A NMR Study of the Interaction of a Three-domain Construct of ATP7A with Copper(I) and Copper(I)-HAH1

THE INTERPLAY OF DOMAINS*1

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ATP7A is a P-type ATPase involved in copper(I) homeostasis in humans. It possesses a long N-terminal tail protruding into the cytosol and containing six copper(I)-binding domains, which are individually folded and capable of binding one copper(I) ion. ATP7A receives copper from a soluble protein, the metallochaperone HAH1. The exact role and interplay of the six soluble domains is still quite unclear, as it has been extensively demonstrated that they are strongly redundant with respect to copper(I) transport in vivo. In the present work, a three-domain (fourth to sixth, MNK456) construct has been investigated in solution by NMR, in the absence and presence of copper(I). In addition, the interaction of MNK456 with copper(I)-HAH1 has been studied. It is proposed that the fourth domain is the preferential site for the initial interaction with the partner. A significant dependence of the overall domain dynamics on the metallation state and on the presence of HAH1 is observed. This dependence could constitute the molecular mechanism to trigger copper(I) translocation and/or ATP7A translocation from the trans-Golgi network to the plasmatic membrane.

Materials and Methods

The three C-terminal metal-binding domains of ATP7A have been expressed as a single construct in Escherichia coli and purified using affinity chromatography based on the use of a His6 tag. The protein has been produced in M9 minimal medium and enriched in 15N or 15O and 13C for NMR studies. The thermal stability of apoMNK456 has been investigated through 1H–15N HSQC spectra (16) at various temperatures, from 298 K up to 323 K. The protein was metallated by titrating it with an acetonitrile complex of copper(I) after reduction with a 5-fold excess of DTT. The titration was followed through 1H–13N HSQC spectra. The MNK56 and MNK4 constructs have been similarly produced in E. coli from rich medium cultures.

The protein showed significant precipitation over a span of 1–2 days at concentrations above 500 μM. To minimize sample aggregation and obtain the best quality spectra, NMR samples were typically 200–300 μM in a 100 mM sodium phosphate buffer at pH 7.0. All spectra were acquired using cryoprobe technology at 500 (for protein dynamics measurements) or 800/900 MHz (for experiments on resonance assignment) spectrometers. The NMR frequencies of backbone nuclei have been assigned using a standard approach based on triple resonance.

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RESULTS AND DISCUSSION

The NMR spectra of MNK456 are quite crowded and showed broadening due to conformational exchange processes, thus complicating the analysis and interpretation of spectra (supplemental Fig. S1). The observed broadening experienced a significant enhancement with increasing protein concentration. The problem was particularly evident for domain 5, as also observed for the isolated domain (15). The complexity and behavior of MNK456 in solution prevented a de novo structural characterization of the protein. As the NMR structure of all of the individual domains is available (15, 19), backbone amide chemical shifts, which are excellent reporters of tertiary structure changes (20), could be compared. Fig. 1 indeed shows that the structure of the three metal-binding domains is conserved in apoMNK456, as significant variations are localized only next to linker regions and are a consequence of the peptide bonds at the N and C termini of each domain (see Equation 1 below).

$$\Delta \delta_{\text{combined}} = \sqrt{\frac{(\Delta \delta^{(1)}(H))^2 + \frac{1}{25}(\Delta \delta^{(15)}(N))^2}{2}}$$  
(Eq. 1)

After the addition of a 1.0 equivalent of copper(I) to apoMNK456, signals from the residues in the loop containing the metal-binding cysteines (loop I) of the metallated fourth and sixth domains were both already observable (supplemental Fig. S2). In the fifth domain, the signals from the residues in loop I are not observed in the apo or in the copper(I) form. The relative intensity of the signals of the metallated fourth and sixth domains indicates that they have similar affinity for copper (within 20%). When increasing the Cu:MNK456 ratio, broadening of several signals within and close to the metal-binding loops of all three domains is observed. In the fifth domain most signals, even far from the metal-binding region, become extremely weak or undetectable.

Based on $^{15}$N relaxation data, a distinct dynamic behavior is observed in different MNK456 regions (Fig. 2). Each of the three domains appears to be individually rigid in solution on the subnanosecond time scale, as shown by the relatively uniform $^{15}$N $R_2$ and NOE values within each domain, whereas the first linker region experiences extensive dynamics experiments and are given in the supplementary material for both the apo and the fully metallated state (Cu$_3$MNK456) (supplemental Tables S1 and S2). The backbone dynamics of MNK456 was studied in the apo form as well as in Cu$_3$MNK456 through the analysis of $^{15}$N $R_1$ and $R_2$ relaxation rates and heteronuclear $^{1}H-^{15}N$ NOEs (17).

The interaction with the human copper(I) chaperone HAH1 has been studied through NMR by titrating isotopically enriched apoMNK456 with unlabeled copper(I)-HAH1 up to a HAH1:MNK456 molar ratio of 8:1. In addition, the backbone dynamics of MNK456 was also studied in the presence of HAH1 at various molar ratios (1:1, 2:1, 4:1). ApoHAH1 was purified from rich medium cultures as described previously (18), reduced with 2-fold DTT, and then metallated by adding a slight excess of [Cu(CH$_3$CN)$_4$]$^{+}$ in a N$_2$ atmosphere chamber. Samples were then passed on a desalting column to remove DTT and acetonitrile. The degree of metallation of HAH1 was checked by NMR and found to be essentially 100%. For titrations, copper(I)-HAH1 was added to reduced apoMNK456 samples from which DTT had been removed using a desalting column, in a N$_2$ atmosphere chamber. Relative ratios of HAH1:MNK456 were measured from the intensity of well resolved NMR signals in the methyl region.

For chemical labeling experiments, 400 pmol of apoMNK456 alone or in the presence of copper(I)-HAH1 were incubated with a 50-fold molar excess of the cysteine-directed reagent 7-diethylamino-3-(4'-maleimidophenyl)-4-methylcoumarin (CPM, Sigma) for 2.5 min in the dark under anaerobic conditions. The reaction was quenched with a 10-fold molar excess of β-mercaptoethanol over CPM. The same experimental setup has been used for MNK56 and MNK4. To investigate the role of the various domains within MNK456, the latter construct was partially proteolyzed by trypsin at a 1:2000 (w/w) ratio for 3 h at room temperature. The reaction was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride (Sigma) protease inhibitor. The proteolyzed MNK456 fragments were separated on a 15% Tricine gel, and the separation of the CPM-labeled peptides was monitored under UV light using a Gel-Doc system. The protein fragments were also stained with Coomassie R250. The present protocol is essentially the same as that described elsewhere (13). In the case of the MNK56 and MNK4 constructs, no digestion was performed.
on the same time scale. The overall dynamics of the multidomain construct is somewhat complex. Domain 4 reorients in solution more freely than the other two domains, being allowed to do so by the long flexible linker 1 (shown in Fig. 2 by the higher $R_1$ and lower $R_2$ values). Indeed, the correlation time for tumbling in solution of domain 4, as calculated from $R_2/R_1$ values, is significantly lower than for domains 5 and 6 ($7.1 \pm 0.3$, $12.8 \pm 1.3$, and $10.8 \pm 1.3$ ns for domains 4, 5, and 6 respectively). Note that the above values for the correlation times should be regarded as estimates because they are based on the assumption of isotropic reorientation in solution. Conformational equilibria are present in the multidomain construct as well. However, the kinetic process is sufficiently rapid to allow for an overall description by a single correlation time, which is obtained by averaging the $R_2$ values. This correlation time is significantly lower for domain 4, as calculated from $R_2/R_1$ values, which is in agreement with the similarity in the tumbling of the two C-terminal domains. The similarity in the tumbling rate of the two C-terminal domains is in agreement with the similarity of the reorientation in solution. Conformational equilibria are present in the multidomain construct as well. However, the kinetic process is sufficiently rapid to allow for an overall description by a single correlation time, which is obtained by averaging the $R_2$ values. This correlation time is significantly lower for domain 4, as calculated from $R_2/R_1$ values, which is in agreement with the similarity in the tumbling of the two C-terminal domains.

When apoMNK456 is presented with copper(I)-HAH1, the metal ion is transferred from the chaperone to the ATPase. As shown in Fig. 4, signals of Cys$^{17}$ and Val$^{18}$ in the metal-binding loop (loop I) of the apo form of domain 4 disappear upon the addition of copper(I)-HAH1 at a HAH1:MNK456 ratio of 1:1 (i.e. when only one copper(I) ion is available per MNK456 molecule), suggesting a slow kinetics of exchange similar to isolated MNK2 and MNK5 domains. A reduction of the intensity of signals from the metal-binding loop of domains 5 and 6 is also observed during the titration, but this is much shallower than for domain 4, suggesting that in the initial stages (HAH1:MNK456 ratio up to 1.0) of the titration copper is preferentially transferred to domain 4. The chemical shift variations observed during the titration are very small. Signals from copper(I) domain 6 appear at a HAH1:MNK456 ratio of 2.1, showing that copper(I) eventually is loaded in this domain. To analyze independently the possible preferential interaction of copper(I)-HAH1 with a single domain within the present three-domain construct, an experiment based on chemical labeling with a fluorophore and proteolysis, as applied to the Wilson protein (13), was performed (Fig. 5). Cysteines become labeled by the fluorophore only when they are not engaged in any bond, i.e. when they are reduced and not bound to the metal. Therefore, the detection of the modification by the chemical label, or absence thereof, is indicative of the copper(I)-binding state (13). The experiment shows that copper(I)-HAH1 does transfer the metal to MNK456, as HAH1 is (partly) labeled by the fluorophore (separate experiments, not shown, demonstrate that copper(I)-HAH1 does not react with the chemical label). In the presence of excess copper(I)-HAH1 (Fig. 5, rightmost lane), one of the bands from the MNK456 digest disappears, demonstrating preferential protection from the cysteine-directed probe caused by copper(I) transfer (13). The molecular weight of the disappearing band is about 8,000 Da, indicating that it contains a single domain. As discussed previously for the Wilson protein (13), this experiment shows that copper(I)-HAH1 selects an individual domain in the present three-domain construct as the initial interaction site. The results of NMR experiments (Fig. 4) are fully con-
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FIGURE 4. Variation of the intensity of selected signals of residues in domain 4 during the titration of 15N-enriched apoMNK456 with unlabeled copper(I)-HAH1. All signal intensities have been referenced to the intensity of the signal of Cys17 in the absence of HAH1.

FIGURE 5. Interaction of MNK456 with copper(I)-HAH1 revealed through chemical labeling of free (i.e. not bound to the metal) cysteines with a fluorescent probe. From left to right: first lane, apoMNK456; second lane, apoMNK456 treated with trypsin; third to fifth lanes, apoMNK456 incubated with increasing concentration of copper(I)-HAH1 (MNK456:HAH1 ratios of 1:1, 3:1, and 5:1, respectively). The disappearing band from an MNK456 fragment and the band corresponding to apoHAH1 are indicated by arrows.

FIGURE 6. Interaction of MNK56 and MNK4 with copper(I)-HAH1 revealed through chemical labeling. From left to right: first lane, apoMNK56; second lane, apoMNK56 in the presence of excess copper(I)-HAH1; third lane, apoMNK4; fourth lane, apoMNK4 in the presence of excess copper(I)-HAH1. The bands of MNK56, MNK4, and apoHAH1 (formed upon copper(I) transfer) are indicated by arrows. Lanes containing only apoMNK are labeled with a minus sign at the top; lanes containing apoMNK incubated with copper(I)-HAH1 are labeled with a plus sign.

NMR shows that the presence of copper(I)-HAH1 affects protein dynamics with respect to isolated apoMNK456 (Fig. 3B), inducing slower tumbling in domain 4 (but to a smaller extent than Cu2+MNK456) as well as enhanced conformational averaging. Instead, in domains 5 and 6 copper(I)-HAH1 induces faster tumbling (tumbling correlation times reduced respectively by 1.4 ± 2.4 and 1.0 ± 1.9 ns). In addition, the presence of HAH1 causes conformational exchange in the linker I region, so that signals from residues in this region are broadened beyond detection.

The present data are consistent with a mechanism for copper(I) transfer to MNK456 from HAH1, where the first entry point for the metal ion into the ATPase is provided by domain 4. This is presumably because of MNK4 having a better electrostatic and/or steric complementarity to the physiological partner. Shielding of domains 5 and 6 by domain 4 in MNK456 is unlikely to play a role in tuning the interaction, as the two-domain construct MNK56 does not interact with HAH1 (Fig. 6). Domain 4 has a surface electrostatic potential relatively similar to that of the two metal-binding domains of Ccc2, the yeast homolog of ATP7A, which form a relatively stable adduct with their partner (22, 23). It is also relevant that the pI of domain 4 in ATP7A, as well as in ATP7B, is the most similar to that of both domains of yeast Ccc2 (4.0–4.5). Other ATP7A domains, in which the surface is somewhat dissimilar from Ccc2, do not form such an adduct and feature a slow kinetic of interaction with HAH1 (15). A role for domain 4 as the preferential interaction site for HAH1 in ATP7B has been proposed recently (14), possibly together with domain 2, whereas earlier work identified only domain 2 (13). Notably, domain 2 has a pI slightly higher than 8.0 in ATP7A and ATP7B, thus quite different from domain 4, but the electrostatic surface at the putative interaction site is still compatible with that of HAH1 (13). The present data show that copper(I) is anyway distributed over the various domains. It is not possible to ascertain whether transfer to domains other than the fourth happens by way of direct interaction with domain 4 or HAH1 or through the release of free copper(I) in solution. The latter mechanism appears quite unlikely to be physiologically relevant.

A second noteworthy conclusion from the present work is that the dynamics of the multidomain MNK456 construct is quite elaborate, depending significantly on the presence of copper(I) and HAH1. This can be the result both of a modulation of interdomain contacts and of the variation of dynamics in the linker I region. There is also some unexpected modulation in the dynamic processes involving the interaction between domains 5 and 6, permitted by the relatively short linker II. It can be suggested that the dependence of domain dynamics on copper(I) and HAH1 (which might be associated also to changes of the time-averaged protein conformation) constitutes a molecular mechanism to signal (internally to the other domains and/or externally to other biomolecules) the status of copper(I) loading of the cytoplasmic tail. Indeed, the present data provide direct experimental support for previous proposals that ATP7A multiple domains could modulate protein relocalization and/or the affinity for copper(I) of the metal binding sites in the trans-membrane region through copper(I)-dependent variations in the conformation and/or dynamics of the cytoplasmic tail (11, 24).

In summary, it is proposed that when interacting with the MNK456 triple domain construct, copper(I)-HAH1 preferentially donates its cargo to domain 4. The present work thus reinforces the view that interaction of ATP7A or ATP7B with its partner metallochaperone does not involve all of the domains equally, with some domains actually interacting more readily than others. This preferential interaction is presumably under kinetic control. Thermodynamically all domains

consistent with this picture and clearly suggest domain 4 as the preferential interaction site. To assess independently the identity of the domain constituting the preferential site for HAH1 docking, we separately produced MNK56 and MNK4 and repeated the chemical labeling experiments (Fig. 6). Fig. 6 clearly shows that copper(I)-HAH1 can transfer its metal cargo to MNK4 but not to MNK56. Transfer to MNK4 is demonstrated by the disappearance of the MNK4 band and the appearance of a lower molecular weight band due to apoHAH1 (Fig. 6, compare the fourth and third lanes). Instead, MNK56 reacts with the fluorophore both in absence and in presence of copper(I)-HAH1 (Fig. 6, first two lanes), demonstrating that it is unable to uptake the metal ion from the chaperone.
have a similar affinity for copper(I) (25). The metal donated by HAH1 is indeed found to be distributed over all domains in MNK4. It might be supposed that in vivo this (re)distribution happens via interdomain contacts. Loading the cytoplasmic tail of ATP7A with copper(I) and the interaction with the partner induce a significant change in ATP7A domain dynamics. This change (or the associated variation of the time-averaged protein conformation) may be relevant to activate copper(I) translocation across the membrane and/or ATP7A relocalization from the trans-Golgi to the cytoplasmic membrane.

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REFERENCES

1. Linder, M. C. (1991) Biochemistry of Copper, Plenum Press, New York
2. O’Halloran, T. V., and Culotta, V. C. (2000) J. Biol. Chem. 275, 25057–25060
3. Harrison, M. D., Jones, C. E., Solioz, M., and Dameron, C. T. (2000) Trends Biochem. Sci. 25, 29–32
4. Puig, S., and Thiele, D. J. (2002) Curr. Opin. Chem. Biol. 6, 171–180
5. Menkes, J. H., Alter, M., Steigleder, G. K., Weakley, D. R., and Sung, J. H. (1962) Pediatrics 29, 764–779
6. Arnesano, F., Banci, L., Bertini, I., and Bonvin, A. M. (2004) Structure (Camb.) 12, 669–676
7. Forbes, J. R., Hsi, G., and Cox, D. W. (1999) J. Biol. Chem. 274, 12408–12413
8. Voskoboinik, I., Strausak, D., Greenough, M., Brooks, H., Petris, M., Smith, S., Mercer, J. F., and Camakaris, J. (1999) J. Biol. Chem. 274, 20008–20012
9. Strausak, D., La Fontaine, S., Hill, J., Firth, S. D., Lockhart, P. J., and Mercer, J. F. (1999) J. Biol. Chem. 274, 11170–11177
10. Huster, D., and Lutsenko, S. (2003) J. Biol. Chem. 278, 32212–32218
11. Pufahl, R. A., Singer, C. P., Pearson, K. L., Lin, S.-J., Schmidt, P. J., Fahrni, C. J., Cizewski Culotta, V., Penner-Hahn, J. E., and O’Halloran, T. V. (1997) Science 278, 853–856
12. Walker, J. M., Huster, D., Ralle, M., Morgan, C. T., Blackburn, N. J., and Lutsenko, S. (2004) J. Biol. Chem. 279, 15376–15384
13. van Dongen, E. M., Klop, L. W., and Merkx, M. (2004) Biochim. Biophys. Res. Commun. 323, 789–795
14. Banci, L., Bertini, I., Chasapis, C., Ciofi-Baffoni, S., Hadjiladas, N., and Rosato, A. (2005) FEBS J. 272, 865–871
15. Bodenhausen, G., and Ruben, D. J. (1980) Chem. Phys. Lett. 69, 185–188
16. Ishima, R., and Torchia, D. A. (2000) Nat. Struct. Biol. 7, 740–743
17. Anastassopoulou, J., Banci, L., Bertini, I., Cantini, F., Katsari, E., and Rosato, A. (2004) Biochemistry 43, 13046–13053
18. Gitschier, J., Moffat, B., Reilly, D., Wood, W. L., and Fairbrother, W. J. (1998) Nat. Struct. Biol. 5, 47–54
19. Wishart, D. S., and Case, D. A. (2001) Methods Enzymol. 338, 3–34
20. Banci, L., Bertini, I., Ciofi-Baffoni, S., Gonnelli, L., and Sa, X. C. (2003) J. Biol. Chem. 278, 50506–50513
21. Arnesano, F., Banci, L., Bertini, I., Cantini, F., Ciofi-Baffoni, S., Huffman, D. L., and O’Halloran, T. V. (2001) J. Biol. Chem. 276, 41365–41376
22. Arnesano, F., Banci, L., Bertini, I., and Bonvin, A. M. (2004) Structure (Camb.) 12, 669–676
23. Voskoboinik, I., Mar, J., Strausak, D., and Camakaris, J. (2001) J. Biol. Chem. 276, 28620–28627
24. Wernimont, A. K., Yatsunyk, L. A., and Rosenzweig, A. C. (2004) J. Biol. Chem. 279, 12269–12276
25. Garrett, D. S., Seok, Y. J., Peterkofsky, A., Clore, G. M., and Gronenborn, A. M. (1997) Biochemistry 36, 4393–4398