidence for the important role of N-ATP7B structural dynamics in regulation of trafficking.

Background: Copper regulates the intracellular localization of a copper transporter ATP7B; the molecular mechanism of this process is unclear.

Results: Nanobodies detected transient interactions between the metal-binding domains (MBDs) and modulated intracellular localization of ATP7B.

Conclusion: MBDs 1–3 form a dynamic structural module of ATP7B involved in regulation of trafficking.

Significance: Nanobodies demonstrate the importance of protein dynamics in ATP7B localization in cells.

Human Cu(I)-transporting ATPase ATP7B plays an essential role in maintaining cellular copper homeostasis. ATP7B is located in the trans-Golgi network (TGN), where it transports copper to metalloenzymes undergoing functional maturation in this compartment (Fig. 1A). Upon copper elevation, ATP7B moves from the TGN to specialized vesicles (1). In the vesicles, ATP7B sequesters excess copper for further export, which occurs via vesicle fusion at the plasma membrane. Following copper depletion, ATP7B returns from vesicles to the TGN to resume its function in the biosynthesis of cuproenzymes (1). The loss of ATP7B activity is associated with Wilson disease, a severe hepato-neurological disorder.

ATP7B has a complex multidomain architecture (Fig. 1B). The key biochemical properties of ATP7B have been characterized, and structures of several individual domains have been determined (2–8). It has also been established that ATP7B trafficking between the TGN and the vesicles is associated with changes in kinase-mediated phosphorylation (9, 10). However, the molecular mechanism that underlies ATP7B movement between intracellular compartments remains unclear. The N-terminal copper-sensing domain was shown to undergo conformational transitions upon copper binding (11), suggesting that structural changes could be important for initiation of ATP7B trafficking. Yet, the deletion of a large portion of this domain, including four metal-binding domains (MBDs) does not disrupt the ability of ATP7B to traffic. The removal of the entire N-ATP7B abrogates trafficking but also inactivates the pump (12, 13).

Mutational analysis revealed importance of several protein regions in regulated trafficking response. Mutations within the first transmembrane segment, which is directly linked to the N-terminal copper-sensing domain of ATP7B (N-AT7B), are inhibitory to trafficking (14), whereas mutations of residues Ser-340/341 in N-ATP7B produce a constitutive relocalization of ATP7B to vesicles (15). These and other studies led to the
suggestion that inter-domain communication plays a significant role in ATP7B trafficking. Stabilization of distinct conformation(s) during catalysis or inhibition of copper release from ATP7B upon saturation of acceptor sites in the TGN lumen are also thought to be important for copper-ATPase exit from the TGN (1, 15, 18). Testing these hypotheses and determining the role of conformational dynamics in ATP7B trafficking has been exceedingly difficult.

Nanobodies have emerged as important tools for studies of proteins in solution and at the cell surface (19). Their relatively small size, high binding affinity, and ease of chemical and genetic derivatization have triggered rapidly increasing interest in using nanobodies for various research and clinical applications (20). We generated the panel of nanobodies directed against the N-terminal copper-sensing domain of ATP7B and used the nanobodies to clarify the structural organization of N-ATP7B and the role of MBD packing in regulation of ATP7B in a cell. The strategies employed in our study should be transferable to characterization of other membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Immunization of an Alpaca and Construction of a VHH Library**—Expression and purification of the N-terminal domain of human ATP7B (N-ATP7B) fused to maltose-binding protein (MBP) was done as previously described (21). An alpaca was injected subcutaneously on days 0, 7, 14, 21, 28, and 35, each time with about 300 μg of human recombinant copper-loaded human N-ATP7B (N-ATP7B-MBP_Cu). On day 39, anticoagulated blood was collected for lymphocyte preparation. A variable heavy chain (VHH) library was constructed and screened for the presence of antigen-specific nanobodies. A VHH library of about 4 × 10^8 independent transformants was obtained. About 76% of the transformants harbored the vector with the expected insert size.

**Isolation of the Antigen-specific Nanobodies**—The library was subject to two consecutive rounds of panning, performed on a solid-phase plate coated with human N-ATP7B-MBP_Cu used as an antigen (concentration: ~100 μg/ml, ~10 μg/well). Binding of nanobodies to coated antigen was competed with 350 nM of an antigen (concentration: 1 g/ml). The eluted nanobodies were dialyzed overnight at 4 °C and His-tagged nanobodies were purified on His-select resin in regulation of ATP7B in a cell. The strategies employed in our study should be transferable to characterization of other membrane proteins.

**Expression and Purification of the Recombinant Nanobodies—E. coli** WK6 cells transformed with a nanobody-encoding plasmid were grown at 37 °C until A_{600} of 0.6–0.9, then protein expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 28 °C for 16–18 h. Cells were harvested by centrifugation at 8,000 × g, resuspended in 12 ml of TES (0.2 M Tris, pH 8.0, 0.5 mM EDTA, and 0.5 mM sucrose), shaken for 1 h on ice, diluted with 18 ml of TES/4 (4 times diluted TES), and incubated for an additional 1 h on ice. The periplasmic proteins were collected by centrifugation at 8,000 × g for 30 min at 4 °C and His-tagged nanobodies were purified on His-select resin (Sigma). The eluted nanobodies were dialyzed overnight at 4 °C against 1 liter of PBS to remove imidazole. The amount of protein was estimated by absorbance at 280 nm (A_{280}) using an extinction coefficient calculated based on protein sequence of each clone.

**Conjugation of Nanobodies with Rhodamine (Rho)**—The purified nanobodies were labeled with NHS-Rho (Pierce) following the manufacturer’s protocol. The concentration of Rho-conjugated nanobodies was determined using the following formula: [Protein] = [A_{280} – 0.34A_{555}] / ε_{prot}. Protein extinction coefficient (ε_{prot}) was calculated based on the nanobody amino acid sequence. For Western blot analysis, membrane proteins were separated on 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Following blocking in 5% nonfat milk, blots were incubated at 4 °C with Rho-labeled nanobodies overnight; the binding was detected by fluorescence imaging (Alpha Innotech).

**NMR Spectroscopy**—The 15N-labeled N-ATP7B including MBD1–6 (residues 1–633) was expressed as a fusion with the chitin binding domain and purified as described previously (5, 22). The protein was additionally purified by ion-exchange chromatography. The NMR samples contained 70–160 μM protein, 50 mM HEPES-Na, pH 7.4, 50 mM NaCl, 5 mM tris(2-carboxyethyl)phosphine, 5% (v/v) D_{2}O, and 0.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate for the chemical shift referencing. The 1H,15N-TROSY (23) experiments were recorded at 310 K on a 900 MHz Varian NMR spectrometer equipped with a triple-resonance cold probe with z axis gradients. The previously published backbone chemical shift assignments (2) were used for mapping the nanobody binding sites. Combined chemical shift change was calculated as |Δδ^{3} NH + Δδ^{5} NS| / 2^{1/3}, where Δδ^{3} NH and Δδ^{5} NS are the chemical shift changes of the amide proton and nitrogen, respectively. Nanobodies were added to the protein at 1:1 molar ratio.

For the backbone dynamics studies the longitudinal (R_{1}) and transverse (R_{2}) relaxation rates and heteronuclear 15N(1H)-NOEs were measured using TROSY-based experiments as described elsewhere (24). For the R_{1} measurements, spectra were collected with seven relaxation delay values in the range from 0 to 1200 ms; for the R_{2} measurements spectra were collected using seven relaxation delay values from 0 to 140 ms. The 15N(1H)-heteronuclear NOE values were determined from the ratio of the peak intensities measured with and without proton saturation. Data were processed using NMRPipe (25) and analyzed using NMRView (26). Relaxation rates were obtained from exponential fits of peak intensities in the
Nanobodies Reveal ATP7B Dynamics in Vitro and in Cells

corresponding series of two-dimensional $^1$H,$^{15}$N-TROSY spectra.

The overall tumbling times and the rotational diffusion tensors of the individual domains of MBD 1–6 were determined from the relaxation rates using TENSOR V2 program (27). Model-free analysis of the $^{15}$N $R_{1}$, $R_{2}$, and $^{15}$N[3H]-heteronuclear NOE parameters was performed using TENSOR V2 with five models of spectral density function: [$S^{2}$], [$S^{2}$, $R_{ex}$], [$S^{2}$, $\tau_{c}$], [$S^{2}$, $\tau_{c}$, $R_{ex}$], and [$S^{2}$, $S^{2}$, $\tau_{c}$, $\tau_{c}$], where $S^{2}$ and $S^{2}$, $S^{2}$ are generalized order parameters, $\tau_{c}$ and $\tau_{c}$ are the correlation times of the internal motions and $R_{ex}$ is the exchange contribution to the $R_{2}$ relaxation rates.

In Vitro Binding to the Full-length ATP7B—HEK293 T-Rex cells were co-transfected with the plasmids expressing human FLAG-ATP7B under an inducible tet-promoter and a plasmid constitutively expressing GFP or GFP-2R50; transfection was performed using FuGENE HD (Roche Applied Science) reagent. One day post-transfection, expression of FLAG-ATP7B was induced by 40 ng/ml of doxycycline for 24 h, then cells were harvested for membrane protein preparation. Membrane proteins were solubilized in 0.5% dodecylmaltoside, 50 mM BisTris, pH 7.0, 10% glycerol, 50 mM NaCl, incubated on ice for 30 min, and centrifuged at 20,000 × g for 10 min. Supernatants were transferred to a fresh tube, then 10% of ×10 dye (0.5 mM amino-caproic acid and 5% Coomassie Brilliant Blue G-250) was added, and the samples containing 30 µg of total protein were separated using NativePAGE™ Novex® 4–16% BisTris Gels (15 well) in a cold room. Coomassie Brilliant Blue G-250, 0.002%, was added to the cathode buffer. Following separation, the samples were transferred to PVDF membrane for Western blotting. The presence of nanobody was detected by immunostaining with rabbit anti-GFP antibody (Invitrogen); the membranes were then re-probed with rat anti-N-ATP7B antibody.

Binding of Nanobodies to ATP7B in Cells—HEK293 T-Rex cells were co-transfected with the plasmid encoding GFP or GFP-tagged nanobody (2R21, 2R50, or 4A19) and the plasmid coding for Cherry-ATP7B (kindly provided by Dr. Lelita Braiterman) using Lipofectamine 2000 (Invitrogen). After overnight transfection, cells were treated with 100 µM bathocuproine disulfonic acid (BCS) or 100 µM CuCl$_2$ for 2 h at 37 °C, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 1% gelatin and 1% BSA in PBS over-night at 4 °C. Cells were mounted with 1:1 of Fluoromount-G T:DAPI-fluoromount G (EMS) and imaged using confocal microscopy.

RESULTS

Generation of Anti-ATP7B Nanobodies—To generate nanobodies for the studies of ATP7B, we used a purified recombinant copper-bound N-ATP7B fused to MBP. Following immunization and lymphocyte isolation, total RNA was extracted to construct a library of cDNA of VHH antibodies; the library was then screened using phage display (see “Experimental Procedures”). In total, 115 colonies from two panning rounds (20 and 95 from the 1st and 2nd rounds, respectively) were selected and analyzed by ELISA for the presence of antigen-specific nanobodies in the periplasmic extracts. A clone was considered positive if it produced a signal on the N-ATP7B-MBP-coated wells (either apo- or copper-containing), which was at least 2-fold higher than the signal obtained with the MBP-coated well. Fig. 1C illustrates the ELISA results for periplasmic extracts of the 15 colonies selected for further studies. All selected clones were sequenced (Fig. 2).

Nanobodies 2R21 and 2R50 Bind to Distinct Regions of N-ATP7B—For further characterization, we initially used two nanobodies. In the ELISA screen, 2RNDCU21 (hereafter 2R21) recognized apo- and copper-bound N-ATP7B equally well, whereas 2RNDCU50 (hereafter 2R50) showed some preference for the copper-bound form of N-ATP7B (Fig. 1C). We generated homology models of 2R21 and 2R50 using their primary sequence (Fig. 1D) and the atomic coordinates of the previously characterized nanobodies (Protein Data Bank codes 2X1P and 2X1Q) as templates for Phyre2 (37). Comparison of the structural models for 2R21 and 2R50 revealed that their variable residues cluster on one side of the protein (Fig. 1E), presumably the region involved in antigen recognition.

To directly identify the regions of interactions between the nanobodies and ATP7B, we generated a $^{15}$N-labeled non-tagged soluble fragment (Met1–Gln633) that encompassed the entire N-ATP7B used for immunization including all 6 MBDs. Because of the largely independent dynamics of its individual MBDs, N-ATP7B produces very well resolved NMR spectra despite its large size (Fig. 3, a and b). The previously determined backbone chemical shift assignments for MBD1–6 (2) were used for the chemical shift perturbation analysis of TROSY spectra to map the binding sites of several nanobodies, including 2R21 and 2R50, onto N-ATP7B. The addition of 2R50 caused large chemical shift changes clustered almost exclusively in MBD3 (Fig. 3c). From the distribution of changes in the three-dimensional structure of MBD3, 2R50 appears to bind to the C-terminal region of helix α1 and the adjacent loop (Fig. 3e). The addition of 2R21 led to much smaller spectral changes (Fig. 3d); most of these were localized to MBD4, suggesting that this domain is the primary binding site for 2R21 (Fig. 3f). Assignment of the nanobody binding sites to MBD3 and MBD4 was supported by Western blot analysis, which showed that both nanobodies bind within the MBD1–4 region of the N-terminal domain excluding MB2 (Fig. 4). The two nanobodies exhibited other differences in their properties. In solution NMR studies of N-ATP7B, chemical shift perturbations caused by binding of 2R21 were much smaller compared with perturbations caused by 2R50. In contrast, Western blot detection of N-ATP7B showed stronger signals for 2R21 than 2R50. Given significant similarity of sizes of the two nanobodies, these results suggest that access of 2R21 to its epitope may be sterically obstructed in the folded protein under the conditions used for NMR, whereas the epitope is more available when the protein is partially unfolded or completely denatured. The lower reactivity of 2R50 on the Western blot may also be explained by disruption of a discontinuous epitope in the denatured protein. We have also mapped binding sites for the 1R1, 4A19, and 5A51 nanobodies, which all bind to MBD4 (Fig. 5). This result suggested a higher surface exposure and/or antigenicity of this domain.

Unlike 2R50 and 2R21, the 1R1, 4A19, and 5A51 nanobodies, when mixed with N-ATP7B at the equimolar ratio,
FIGURE 1. Main properties of ATP7B and anti-ATP7B nanobodies. A, ATP7B is targeted to the trans-Golgi network where it delivers copper to secreted copper-dependent enzymes such as ceruloplasmin (CP). Upon copper elevation, ATP7B traffics to vesicles where it facilitates copper sequestration for subsequent extrusion. B, ATP7B is a polytopic membrane-bound protein with a large N-terminal domain (orange) that consists of 6 MBD, an actuator domain (yellow), and an ATP-binding domain (light blue). ATP hydrolysis supplies energy for copper transport; both activities as well as trafficking involve complex interdomain interactions. Dynamic interactions between individual MBDs identified previously (MBD5–6) and in this work (MBD1–3) are shown by the arrows. C, ELISA testing of nanobody binding using periplasmic extracts of positive colonies. The values shown are absorbances (Abs) obtained with N-ATP7B-MBP_Cu-coated, N-ATP7B-MBP-apo-coated, and MBP-coated wells; stars point to the values for 2R21 and 2R50. D, amino acid sequences of 2R21 and 2R50 nanobody selected for this study. E, structural models of 2R21 and 2R50 with variable residues shown in color.

FIGURE 2. Protein sequence alignment for anti-ATP7B nanobodies. The alignment has been generated using ClustalW. The sequences of the nanobodies 2R21, 2R50, and 4A19 used for characterization of ATP7B in this work are highlighted with gray, italics, and bold, respectively.
caused disappearance of many NMR peaks, primarily in MBD4, rather than chemical shift changes. This result may reflect stronger binding of these nanobodies to MBD4 compared with 2R21 and the intermediate exchange regime. Complete immobilization of the MBD4-nanobody complex seems unlikely because in the case of a similarly sized 2R50 bound to MBD3 (τ_e = 8.26 ± 0.68 ns), no major peak intensity loss was observed. 

**Nanobodies Reveal Transient Inter-domain Interactions in N-ATP7B**—We have previously found that the CXXC to AXXA mutation of a single copper-binding site in either MBD2 or MBD3 triggers oxidation in two other N-terminal MBDs and a total loss of copper binding by three or four MBDs, whereas similar mutations of MBD4 or MBD6 do not affect copper binding characteristics of other MBDs (24). These results suggested that MBD2 and MBD3 work together to regulate properties of 

**FIGURE 3. Nanobodies bind to the distinct regions of N-ATP7B: 2R50 to MBD3 and 2R21 to MBD4.** 900 MHz 1H,15N-TROSY spectra of MBD1–6 were recorded with (red) and without (black) 2R50 (a) or 2R21 (b). Spectral regions containing most of the changes are shown. Signals of the residues showing significant chemical shift changes in the presence of nanobody are labeled. The combined 1H,15N chemical shift change (Δδ) caused by 2R50 (c) and 2R21 (d) binding is shown as a function of the amino acid residue sequence number. Locations of individual MBDs in the primary sequence are shown below. The chemical shift change (Δδ) caused by 2R50 (e) and 2R21 (f) binding mapped on the structures of MBD3 (2ROP) and MBD4 (2ROP) with metal binding Cys in cyan.
Nanobodies Reveal ATP7B Dynamics in Vitro and in Cells

2R50 to MBD3 caused marked alterations in the N-ATP7B dynamics, as demonstrated by the distinct changes in the transverse relaxation rates $R_2$ of the individual domains (Fig. 7A). The tumbling rate of MBD3 decreased, as shown by the increase of its correlation time $\tau_c$ from 6.4 to 8.3 ns. Simultaneously, the tumbling rates of MBD1 and MBD2 increased ($\tau_c$ decreased from 7.8 to 5.6 ns for each of these domains). In addition, the ratio of diffusion tensor components (Table 1) suggested a switch to a highly anisotropic tumbling for MBD3, and concomitant changes in the motions of MBD2. In contrast to MBD1–3, MBD4 showed only a marginal decrease in the overall correlation time, whereas the correlation time of MBD5 and MBD6 was largely unaffected.

Previous biochemical and NMR structural studies have shown that MBD5–6 domains form a distinct structural and functional unit. Consistent with this finding, the diffusion tensor ratio showed very similar changes for both domains upon R50 binding to MBD3. Changes in the motion anisotropy of MBD4–6 likely reflect spatial restrictions imposed by nanobody binding to MBD3, although changes in MBD1–3–4 interactions in the presence of the nanobody can also be a contributing factor. Altogether, these results suggested that transient interactions existed between MBD1, MBD2, and MBD3 and that these interactions were disrupted by 2R50 binding to MBD3. The $\tau_c$ values for MBD1 and MBD2 in the presence of 2R50 bound to MBD3 were similar to $\tau_c$ of the isolated MBD2 measured in a separate experiment (5.6 and 4.6 ns, respectively). This result suggests that interactions between MBD1 and MBD2 are insignificant and that, in the absence of the nanobody, both MBD2 and MBD1 interact directly with MBD3. By comparison, the addition of 1R1, which binds to MBD4, led to a large increase in the relaxation rates of MBD4 and a smaller increase in the relaxation rates of the other MBDs (Fig. 7B). This indicates that the slower tumbling of MBD4 when bound to the nanobody partially propagates to the other domains of N-ATP7B. Altogether, nanobodies allowed identification of interdomain interactions that were difficult to detect using standard approaches, helped to clarify discrepancy between existing structural and functional data, and enabled us to modulate behavior of ATP7B in cells by changing the domain arrangement in N-ATP7B in cells (see below).

Direct Detection of N-ATP7B and the Full-length ATP7B in Cell Lysates Using Nanobodies—Robust binding of nanobodies to the purified N-ATP7B enabled further studies of this domain as well as the full-length ATP7B in a more complex native-like environment. To examine whether nanobodies can be derivatized for detection of N-ATP7B and ATP7B in cell lysates, we used several approaches. First, we generated His-tagged versions of the nanobodies and expressed them in E. coli. Cell lysates containing 2R21-His$_6$, or 2R50-His$_6$ were then mixed with the E. coli cell lysates containing N-ATP7B-MBP, and protein interactions were assayed by co-purification on amylose resin (Fig. 8A). Both N-ATP7B-MBP and a His-tagged nanobody were detected by Coomassie staining in the protein fraction eluted from the amylose-resin column (Fig. 8A, top). The protein identity was verified by immunostaining with polyclonal anti-ATP7B and anti-His tag antibodies (Fig. 8A, bottom).
Second, to examine nanobody binding to the full-length ATP7B, we generated a GFP-tagged version of 2R50. Following co-transfection of the plasmids encoding GFP-2R50 and a FLAG-tagged ATP7B into HEK293 cells and protein expression, we isolated the membrane fraction and tested it for the presence of 2R50-ATP7B complexes. A stable complex between the nanobody and ATP7B was identified by immunostaining after separating the detergent-solubilized membrane proteins on a Blue Native gel. A 300-kDa band (the apparent mass of detergent-solubilized ATP7B in native gels) was detected by both polyclonal anti-ATP7B and anti-GFP antibodies. In addition, a slight increase in the apparent molecular weight was detected for ATP7B incubated with 2R50 compared with ATP7B in the absence of nanobody (Fig. 8B). Co-transfection of ATP7B with GFP alone was used as a negative control, and, as expected, GFP did not bind to ATP7B.

Finally, we conjugated nanobodies with rhodamine and used these nanobodies for direct one-step detection of the endogenous ATP7B in HEK293 and HepG2 cell lysates by Western blotting (Fig. 8C). The rhodamine-conjugated nanobodies (4A19 and 2R21) detected ATP7B in lysates from both types of cell lines.

**TABLE 1**
Dynamic characteristics of individual metal-binding domains in the context of MBD1–6

| Domain | Structure | Correlation time (τc) | Diffusion tensor component ratio (D⊥/D∥) |
|--------|-----------|-----------------------|------------------------------------------|
|        |           | Free +R50             | Free +R50                                |
| MBD1   | NA*       | 7.83 ± 0.32           | 5.65 ± 0.11                              |
| MBD2   | 2LQB      | 7.65 ± 0.35           | 5.63 ± 0.18                              |
| MBD3   | 2ROP      | 6.37 ± 0.28           | 8.26 ± 0.68                              |
| MBD4   | 2ROP      | 5.97 ± 0.26           | 5.44 ± 0.08                              |
| MBD5   | 2EW9      | 7.19 ± 0.24           | 6.94 ± 0.19                              |
| MBD6   | 2EW9      | 5.52 ± 0.08           | 5.17 ± 0.08                              |

* NA, not applicable.
Nanobodies Reveal ATP7B Dynamics in Vitro and in Cells

FIGURE 7. Transverse relaxation rates in MBD1–6 bound to 2R50 or 1R1 reveal distinct dynamic behavior of individual MBDs. The difference between the transverse relaxation rates $R_2$ in the absence and presence of the 2R50 (A) or 1R1 (B) nanobody is plotted as a function residue sequence number. Location of individual MBDs in the primary protein sequence is shown. Negative difference indicates faster relaxation (slower tumbling) with nanobody bound. Conversely, positive difference corresponds to slower relaxation in the presence of nanobody and thus faster tumbling.

Transverse relaxation rates $R_2$ in MBD1–6 bound to 2R50 or 1R1 reveal distinct dynamic behavior of individual MBDs. Location of individual MBDs in the primary protein sequence is shown.

Negative difference indicates faster relaxation (slower tumbling) with nanobody bound. Conversely, positive difference corresponds to slower relaxation in the presence of nanobody and thus faster tumbling.

Nanobodies Bind to ATP7B in Cells in Low and High Copper—

cells, and the relative intensity of signal reflected the higher natural abundance of ATP7B in HepG2 compared with HEK293. The nanobody showed a less intense signal than the polyclonal antibody against N-ATP7B, which was used as a control, likely because staining with the polyclonal antibody was enhanced by the secondary enzymatic signal amplification. A similar signal enhancement could be achieved in the future by generating a nanobody variant fused to horseradish peroxidase.

Overall, these experiments illustrated that nanobodies can be used successfully in vitro to detect ATP7B in complex, multi-component mixtures. Because milligram quantities of nanobodies can be produced in a highly purified form, it is now possible to design strategies for ATP7B purification from native sources as well as for mapping the positions of MBD3 and MBD4 in the structural studies of the full-length ATP7B.

Nanobody Effect on Intracellular Distribution of ATP7B Suggests the Role for Domain Dynamics in ATP7B Trafficking—
The difference in GFP-2R21 patterns in the absence and presence of a recombinant ATP7B suggested that ATP7B levels might be critical for GFP-2R21 binding and/or that this nanobody only detects ATP7B during biosynthesis, prior to ATP7B final folding. The results of the Western blot indicate that 2R21 and 4A19 bind well to the unfolded endogenous ATP7B in cell lysates (Fig. 8C). Consequently, to facilitate interactions of nanobodies with the endogenous ATP7B, we generated HEK293 cells that stably expressed GFP-2R21 (low affinity binder), GFP-4A19 (moderate affinity binder), or GFP (as a control). We then examined how the constitutive presence of nanobody during biosynthesis of endogenous ATP7B influences targeting and trafficking of the transporter. Fig. 10 illustrates that nanobody did not disrupt normal targeting of endogenous ATP7B to the TGN or its ability to traffic in response to copper elevation. Instead, in cells expressing GFP-2R21 or GFP-4A19, more ATP7B was detected in vesicles and plasma membrane compared with cells expressing GFP (Fig. 10A). Quantitative analysis of cells with different ATP7B localization patterns (TGN alone, TGN and vesicles, or vesicles and plasma membrane) confirmed a statistically significant increase in the number of cells with the plasma membrane localization of ATP7B in the presence of nanobodies (Fig. 10B). We conclude that nanobody-induced changes in the N-ATP7B structure or dynamics modulate the trafficking response of ATP7B.

DISCUSSION

Intracellular trafficking is essential for physiological function of ATP7B but the precise molecular mechanism of this process remains poorly understood. Our results illustrate the important residue substitutions invariably change protein structure, complicating interpretation of the results. Nanobodies offer the opportunity to study and modulate intracellular behavior of unmodified ATP7B. As a starting point for such experiments, we examined intracellular interactions of the GFP-tagged nanobodies with the recombinant and endogenous ATP7B.

Transient transfection of GFP-2R50 into HEK293 cells showed diffuse cytosolic distribution, which was indistinguishable from that of GFP. GFP-2R21 was expressed at a lower level than GFP-2R50 and showed a tight perinuclear localization, which resembled the TGN pattern, the expected localization of ATP7B under basal and low copper conditions (not shown). However, following treatment of cells with high copper (50–200 μM), which caused partial relocalization of ATP7B to the vesicles, relocalization of GFP-2R21 was not observed. This behavior was consistent with GFP-2R21 either forming aggregates or interacting only with a subpopulation of ATP7B, such as newly synthesized proteins. To provide an independent verification of the ability of GFP-2R21 to detect ATP7B in cells, we co-expressed GFP-2R21 with Cherry-ATP7B (Fig. 9A). In these experiments, we observed significant co-localization of GFP-2R21 and ATP7B. Furthermore, copper elevation caused very similar changes in the patterns of intracellular distribution of the nanobody and ATP7B (Fig. 9B), indicating that in cells the nanobody recognizes both the apo- and copper-bound forms of the protein and traffics with ATP7B.

Nanobody Effect on Intracellular Distribution of ATP7B Suggests the Role for Domain Dynamics in ATP7B Trafficking—The difference in GFP-2R21 patterns in the absence and presence of a recombinant ATP7B suggested that ATP7B levels might be critical for GFP-2R21 binding and/or that this nanobody only detects ATP7B during biosynthesis, prior to ATP7B final folding. The results of the Western blot indicate that 2R21 and 4A19 bind well to the unfolded endogenous ATP7B in cell lysates (Fig. 8C). Consequently, to facilitate interactions of nanobodies with the endogenous ATP7B, we generated HEK293 cells that stably expressed GFP-2R21 (low affinity binder), GFP-4A19 (moderate affinity binder), or GFP (as a control). We then examined how the constitutive presence of nanobody during biosynthesis of endogenous ATP7B influences targeting and trafficking of the transporter. Fig. 10 illustrates that nanobody did not disrupt normal targeting of endogenous ATP7B to the TGN or its ability to traffic in response to copper elevation. Instead, in cells expressing GFP-2R21 or GFP-4A19, more ATP7B was detected in vesicles and plasma membrane compared with cells expressing GFP (Fig. 10A). Quantitative analysis of cells with different ATP7B localization patterns (TGN alone, TGN and vesicles, or vesicles and plasma membrane) confirmed a statistically significant increase in the number of cells with the plasma membrane localization of ATP7B in the presence of nanobodies (Fig. 10B). We conclude that nanobody-induced changes in the N-ATP7B structure or dynamics modulate the trafficking response of ATP7B.

DISCUSSION

Intracellular trafficking is essential for physiological function of ATP7B but the precise molecular mechanism of this process remains poorly understood. Our results illustrate the important
role of interdomain interactions in intracellular trafficking of ATP7B. We have previously demonstrated that mutations in the flexible region connecting MBD3 and MBD4 trigger exit of ATP7B from the TGN (15). We hypothesized that these mutations produced an “open” conformation of ATP7B necessary for recruitment of cellular trafficking machinery (15). Directly demonstrating the relationship between protein molecular dynamics, conformation, and intracellular localization is challenging, especially for endogenous proteins. Therefore, the effect of nanobodies that bind to and alter dynamic characteristics of N-ATP7B on trafficking behavior of endogenous ATP7B is highly significant. Increased relocalization of ATP7B to the vesicles and plasma membrane in the presence of nanobodies is consistent with the notion that structural dynamics of N-ATP7B represent an essential component of molecular mechanism involved in trafficking of ATP7B between cellular compartments.

We further demonstrate that nanobodies can be used in the in vitro and cellular studies of a complex multidomain membrane protein to identify transient interdomain interactions and decipher the role of structural changes in the behavior of the protein in cells. Nanobodies have been employed previously to characterize membrane proteins at the cell surface and inside cells following endocytosis (31, 32). Our identification of stable complexes between nanobodies and ATP7B paves the way for purification of native ATP7B from cells and tissues. The ease with which milligram quantities of purified nanobodies can be generated in E. coli promises a reliable supply of reagents for immunodetection, including super-resolution microscopy (33), which can be further enhanced by generating fusions between nanobodies and signal amplifying enzymes. Similarly, tagging nanobodies with various intracellular trafficking and/or degradation signals should permit the manipulation of ATP7B molecules in cells.

The unique properties of nanobodies (small size and availability as recombinant protein) make them a much more versatile probe for NMR investigations of protein dynamics than conventional antibodies. The high molecular mass of IgG (~160 kDa) or even its Fab fragment (~50 kDa) hinders analysis of antibody-antigen complexes, whereas the lower molecular mass of nanobodies (~13 kDa) is ideal for NMR relaxation measurements. Nanobodies are sufficiently large to cause an easily detectable change in the tumbling rate of their target proteins, or protein domains and yet sufficiently small to obtain high-resolution NMR spectra of the complex. The availability of a palette of nanobodies with the same target region but var-

---

**FIGURE 8.** Nanobodies form stable complexes with N-ATP7B and the full-length ATP7B in cell lysates. A, cell lysates containing N-ATP7B-MBP were mixed with cell lysates containing His6-tagged nanobody, and, following a brief incubation, the complex was purified on amylose resin. Complex formation was verified by separating proteins by SDS-PAGE and subsequent Coomassie staining (top) and Western blotting (bottom). B, HEK293 cells were transfected with GFP alone, GFP-2R50, and FLAG-ATP7B; membrane fractions were isolated, and the presence of ATP7B-2R50 complexes were analyzed by immunostaining following separation of membrane proteins on a Blue Native gel. The arrow points to the 300-kDa band that is positive for both anti-GFP and polyclonal anti-ATP7B antibodies. C, direct detection of ATP7B in cell lysates using nanobody (example with 4A19 is shown, top) or polyclonal anti-N-ATP7B antibody used as a control (bottom).

---

**FIGURE 9.** GFP-2R21 binds to and traffics with Cherry-ATP7B in cells in response to copper elevation. A, HEK293 cells were co-transfected with plasmids encoding Cherry-ATP7B (red) and GFP-2R21 or GFP (green); only GFP-2R21 shows co-localization with ATP7B (merge). B, HEK293 cells were co-transfected with plasmids encoding Cherry-ATP7B and GFP-2R21, and co-localization of the two proteins was monitored under basal growth conditions or following treatment with 10 μM CuCl₂ for 24 h.
Nanobodies Reveal ATP7B Dynamics in Vitro and in Cells

Our results also suggest the possibility of using nanobodies in live cells for studies of the full-length ATP7B. The conventional way of monitoring the localization and trafficking of proteins in live cells is through the generation of GFP-tagged variants. GFP-tagged nanobodies offer a complementary approach, especially when the N- or C-terminal tags affect localization or activity of the membrane protein of interest. Regulating the activity of the membrane protein of interest. Regulating the activity of the membrane protein of interest. Regulating the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and changes that may occur during copper binding to N-ATP7B or post-translational modification of the latter. It is well established that the copper chaperone Atox1 transfers copper to N-ATP7B (34), and that MBD4 and MBD2 are the preferred docking sites for the chaperone (2). Under copper limiting conditions, Atox1 transfers copper to MBD2; this triggers structural changes in the loop connecting MBD3 and MBD4 (34). We propose that copper-Atox1 docking to MBD2 disrupts the interactions between MBD1, MBD2, and MBD3 in a fashion similar to 2R50 binding to MBD3. The loss of interdomain interactions and the changes in the connecting loops would increase dynamic independence of MBD1 and MBD3 and facilitate copper transfer from Atox1 to these domains. Indeed, at increasing molar ratios, Atox1 can transfer copper to all MBDs (2, 35, 36). Finally, unlike MBD2 and MBD3, MBD4 moves mostly independently as shown previously (2, 4) and in this work, does not influence copper-binding characteristics of other MBDs (36) and, in rat, this domain lacks copper binding function entirely (17). These properties suggest that in the context of the full-length ATP7B, MBD4 may play a purely structural role. The lower correlation time compared with most MBDs (Table 1) and a moderate decrease in the tumbling rates of MBD1–3 and MBD5–6 upon 1R1 nanobody binding to MBD4 is consistent with the role of MBD4 as a link between these two groups of dynamically correlated metal-binding domains.

Our analysis focused on the molecular motions and interactions of individual MBDs treated as distinct structural units. Their dynamic behavior suggests that the connecting loops between MBDs are highly flexible and largely disordered. Still, the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and require further studies, especially in view of reported regulatory phosphorylation in the loop regions between MBD3 and MBD4 (13) and MBD4 and MBD5 (12).

Our results also suggest the possibility of using nanobodies in live cells for studies of the full-length ATP7B. The conventional way of monitoring the localization and trafficking of proteins in live cells is through the generation of GFP-tagged variants. GFP-tagged nanobodies offer a complementary approach, especially when the N- or C-terminal tags affect localization or activity of the membrane protein of interest. Regulating the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and changes that may occur during copper binding to N-ATP7B or post-translational modification of the latter. It is well established that the copper chaperone Atox1 transfers copper to N-ATP7B (34), and that MBD4 and MBD2 are the preferred docking sites for the chaperone (2). Under copper limiting conditions, Atox1 transfers copper to MBD2; this triggers structural changes in the loop connecting MBD3 and MBD4 (34). We propose that copper-Atox1 docking to MBD2 disrupts the interactions between MBD1, MBD2, and MBD3 in a fashion similar to 2R50 binding to MBD3. The loss of interdomain interactions and the changes in the connecting loops would increase dynamic independence of MBD1 and MBD3 and facilitate copper transfer from Atox1 to these domains. Indeed, at increasing molar ratios, Atox1 can transfer copper to all MBDs (2, 35, 36). Finally, unlike MBD2 and MBD3, MBD4 moves mostly independently as shown previously (2, 4) and in this work, does not influence copper-binding characteristics of other MBDs (36) and, in rat, this domain lacks copper binding function entirely (17). These properties suggest that in the context of the full-length ATP7B, MBD4 may play a purely structural role. The lower correlation time compared with most MBDs (Table 1) and a moderate decrease in the tumbling rates of MBD1–3 and MBD5–6 upon 1R1 nanobody binding to MBD4 is consistent with the role of MBD4 as a link between these two groups of dynamically correlated metal-binding domains.

Our analysis focused on the molecular motions and interactions of individual MBDs treated as distinct structural units. Their dynamic behavior suggests that the connecting loops between MBDs are highly flexible and largely disordered. Still, the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and require further studies, especially in view of reported regulatory phosphorylation in the loop regions between MBD3 and MBD4 (13) and MBD4 and MBD5 (12).

Our results also suggest the possibility of using nanobodies in live cells for studies of the full-length ATP7B. The conventional way of monitoring the localization and trafficking of proteins in live cells is through the generation of GFP-tagged variants. GFP-tagged nanobodies offer a complementary approach, especially when the N- or C-terminal tags affect localization or activity of the membrane protein of interest. Regulating the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and changes that may occur during copper binding to N-ATP7B or post-translational modification of the latter. It is well established that the copper chaperone Atox1 transfers copper to N-ATP7B (34), and that MBD4 and MBD2 are the preferred docking sites for the chaperone (2). Under copper limiting conditions, Atox1 transfers copper to MBD2; this triggers structural changes in the loop connecting MBD3 and MBD4 (34). We propose that copper-Atox1 docking to MBD2 disrupts the interactions between MBD1, MBD2, and MBD3 in a fashion similar to 2R50 binding to MBD3. The loss of interdomain interactions and the changes in the connecting loops would increase dynamic independence of MBD1 and MBD3 and facilitate copper transfer from Atox1 to these domains. Indeed, at increasing molar ratios, Atox1 can transfer copper to all MBDs (2, 35, 36). Finally, unlike MBD2 and MBD3, MBD4 moves mostly independently as shown previously (2, 4) and in this work, does not influence copper-binding characteristics of other MBDs (36) and, in rat, this domain lacks copper binding function entirely (17). These properties suggest that in the context of the full-length ATP7B, MBD4 may play a purely structural role. The lower correlation time compared with most MBDs (Table 1) and a moderate decrease in the tumbling rates of MBD1–3 and MBD5–6 upon 1R1 nanobody binding to MBD4 is consistent with the role of MBD4 as a link between these two groups of dynamically correlated metal-binding domains.

Our analysis focused on the molecular motions and interactions of individual MBDs treated as distinct structural units. Their dynamic behavior suggests that the connecting loops between MBDs are highly flexible and largely disordered. Still, the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and require further studies, especially in view of reported regulatory phosphorylation in the loop regions between MBD3 and MBD4 (13) and MBD4 and MBD5 (12).

Our results also suggest the possibility of using nanobodies in live cells for studies of the full-length ATP7B. The conventional way of monitoring the localization and trafficking of proteins in live cells is through the generation of GFP-tagged variants. GFP-tagged nanobodies offer a complementary approach, especially when the N- or C-terminal tags affect localization or activity of the membrane protein of interest. Regulating the conformation and dynamic properties of the linker segments would affect the domain dynamics to some extent and changes that may occur during copper binding to N-ATP7B or post-translational modification of the latter. It is well established that the copper chaperone Atox1 transfers copper to N-ATP7B (34), and that MBD4 and MBD2 are the preferred docking sites for the chaperone (2). Under copper limiting conditions, Atox1 transfers copper to MBD2; this triggers structural changes in the loop connecting MBD3 and MBD4 (34). We propose that copper-Atox1 docking to MBD2 disrupts the interactions between MBD1, MBD2, and MBD3 in a fashion similar to 2R50 binding to MBD3. The loss of interdomain interactions and the changes in the connecting loops would increase dynamic independence of MBD1 and MBD3 and facilitate copper transfer from Atox1 to these domains. Indeed, at increasing molar ratios, Atox1 can transfer copper to all MBDs (2, 35, 36). Finally, unlike MBD2 and MBD3, MBD4 moves mostly independently as shown previously (2, 4) and in this work, does not influence copper-binding characteristics of other MBDs (36) and, in rat, this domain lacks copper binding function entirely (17). These properties suggest that in the context of the full-length ATP7B, MBD4 may play a purely structural role. The lower correlation time compared with most MBDs (Table 1) and a moderate decrease in the tumbling rates of MBD1–3 and MBD5–6 upon 1R1 nanobody binding to MBD4 is consistent with the role of MBD4 as a link between these two groups of dynamically correlated metal-binding domains.
help to produce nanobodies against the post-translationally modified regions of ATP7B and further aid in-cell work. ATP7B undergoes kinase-mediated phosphorylation at multiple sites including the region between MBD3 and MBD4 (10, 16) near the nanobody binding sites. Because our nanobodies were produced against a non-phosphorylated N-ATP7B, they may have lower affinity for the phosphorylated ATP7B. If further studies confirm this hypothesis, then nanobodies could be a useful tool for distinguishing modified and non-modified ATP7B.

Acknowledgments—We thank Christopher O’Grady and Eva-Maria Uhlemann for expert technical assistance. NMR data were collected at the National Magnetic Resonance at Madison (NMRFAM), which is supported by National Institutes of Health Grant P41 GM103399 and the University of Wisconsin-Madison.

REFERENCES

1. Hasan, N. M., and Lutsenko, S. (2012) Regulation of copper transporters in human cells. Curr. Top. Membr. 69, 137–161
2. Banci, L., Bertini, I., Cantini, F., Massagni, C., Migliardi, M., and Rosato, A. (2009) An NMR study of the interaction of the N-terminal cytoplasmic tail of the Wilson disease protein with copper(I)-HAH1. J. Biol. Chem. 284, 9354–9360
3. Banci, L., Bertini, I., Cantini, F., Migliardi, M., Natile, G., Nushi, F., and Rosato, A. (2009) Solution structures of the actuator domain of ATP7A and ATP7B, the Menkes and Wilson disease proteins. Biochemistry 48, 7849–7855
4. Banci, L., Bertini, I., Cantini, F., Rosenzweig, A. C., and Yatsunyk, L. A. (2008) Metal binding domains 3 and 4 of the Wilson disease protein: solution structure and interaction with the copper(I) chaperone HAH1. Biochemistry 47, 7423–7429
5. Dmitriev, O., Tsivkovskii, R., Ahldgaard, F., Morgan, C. T., Markley, J. L., and Lutsenko, S. (2006) Solution structure of the N-domain of Wilson disease protein: distinct nucleotide-binding environment and effects of disease mutations. Proc. Natl. Acad. Sci. U.S.A. 103, 5302–5307
6. Tsivkovskii, R., Eisses, J. F., Kaplan, J. H., and Lutsenko, S. (2002) Functional properties of the copper-transporting ATPase ATP7B (the Wilson’s disease protein) expressed in insect cells. J. Biol. Chem. 277, 976–983
7. Dolgova, N. V., Nokhrin, S., Yu, C. H., George, G. N., and Dmitriev, O. Y. (2013) Copper chaperone Atx1 interacts with the metal-binding domain of Wilson’s disease protein in cisplatin detoxification. Biochem. J. 454, 147–156
8. Achila, D., Banci, L., Bertini, I., Bunce, J., Giofi-Baffoni, S., and Huffman, D. L. (2006) Structure of human Wilson protein domains 5 and 6 and their interplay with domain 4 and the copper chaperone HAH1 in copper uptake. Proc. Natl. Acad. Sci. U.S.A. 103, 5729–5734
9. Vanderwerf, S. M., Cooper, M. J., Stetsen, I. V., and Lutsenko, S. (2001) Copper specifically regulates intracellular phosphorylation of the Wilson’s disease protein, a copper-carrying ATPase. J. Biol. Chem. 276, 36289–36294
10. Filankatta, R., Lewis, D., and Inesi, G. (2011) Involvement of protein kinase D in expression and trafficking of ATP7B (copper ATPase). J. Biol. Chem. 286, 7389–7396
11. DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W., and Sarkar, B. (1997) Expression, purification, and metal binding properties of the N-terminal domain of the Wilson disease putative copper-transporting ATPase (ATP7B). J. Biol. Chem. 272, 33279–33282
12. Cater, M. A., Forbes, J., La Fontaine, S., Cox, D., & Mercer, J. F. (2004) Intracellular trafficking of the human Wilson protein: the role of the six N-terminal metal-binding sites. Biochem. J. 380, 805–813
13. Forbes, J. R., Hsi, G., and Cox, D. W. (1999) Role of the copper-carrying domain in the copper transport function of ATP7B, the P-type ATPase defective in Wilson disease. J. Biol. Chem. 274, 12408–12413
14. Braiterman, L. T., Murthy, A., Jayakanthan, S., Nysae, L., Tzeng, E., Gro-

madza, G., Woolf, T. B., Lutsenko, S., Hubbard, A. L. (2014) Distinct phenotype of a Wilson disease mutation reveals a novel trafficking determinant in the copper transporter ATP7B. Proc. Natl. Acad. Sci. U.S.A. 111, E1364–E1373
15. Hasan, N. M., Gupta, A., Polischuk, E., Yu, C. H., Polischuk, R., Dmitriev, O. Y., and Lutsenko, S. (2012) Molecular events initiating exit of a copper-transporting ATPase ATP7B from the trans-Golgi network. J. Biol. Chem. 287, 36041–36050
16. Barnes, N., Barlow, M. Y., Braiterman, L., Gupta, A., Ustianyi, V., Zuzel, V., Kaplan, J. H., Hubbard, A. L., and Lutsenko, S. (2009) Cell-specific trafficking suggests a new role for renal ATP7B in the intracellular copper storage. Traffic 10, 767–779
17. Tsay, M. J., Fatemi, N., Narindrasorasak, S., Forbes, J. R., and Sarkar, B. (2004) Identification of the “missing domain” of the rat copper-transporting ATPase, atp7b: insight into the structural and metal binding characteristics of its N-terminal copper-binding domain. Biochim. Biophys. Acta 1688, 78–85
18. Petris, M. J., Voskoboinik, I., Carter, M., Smith, K., Kim, B. E., Llanos, R. M., Strausak, D., Camakaris, J., and Mercer, J. F. (2002) Copper-regulated trafficking of the Menkes disease copper ATPase is associated with formation of a phosphorylated catalytic intermediate. J. Biol. Chem. 277, 46736–46742
19. Muylvermier, S. (2013) Nanobodies: natural single-domain antibodies. Annu. Rev. Biochem. 82, 775–797
20. Hassanzadeh-Ghassabeh, G., Devoogdt, N., De Pauw, P., Vincke, C., and Muylvermier, S. (2013) Nanobodies and their potential applications. Nanomedicine 8, 1013–1026
21. Lutsenko, S., Petrukhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1997) N-terminal domains of human copper-transporting adenosine triphosphatases (the Wilson’s and Menkes disease proteins) bind copper selectively in vivo and in vitro with stoichiometry of one copper per metal-binding repeat. J. Biol. Chem. 272, 18939–18944
22. Morgan, C. T., Tsivkovskii, R., Kosinsky, Y. A., Efremov, R. G., and Lutsenko, S. (2004) The distinct functional properties of the nucleotide-binding domain of ATP7B, the human copper-transporting ATPase: analysis of the Wilson disease mutations E1064A, H1069Q, R1151H, and C1104F. J. Biol. Chem. 279, 36363–36371
23. Pervushin, K., Riek, R., Wider, G., and Wühlrich, K. (1997) Attenuated T2 relaxation by mutual cancellation of dipole–dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. Proc. Natl. Acad. Sci. U.S.A. 94, 12366–12371
24. Zhu, G., Xia, Y., Nicholson, L. K., and Sze, K. H. (2000) Protein dynamics measurements by TROSY-based NMR experiments. J. Magn. Reson. 143, 423–426
25. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biol. Chem. 269, 277–293
26. Johnson, B. A., and Blevins, R. A. (1994) NMR View: a computer program for the visualization and analysis of NMR data. J. Biol. Chem. 4, 603–614
27. Dossert, P., Hus, J. C., Blackledge, M., and Marion, D. (2000) Efficient analysis of macromolecular rotational diffusion from heteronuclear relaxation data. J. Biomol. NMR 16, 23–28
28. Dayie, K. T., Wagner, G., and Leleuvre, J. F. (1996) Theory and practice of nuclear spin relaxation in proteins. Annu. Rev. Phys. Chem. 47, 243–282
29. DiDonato, M., Hu, H. F., Narindrasorasak, S., Que, L., Jr., and Sarkar, B. (2000) Copper-induced conformational changes in the N-terminal domain of the Wilson disease copper-transporting ATPase. Biochemistry 39, 1890–1896
30. Cater, M. A., La Fontaine, S., and Mercer, J. F. (2007) Copper binding to the N-terminal metal-binding sites or the CPC motif is not essential for copper-induced trafficking of the human Wilson protein (ATP7B). Biochem. J. 401, 143–153
31. Altintas, I., Heukers, R. van der Meel, R., Lacombe, M., Amidi, M., van Bergen En Henegouwen, P. M., Hennink, W. E., Schifferers, R. M., and Kok, R. J. (2013) Nanobody-albumin nanoparticles (NANAPs) for the delivery of a multikinase inhibitor 17864 to EGFR overexpressing tumor cells. J. Control Rel. 165, 110–118
32. Zhu, J., Declercq, J., Roucourt, B., Ghassabeh, G. H., Meulemans, S., Kinne, J., David, G., Vermorken, A. J., Van de Ven, W. J., Lindberg, I., Muyldermans, S., and Creemers, J. W. (2012) Generation and characterization of non-competitive furin-inhibiting nanobodies. Biochem. J. 448, 73–82
33. Ries, J., Kaplan, C., Platonova, E., Eghlidi, H., and Ewers, H. (2012) A simple, versatile method for GFP-based super-resolution microscopy via nanobodies. Nat. Methods 9, 582–584
34. Walker, J. M., Tsivkovskii, R., and Lutsenko, S. (2002) Metallochaperone Atox1 transfers copper to the NH2-terminal domain of the Wilson’s disease protein and regulates its catalytic activity. J. Biol. Chem. 277, 27953–27959
35. Yatsunyk, L. A., and Rosenzweig, A. C. (2007) Cu(I) binding and transfer by the N terminus of the Wilson disease protein. J. Biol. Chem. 282, 8622–8631
36. LeShane, E. S., Shinde, U., Walker, J. M., Barry, A. N., Blackburn, N. J., Ralle, M., and Lutsenko, S. (2010) Interactions between copper-binding sites determine the redox status and conformation of the regulatory N-terminal domain of ATP7B. J. Biol. Chem. 285, 6327–6336
37. Kelley, L. A., and Sternberg, M. J. E. (2009) Protein structure prediction on the web: a case study using the Phyre server. Nat. Protoc. 4, 363–371