Binding of Copper(I) by the Wilson Disease Protein and Its Copper Chaperone*

Received for publication, October 13, 2003, and in revised form, December 18, 2003
Published, JBC Papers in Press, January 6, 2004, DOI 10.1074/jbc.M311213200

Amy K. Wernimont†, Liliya A. Yatsunyk, and Amy C. Rosenzweig‡§
From the Departments of Biochemistry, Molecular Biology, and Cell Biology, and Chemistry, Northwestern University, Evanston, Illinois 60208

The Wilson disease protein (WND) is a transport ATPase involved in copper delivery to the secretory pathway. Mutations in WND and its homolog, the Menkes protein, lead to genetic disorders of copper metabolism. The WND and Menkes proteins are distinguished from other P-type ATPases by the presence of six soluble N-terminal metal-binding domains containing a conserved CXXC metal-binding motif. The exact roles of these domains are not well established, but possible functions include exchanging copper with the metallochaperone Atox1 and mediating copper-responsive cellular relocalization. Although all six domains can bind copper, genetic and biochemical studies indicate that the domains are not functionally equivalent. One way the domains could be tuned to perform different functions is by having different affinities for Cu(I). We have used isothermal titration calorimetry to measure the association constant (Kₐ) and stoichiometry (n) values of Cu(I) binding to the WND metal-binding domains and to their metallochaperone Atox1. The association constants for both the chaperone and target domains are $10^{5}$ to $10^{6}$ M⁻¹, suggesting that the handling of copper by Atox1 and copper transfer between Atox1 and WND are under kinetic rather than thermodynamic control. Although some differences in both n and Kₐ values are observed for variant proteins containing less than the full complement of six metal-binding domains, the data for domains 1–6 were best fitted with a single site model. Thus, the individual functions of the six WND metal-binding domains are not conferred by different Cu(I) affinities but instead by fold and electrostatic surface properties.

Wilson disease and Menkes syndrome are human diseases of copper metabolism caused by mutations in the genes for two closely related proteins, the Wilson (1) and Menkes (2) ATPases. These proteins couple ATP hydrolysis to copper translocation across membranes. Menkes syndrome is caused by decreased copper uptake across the small intestine as well as impaired copper distribution to a variety of tissues, including the brain. By contrast, Wilson disease is characterized primarily by copper overload in the liver because of reduced biliary excretion (3). The Wilson disease protein (WND) is usually localized in the trans-Golgi network of hepatocytes where it delivers copper to key metalloenzymes, including ceruloplasmin, a multicopper oxidase involved in high affinity iron uptake (4, 5). This process is dependent on interaction with Atox1, a copper chaperone that delivers Cu(I) ions to WND (6, 7). At elevated copper concentrations, WND redistributes to cytoplasmic vesicles and mediates copper efflux from the cell (4, 8). Similarly, the Menkes disease protein (MNK) relocates from the trans-Golgi network to the plasma membrane in response to potentially toxic copper concentrations (9, 10).

Both proteins consist of eight transmembrane domains, of which the sixth contains an invariant CPC motif proposed to bind metal ions. Phosphatase- and ATP-binding domains and a phosphorylation site are conserved as well (11). Metal transport ATPases are distinguished from other P-type ATPases by the presence of multiple soluble N-terminal domains containing a conserved CXXC metal-binding motif (12) (Fig. 1). Both WND and MNK contain six such domains, whereas the yeast homolog, Ccc2 (13), contains only two repeats and the Drosophila melanogaster homolog contains four. These domains are present not only in the copper ATPases but also occur in Zn(II), Cd(II), and Pb(II) transporters (11, 12). The Atox1 metallochaperone and its homologs, both eukaryotic and prokaryotic, contain a single CXXC motif (14). Crystal and solution structures of Atox1 (15), yeast Atx1 (16, 17), bacterialCopZ (18, 19), and single domains of MNK (20) and Ccc2 (21) reveal a conserved βαββββ-fold with the cysteines from the CXXC motif coordinating metal ions on a surface-exposed loop. Notably, the CXXC motifs from two Atox1 molecules coordinate a single Cu(I) ion in the x-ray structure (15). Although there are no structures of polypeptides comprising all six repeats from WND or MNK, their metal-binding properties have been studied in some detail. Both the WND and MNK N termini bind 5–6 copper ions (22), and x-ray absorption spectroscopic studies indicate that the copper is present as Cu(I), ligated by the sulfurs from two cysteines (23–25).

The roles of the six WND and MNK metal-binding domains in copper metabolism are not well established. Numerous genetic and biochemical studies suggest that the domains are not functionally equivalent. For WND, yeast complementation assays indicate that the sixth domain alone is sufficient for copper loading of Fet3, the yeast homolog of ceruloplasmin (26, 27). Furthermore, the second or third metal-binding domain cannot substitute for the sixth domain, supporting distinct functions for the individual domains (27). Recently, a more specific function has been assigned to domains 5 and 6. These domains are responsible for the cooperative effect of copper on WND catalytic phosphorylation activity (28). In contrast to

* This work was supported by National Institutes of Health Grant GM58518 and a Camille Dreyfus Teacher-Scholar Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported in part by National Institutes of Health Training Grant GM008061.
‡ To whom correspondence should be addressed. Tel.: 847-467-5301; Fax: 847-467-6489; E-mail: amy@northwestern.edu.
§ The abbreviations used are: WND, Wilson disease protein; MNK, Menkes disease protein; ITC, isothermal titration calorimetry; WD12, -34, -56, -16, WND metal-binding domains 1–2, 3–4, 5–6, and 1–6, respectively; MES, 2-(N-morpholino)ethanesulfonic acid.
WND, yeast complementation studies on MNK indicate that the first four N-terminal domains are important for copper transport (29). It may be that the domains function differently in the two ATPases. Another potential function for the metal-binding domains is mediating copper-responsive cellular relocation. For example, mutations in MNK domains 4–6 interfere with the ability to traffic to the plasma membrane in response to elevated copper (30, 31). Finally, some of the domains may interact specifically with the copper chaperone Atox1. According to yeast two-hybrid assays using both WND (32) and MNK (33), Atox1 interacts most strongly with domains 1–4 and not at all with domains 5–6, although an interaction with MNK domains 5–6 has been detected by surface plasmon resonance (33). These interactions are metal-dependent and specific for copper.

Taken together, these data suggest that the six domains play discrete roles in the function and regulation of WND. The metal-binding domains most important for interaction with Atox1 (domains 1–4) are clearly distinct from those required for copper transport (domains 5–6). Domains 1–4 of WND have also been proposed to function in copper-responsive localization (27, 28). Although all six metal-binding domains can bind copper (22, 34), their copper-binding affinities are not known. Furthermore, the copper-binding affinity of Atox1 has not been reported. Such quantitative data are crucial to understanding the functions of individual domains. We have used isothermal titration calorimetry (ITC) to measure the association constant \( K_a \) and stoichiometry \( n \) values of Cu(I) binding to the WND metal-binding domains and to Atox1. These data provide new insight into the energetics of copper transfer and into how the individual functions of the WND domains are defined.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning and Expression**—Plasmids containing the cDNA for the entire WND and for Atox1 were generously supplied by Dr. Jonathan Gitlin (Washington University School of Medicine). Individual constructs comprising domains 1–2 (WD12), domains 3–4 (WD34), domains 5–6 (WD56), domains 1–4 (WD14), and domains 1–6 (WD16) were generated by PCR using the primers listed in Table I. The PCR products for the WND proteins were gel-purified and inserted into the pET32 Xa/LIC vector following the ligation independent cloning protocol from Novagen. This vector includes an N-terminal thioredoxin tag followed by a His6 tag, a thrombin cleavage site, an S-tag, and a Factor Xa cleavage site. Plasmids were then transformed into BL21(DE3)pLysS cells for induction tests. The primers for Atox1 incorporate an NdeI sequence at the N terminus and a stop codon and EcoRI sequence at the C terminus. The Atox1 PCR product was gel-purified and cloned into the pET21b vector (Novagen) using conventional methods.

For the expression of WD12, WD34, WD56, and WD14, a single colony was used to inoculate a 50- or 100-ml culture of LB medium containing 1 mg/ml carbenicillin and 0.4 mg/ml chloramphenicol (LB/carb/chlor). After incubating overnight, 8 ml of this culture was used to inoculate multiple 2-liter flasks containing 1 liter of LB/carb/chlor, and these flasks were incubated at 250 rpm and 37 °C. When the optical density at 600 nm \( (A_{600}) \) reached 0.6–0.8, protein expression was induced with 0.9 mM isopropyl-\(\beta\)-D-thiogalactopyranoside at an \( A_{600} \) of 0.3. Protein expression was induced with 0.9 mM isopropyl-\(\beta\)-D-thiogalactopyranoside at an \( A_{600} \) of 0.7–0.9, and the cells were harvested after 20 h. This
protocol was necessary for optimal solubility of WD16. Atox1 was expressed using the same procedures as for WD12, WD34, WD56, and WD14 except that chloramphenicol was not included in the medium.

**Protein Purification**—For purification of WD12, WD34, WD56, and WD14, cells were thawed in 50 m M Hepes, pH 7.5, 500 m M NaCl, and 5% glycerol (buffer A). EDTA-free protease inhibitor tablets (Roche Applied Science) and a small amount of solid DNase I were added, and protein was extracted with stirring for 30 min to 1 h at room temperature. This solution was then centrifuged at 9000 × g for 30 min, and the supernatant was applied to a 20-ml pre-equilibrated, nickel-loaded chelating-Sepharose column (Amersham Biosciences), rinsed with buffer A, and eluted with an 8-column volume gradient of 300 m M imidazole, pH 7.5, and 500 m M NaCl (buffer B). For WD12, WD34, and WD56, fractions containing the tagged protein of interest were pooled and dialyzed versus 2 liters of buffer A, and eluted with a 6-column volume gradient into buffer B.

Cleavage reactions were reloaded onto the nickel column, rinsed with buffer A, and eluted with a 6-column volume gradient into buffer B. Cleaved protein without the tags eluted at 0.15 M NaCl, and 500 m M imidazole. For WD14, the tagged protein from the nickel column was concentrated, cleaved with 100 units of Factor Xa (Novagen) and then diluted to remove excess imidazole.

Cleavage reactions were reloaded onto the nickel column, rinsed with buffer A, and eluted with a 6-column volume gradient into buffer B. Cleaved protein without the tags eluted at 0.15 M NaCl, and 500 m M imidazole. Fractions containing the cleaved protein were concentrated with a Centriprep 10 (Amicon) to 5 ml, loaded onto a Superdex 75 gel filtration column (Amersham Biosciences), and eluted with buffer A.

**Table I**

| Primer | Sequence |
|--------|----------|
| WD14, WD16 forward | 5′-GGTATTTGGAGCTTGCCATGCTGCAAGGAGAGACAC3′ |
| WD14 reverse | 5′-AGAGGAGGATGATGAGGCCCTGAGAAGGCTACATG3′ |
| WD34 forward | 5′-GGTATTTGGAGCTTGCCATGCTGCAAGGAGAGACAC3′ |
| WD34 reverse | 5′-AGAGGAGGATGATGAGGCCCTGAGAAGGCTACATG3′ |
| WD12 forward | 5′-GGTATTTGGAGCTTGCCATGCTGCAAGGAGAGACAC3′ |
| WD12 reverse | 5′-AGAGGAGGATGATGAGGCCCTGAGAAGGCTACATG3′ |
| WD56 forward | 5′-GGTATTTGGAGCTTGCCATGCTGCAAGGAGAGACAC3′ |
| WD56 reverse | 5′-AGAGGAGGATGATGAGGCCCTGAGAAGGCTACATG3′ |
| Atox1 forward | 5′-TGGATTCGACATAGCTGAGCGTTGGA3′ |
| Atox1 reverse | 5′-ATCTCGACATATGCAGAGAACTTAA3′ |

*WD16 refers to a polypeptide consisting of all six metal-binding domains. The other proteins are named similarly.*

**Copper(I) Binding by the Wilson Disease Protein and Atox1**

**Isothermal Titration Calorimetry Measurements**—Starting stock protein concentrations were measured using extinction coefficients at 280 nm, determined by amino acid hydrolysis (WD12, 25,588 m M-1 cm-1; WD14, 7421 m M-1 cm-1; WD56, 4140 m M-1 cm-1; WD18, 36,554 m M-1 cm-1; WD14, 19,840 m M-1 cm-1; and Atox1, 3884 m M-1 cm-1). Protein was concentrated or diluted to 50 µ M in 3-ml amounts, and 10 µ M EDTA was added. This stock protein solution was incubated for 30 min on ice and then transferred to a Slide-A-Lyzer dialysis cassette (Pierce) with a 3000-Da molecular mass cutoff. After three successive rounds of dialysis versus 500 m L of chelxed 100 m M MES, pH 6.5, 100 m M NaCl, and 0.6 m M tris-2-carboxyethylphosphine, the concentration of EDTA was estimated to be ~0.36 µ M. The sample was then transferred to a Coy anaerobic chamber and dialyzed three times versus 500 m L de-gassed, chelxed 100 m M MES, pH 6.5, and 100 m M NaCl (buffer M), at which point the concentration of tris-2-carboxyethylphosphine was estimated to be ~130 µ M. After this treatment, the residual copper content was determined by atomic absorption spectroscopy using a PerkinElmer Life Sciences AAAnalyst 700 with a high performance flame (detection limit for copper, 1.5 ppb) and copper lumina hollow cathode lamp. A standard curve was generated using a copper atomic absorption standard (Aldrich). All the samples contained ~30 ppb copper as did buffer M. Taking into account the protein concentration, the average concentration prior to the ITC experiments was 0.03, 0.04, 0.05, 0.05, 0.03, and 0.09 copper ions per protein molecule for Atox1, WD12, WD14, WD56, WD16, respectively. The number of reduced cysteines was measured using the thiol and sulfide quantitation kit from Molecular Probes.

A stock solution of 9 m M CuCl in 10 m M HCl and 1.0 m M NaCl was degassed extensively on a vacuum line, backfilled with argon, and stored in the anaerobic chamber. The concentration of this stock solution was determined by inductively coupled plasma optical emission spectroscopic analysis by Galbraith Laboratories (Knoxville, TN). For all copper-binding experiments both protein and copper stock solutions were diluted with buffer M immediately before the run. Appropriate amounts of copper stock solution and protein stock solution were added to the sample cell with 6 min between injections to allow for the equilibration. A standard curve was generated using a copper atomic absorption standard (Aldrich). All the samples contained ~30 ppb copper as did buffer M. Taking into account the protein concentration, the average concentration prior to the ITC experiments was 0.03, 0.04, 0.05, 0.05, 0.03, and 0.09 copper ions per protein molecule for Atox1, WD12, WD14, WD56, WD16, respectively. The number of reduced cysteines was measured using the thiol and sulfide quantitation kit from Molecular Probes.

A stock solution of 9 m M CuCl in 10 m M HCl and 1.0 m M NaCl was degassed extensively on a vacuum line, backfilled with argon, and stored in the anaerobic chamber. The concentration of this stock solution was determined by inductively coupled plasma optical emission spectroscopic analysis by Galbraith Laboratories (Knoxville, TN). For all copper-binding experiments both protein and copper stock solutions were diluted with buffer M immediately before the run. Appropriate amounts of copper stock solution and protein stock solution were added to the sample cell with 6 min between injections to allow for the equilibration. A standard curve was generated using a copper atomic absorption standard (Aldrich). All the samples contained ~30 ppb copper as did buffer M. Taking into account the protein concentration, the average concentration prior to the ITC experiments was 0.03, 0.04, 0.05, 0.05, 0.03, and 0.09 copper ions per protein molecule for Atox1, WD12, WD14, WD56, WD16, respectively. The number of reduced cysteines was measured using the thiol and sulfide quantitation kit from Molecular Probes.
controls. First, Mg(II) was titrated into both Atox1 (20 μM) and WD12 (10 μM) using 300 μM MgCl₂ prepared in the same buffer as the Cu(I) stock solution. Second, Cu(I) was titrated into 20–35 μM solutions of lysozyme (14,296 kDa, Research Organics). These controls were performed in triplicate under the same conditions used for Atox1 and the WND proteins.

RESULTS

ITC is an excellent technique for determining the thermodynamic properties of a reaction under defined solution conditions. Measuring the heat generated or absorbed on ligand binding to a protein can yield values for the stoichiometry, n, as well as for $K_r$, $\Delta H^\circ$, $\Delta G^\circ$, and $\Delta S^\circ$. To obtain useful ITC data, every aspect of the experiment must be considered carefully (35, 36). Studies of WND and Atox1 are further complicated by the presence of multiple cysteine residues, oxidation of which can cause aggregation, and by the air sensitivity and low solubility of Cu(I). As described above, we have developed protocols for preparing both the protein sample and the Cu(I) titrant solution for loading the calorimeter cell and for conducting the titration anaerobically. The validity of these procedures was tested by titrating Mg(II) into Atox1 (Fig. 2) and WD12, which do not bind Mg(II), and by titrating Cu(I) into lysozyme (Fig. 3), which does not bind Cu(I) specifically. None of these control experiments showed specific Cu(I) binding. By contrast, we obtained interpretable and reproducible data for Cu(I) binding to the WND metal-binding domains and Atox1. In each experiment, titrations of Cu(I) into solutions of metal-free, reduced protein resulted in large exothermic peaks, which eventually diminished to just the heat of dilution (see Figs. 4–9).

Data for Atox1 (Fig. 4), WD34 (Fig. 5), and WD56 (Fig. 6) were fitted well with a one-site binding model. The n value of 1.41 ± 0.22 for Atox1 is consistent with binding a single Cu(I) ion. For both WD34 and WD56, n values very close to 2 were obtained, indicating that each CXXC motif in these polypeptides can bind a Cu(I) ion. The $K_r$ values for WD34 and WD56, $10^6$ M⁻¹, are higher than that for Atox1, $10^5$ M⁻¹. In particular, WD34 binds Cu(I) approximately an order of magnitude more tightly than the chaperone (Table II). Consideration of the experimental error for each measurement suggests that the binding constants are roughly similar, however. Fitting the data for WD12 (Fig. 7) with a one-site model yielded a stoichiometry of 0.95 ± 0.38 Cu(I) ions per protein molecule, an unexpected result for a protein containing two CXXC metal-binding domains. A possible explanation is that one of the domains is especially sensitive to oxidation and thus does not bind copper under the experimental conditions. However, thiol detection indicates that WD12 contains 4.0 ± 0.5 free thiols, suggesting that both domains retain the ability to bind metal. The stoichiometry could also be attributed to the presence of a copper ion already bound in one of the sites, but atomic absorp-
from titration of 8
from titration of 8

shown in Fig. 8, slight dips in the curves were evident, sug-
that measured for Atox1. In several titrations, including that
one Cu(I) ion to WD12. The
with the binding of 2 Cu(I) ions to WD34 and WD56 but just
just
model as well. The stoichiometry of 4.92
contains
model as well. The stoichiometry of 4.92

was evident, sug-
gesting that more than one type of site may be present. We
tried to account for these dips with a two-site model, but un-
reasonable stoichiometries (>10 copper ions) inconsistent with
the number of possible ligands were obtained. Fixing the n
values did not improve the fits and gave poor agreement among
multiple data sets. Therefore, the single site model is most
appropriate for WD16 and suggests that the binding sites in
WD16 have the same affinity for Cu(I) as one another and as
Atox1.

Unlike WD16, the presence of multiple binding sites in
WD14 was clear from the titration curve (Fig. 9) and was
completely reproducible. When fitted to a one-site model, the
stoichiometry was unreasonably low and the fit obviously poor.
By contrast, a two-site model gave $n_1 - 1$ and $n_2 - 3$ (Table II).
The binding constants have large standard deviations but sug-
that the site with $n_1 - 1$ has a higher Cu(I) affinity than
the other three sites in WD14. Moreover, this site binds copper
more tightly than the sites in all the other WND variants
studied (Table II). The class of sites with $n_2 - 3$ is comparable
in affinity to WD34 and WD56. Although the stoichiometry is
reasonable for a protein containing four CXXC motifs, it is not
consistent with the stoichiometries of ~1, ~2, and ~5 mea-
sured for WD12, WD34, and WD16, respectively. Based on these
values, WD14 might be expected to bind 3 rather than 4 Cu(I)
ions.

**DISCUSSION**

The ITC results presented here provide the first thermody-
namic parameters for Cu(I) binding to the Wilson disease pro-
tein and its copper chaperone. Values of $n$, $K_a$, and the appar-
ent $\Delta H^\circ$ have been obtained for Atox1 and five variants of the
Wilson protein, WD12, WD34, WD56, WD16, and WD14 (Table
II). The measured stoichiometries are generally correlated with
the number of CXXC motifs in each polypeptide. Atox1 binds
one Cu(I) ion, indicating that the metal-bridged dimer observed
in the crystal structure (15) does not form at low concentrations
in solution. The measured $n$ value of 1.41 ± 0.22 could also be
construed as binding more than one Cu(I) ion but is most likely
due to an error in the protein concentration determination. The
stoichiometry of ~2 for WD34 and WD56 is consistent with the
presence of two CXXC motifs, and the two classes of sites for
WD14 have $n$ values of ~1 and ~3, reflecting the presence of
four CXXC motifs. By contrast, WD12 binds only one Cu(I) ion,
and WD16 binds closer to 5 Cu(I) ions, consistent with WD12
binding only one Cu(I) ion.

The binding of a single Cu(I) ion by WD12 is unexpected and
cannot be explained by oxidation or a tightly bound Cu(I) ion
present prior to the ITC experiments. It may be that one of the
domains does not bind copper at all. Alternatively, the two
domains could coordinate a single Cu(I) ion with the cysteines
from two CXXC motifs, analogous to what is observed in the
crystal structure of Cu(I)-loaded Atox1 (15). Domains 1 and 2
are separated by a 25 residue linker region, which could allow
the two domains to orient properly for this type of coordination.
This model could also explain surface plasmon resonance data
indicating that MNK domains 1 and 2 have a lower affinity for
Atox1 than the other domains (33). Although this finding may
be because of the presence of a glutathione S-transferase tag
(33), it could also result from the two domains coordinating a
single Cu(I) ion. Because the binding surface of the target
domain is proposed to involve regions near the CXXC motif (15,
37), coordination of a single Cu(I) ion between the CXXC motifs
in MNK domains 1 and 2 could hinder Atox1 access.

The $K_a$ value of ~$10^5$ M$^{-1}$ for Atox1 represents the first
report of a Cu(I) binding constant for a copper chaperone. For
comparison, the apparent $K_a$ for the first Cu(II) ion binding to
the Wilson protein, WD12, WD34, and WD16, respectively. Based on these
values, WD14 might be expected to bind 3 rather than 4 Cu(I)
ions.
Copper(I) Binding by the Wilson Disease Protein and Atox1

| Protein | \( n^a \) | \( K_a \) (m\(^{-1} \)) | \( \Delta H^a \) (kcal/mol) |
|---------|---------|-----------------|-------------------|
| Atox1   | 1.41 \( \pm \) 0.22 | \((2.45 \pm 1.23) \times 10^5 \) | \(-12.96 \pm 1.20 \) |
| WD12    | 0.95 \( \pm \) 0.38 | \((2.07 \pm 1.57) \times 10^5 \) | \(-11.78 \pm 4.17 \) |
| WD34    | 2.23 \( \pm \) 0.19 | \((4.67 \pm 2.72) \times 10^5 \) | \(-9.76 \pm 3.66 \) |
| WD56    | 1.92 \( \pm \) 0.02 | \((1.13 \pm 0.21) \times 10^6 \) | \(-11.63 \pm 0.04 \) |
| WD16    | 4.92 \( \pm \) 0.66 | \((1.96 \pm 0.56) \times 10^5 \) | \(-11.50 \pm 0.38 \) |
| WD14\(^b\) | \( n_1 = 3.38 \pm 0.04 \) \( K_{a1} = (1.89 \pm 2.00) \times 10^6 \) \( \Delta H_{a1} = -2.90 \pm 2.25 \) | \( n_2 = 1.22 \pm 0.07 \) \( K_{a2} = (4.98 \pm 5.46) \times 10^6 \) \( \Delta H_{a2} = -9.33 \pm 1.10 \) |

\(^a\) Standard deviations from at least three measurements are included.
\(^b\) The values for \( \Delta H^a \) are apparent and include contributions not only from Cu(I) binding but from associated events such as deprotonation of the two cysteines in the CXXC motif and changes in the buffer ionization state.

The WD14 data were best fit with a two-site model.

| Protein | \( n^a \) | \( K_a \) (m\(^{-1} \)) | \( \Delta H^a \) (kcal/mol) |
|---------|---------|-----------------|-------------------|
| Atox1   | 1.41 \( \pm \) 0.22 | \((2.45 \pm 1.23) \times 10^5 \) | \(-12.96 \pm 1.20 \) |
| WD16    | 0.95 \( \pm \) 0.38 | \((2.07 \pm 1.57) \times 10^5 \) | \(-11.78 \pm 4.17 \) |
| WD34    | 2.23 \( \pm \) 0.19 | \((4.67 \pm 2.72) \times 10^5 \) | \(-9.76 \pm 3.66 \) |
| WD56    | 1.92 \( \pm \) 0.02 | \((1.13 \pm 0.21) \times 10^6 \) | \(-11.63 \pm 0.04 \) |
| WD16    | 4.92 \( \pm \) 0.66 | \((1.96 \pm 0.56) \times 10^5 \) | \(-11.50 \pm 0.38 \) |
| WD14\(^b\) | \( n_1 = 3.38 \pm 0.04 \) \( K_{a1} = (1.89 \pm 2.00) \times 10^6 \) \( \Delta H_{a1} = -2.90 \pm 2.25 \) | \( n_2 = 1.22 \pm 0.07 \) \( K_{a2} = (4.98 \pm 5.46) \times 10^6 \) \( \Delta H_{a2} = -9.33 \pm 1.10 \) |

\(^a\) Standard deviations from at least three measurements are included.
\(^b\) The values for \( \Delta H^a \) are apparent and include contributions not only from Cu(I) binding but from associated events such as deprotonation of the two cysteines in the CXXC motif and changes in the buffer ionization state.

The WD14 data were best fit with a two-site model.

pH 7.0 (38), and the four higher affinity Cu(II) sites in dopamine β-monoxygenase have \( K_a \) values of \( \sim 10^{11} \) M\(^{-1} \) (39). Because Atox1 functions to shuttle copper ions within the cell, it is reasonable that it would have a lower affinity for copper than these metalloenzymes. Metallothionein, which binds Cu(I) with sulfur ligands, has a stability constant of \( 10^{17} \) to \( 10^{19} \) M\(^{-1} \) (40).

Given the moderate affinity of Atox1 for Cu(I) and the limited free copper concentration (<10\(^{-18} \) M) in the cell (41), how is Atox1 able to retain copper ions until it reaches its target WND domains? One likely possibility is that the kinetic rate constant, \( k_{off} \), is slow, preventing the loss of copper to other higher affinity binding sites. In support of this notion, exchange rates between enzymes and copper are typically slow because of the strength of Cu(I) and Cu(II) binding to ligands (42).

It is not known which WND domains receive copper directly from Atox1, although the strongest interaction by the yeast two-hybrid assay is observed for WD14 (32). If transfer is under thermodynamic control, the WND metal domains, or at least the subset that receives Cu(I) from Atox1, would be expected to have a higher affinity for Cu(I). With the exception of WD14, the \( K_a \) values for the Wilson domains are all on the order of \( 10^5 \) to \( 10^6 \) M\(^{-1} \). Affinities of this magnitude have been reported previously for the six MNK domains (43) and are consistent with EC\(_{50}\) values measured for the activation of WND by copper (28, 44). Importantly, the \( K_a \) value for WD16, the most physiologically relevant variant, is essentially the same as that for Atox1 (Table II). The chaperone and target domains thus have similar affinities for Cu(I). This result is consistent with an exchange constant of \( 1.4 \) measured for copper transfer between the yeast chaperone Atx1 and one domain of its target ATPase, Ccc2 (45). If copper delivery from Atox1 to the WND N-terminal domains is followed by rapid transfer to another site in the ATPase, the ultimate driving force could derive from ATP hydrolysis. Because copper transfer between Atox1 and the WND metal-binding domains is not governed by a thermodynamic gradient, it must be under kinetic control. As suggested above, Atox1 may have a slow \( k_{off} \) for copper, allowing it to function in the copper-limiting environment of the cytoplasm. Complexation between Atox1 and WND could alter \( k_{off} \) and lower the kinetic barrier for transfer between metal-binding sites as suggested previously for Atx1 and Ccc2 (45). Similarly, interactions between Atox1 and its copper donor, which could be the membrane transporter Ctr1 (46) or another yet to be identified factor (47), could modulate \( k_{off} \).

Despite the overall similarity in binding constants for Atox1 and the WND domains, some differences between the WND domains are apparent from the data. An approximate hierarchy of binding affinities is WD14 > WD34 > WD56 > WD16 > WD34 > WD56.
points for the six WND domains range from 3.8 to 8.7 (49). It seems unlikely that protein-protein interactions between Atox1 and each of the six different domains could facilitate copper transfer in the same way. Instead, only some of the domains might exchange copper with Atox1. Yeast two-hybrid data indicate that a polypeptide comprising domains 5 and 6, which are proposed to function in copper transport (28), does not interact with Atox1 (32, 33). Domains 5 and 6 could receive copper from domains 1–4, however. Alternatively, in the intact WD16, domains 5 and 6 might interact directly with Atox1. As noted above, the presence of the other domains can affect the behavior of the individual domains, and data acquired on truncated proteins should be interpreted carefully. Domains 1–4 have been suggested to function in copper-responsive localization (27, 28). Their electrostatic properties and/or overall structure could confer such a function by modulating interactions with additional regulatory proteins. A possible candidate is the Murr1 protein. This protein, proposed to be involved in hepatic copper toxicosis, has recently been shown to interact directly with the six WD metal-binding domains (50).

In sum, ITC has been used to measure values of $n$ and $K_a$ for Cu(I) binding to the copper chaperone Atox1 and various combinations of the metal-binding domains of its target transport ATPase WND. The association constants are $-10^5$ to $10^6$ M$^{-1}$ and are similar for chaperone and target domains. These data suggest that copper handling by Atox1 and copper exchange between Atox1 and WND are under kinetic rather than thermodynamic control. Some differences in both $n$ and $K_a$ values are observed for proteins containing less than the full complement of six metal-binding domains, demonstrating that the copper-binding properties of each domain are affected by the presence of the other domains. The data for WD16, however, were best fitted with $-5$ Cu(I) sites all with the same binding constant. Consequently, the functions of the six WND metal-binding domains are not conferred by different Cu(I) affinities but instead by fold and electrostatic surface properties. Detailed characterization of the interactions between WD16 and its partner proteins is therefore critical to delineating the roles of the individual domains.

Acknowledgments—We thank J. Gitlin for providing plasmids, L. Gracey for assistance for protein purification, and J. Spener, I. Klotz, J. Widom, and H. Godwin for valuable discussions. All ITC data were collected in the Keck Biophysics Facility at Northwestern University.

REFERENCES

1. Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R., and Cox, D. W. (1993) Nat. Genet. 3, 327–337
2. Valpe, C., Levinsion, B., Whitney, S., Packman, S., and Gitschier, J. (1993) Nat. Genet. 3, 7–13
3. Llanos, R. M., and Mercer, J. F. B. (2002) DNA Cell Biol. 21, 259–270
4. Hung, I. H., Suzuki, M., Yamaguchi, Y., Yuan, D. S., Klausner, R. D., and Gitlin, J. D. (1997) J. Biol. Chem. 272, 21461–21466
5. Terada, K., Nakako, T., Yang, X.-L., Iida, M., Aiba, N., Minamiya, Y., Nakai, M., Sakaki, T., Miura, N., and Sugiyama, T. (1998) J. Biol. Chem. 273, 1815–1820
6. Klomp, L. W. J., Lin, S.-J., Yuan, D. S., Klausner, R. D., Culotta, V. C., and Gitlin, J. D. (1997) J. Biol. Chem. 272, 9221–9226
7. Hung, I. H., Casarano, R. L. B., Labesse, G., Matthews, P. S., and Gitlin, J. D. (1998) J. Biol. Chem. 273, 1749–1754
8. Schaefer, M., Hopkins, R. G., Failla, M. L., and Gitlin, J. D. (1999) Am. J. Physiol. 276, G639–G646
9. Petris, M. J., Mercer, J. F. B., Culvenor, J. G., Lockhart, P., Gleeson, P. A., and Camakaris, J. (1996) EMBO J. 15, 6083–6089
10. Petris, M. J., Camakaris, J., Greenough, M., LaFontaine, S., and Mercer, J. F. B. (1998) Hum. Mol. Genet. 7, 2063–2071
11. Lutsenko, S., Efremov, R. G., Tsvkovskiy, R., and Walker, J. M. (2002) J. Bioenerg. Biomembr. 34, 351–362
12. Soloz, M., and Vulpe, C. (1996) Trends Biochem. Sci. 21, 237–241
13. Yuan, D. S., Stearman, R., Dancis, A., Dunn, T., Beeler, T., and Klausner, R. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2633–2638
14. Elam, J. S., Thomas, S. T., Holloway, S. P., Taylor, A. B., and Hart, P. J. (2002) Adv. Protein Chem. 60, 151–219
15. Wernimont, A. K., Huffman, D. L., Lamb, A. L., O’Halloran, T. V., and Rosenzweig, A. C. (2000) Nat. Struct. Biol. 7, 766–771
16. Rosenzweig, A. C., Huffman, D. L., Hou, M. Y., Wernimont, A. K., Pufahl, R. A., and O’Halloran, T. V. (1999) Structure (Lond.) 7, 605–617

FIG. 9. Calorimetric titration of WD14 with Cu(I). Top, raw data from titration of 7.3 μM WD14 with 0.3 mM Cu(I). Bottom, plot of integrated heat versus the Cu(I)/protein ratio.
Copper(I) Binding by the Wilson Disease Protein and Atox1

17. Arnesano, F., Banci, L., Bertini, I., Huffman, D. L., and O'Halloran, T. V. (2001) Biochemistry 40, 1528–1539
18. Wimmer, R., Herrmann, T., Seliz, M., and Wuthrich, K. (1999) J. Biol. Chem. 274, 22609–22616
19. Banci, L., Bertini, I., Conte, R. D., Markey, J., and Ruiz-Dueñas, F. J. (2001) Biochemistry 40, 15660–15668
20. Gitschier, J., Moffat, B., Reilly, D., Wood, W. L., and Fairbrother, W. J. (1998) Nat. Struct. Biol. 5, 47–54
21. Banci, L., Bertini, I., Ciofi-Baffoni, S., Huffman, D. L., and O'Halloran, T. V. (2001) J. Biol. Chem. 276, 8415–8426
22. Lutsenko, S., Petrakhin, K., Cooper, M. J., Gilliam, C. T., and Kaplan, J. H. (1998) J. Biol. Chem. 273, 18939–18944
23. Ralle, M., Cooper, M. J., Lutsenko, S., and Blackburn, N. J. (1998) J. Am. Chem. Soc. 120, 13525–13526
24. DiDonato, M., Heu, H.-F., Narindrasorasak, S., Que, L., Jr., and Sarkar, B. (2000) Biochemistry 39, 1890–1896
25. Ralle, M., Lutsenko, S., and Blackburn, N. J. (2003) J. Biol. Chem. 278, 23163–23170
26. Iida, M., Terada, K., Sambongi, Y., Wakabayashi, T., Miura, N., Koyama, K., Futai, M., and Sugiyama, T. (1998) FEBS Lett. 428, 281–285
27. Forbes, J. R., Hsi, G., and Cox, D. W. (1999) J. Biol. Chem. 274, 12498–12413
28. Halloran, T. V., and Lutsenko, S. (2002) J. Biol. Chem. 277, 32212–32218
29. Payne, A. S., and Gitlin, J. D. (1998) J. Biol. Chem. 273, 3765–3770
30. Strausak, D., LaFontaine, S., Hill, J., Firth, S. D., Lockhart, P. J., and Mercer, J. F. B. (1999) J. Biol. Chem. 274, 11170–11177
31. Voskoboinik, I., Strausak, D., Greenough, M., Brooks, H., Petris, M., Smith, S., Mercer, J. F., and Camakaris, J. (1999) J. Biol. Chem. 274, 223068–223012
32. Larin, D., Meekins, C., Das, K., Ross, B., Yang, A.-S., and Gilliam, T. C. (1999) J. Biol. Chem. 274, 28497–28504
33. Strausak, D., Howie, M. K., Firth, S. D., Schlicksupp, A., Pipkorn, R., Multhaup, G., and Mercer, J. F. B. (2003) J. Biol. Chem. 278, 20821–20837
34. DiDonato, M., Narindrasorasak, S., Forbes, J. R., Cox, D. W., and Sarkar, B. (1997) J. Biol. Chem. 272, 33279–33282
35. O'Brien, R. E., L. J., and Chowdhry, B. Z. (2001) in Protein-ligand Interactions: Hydromechanics and Calorimetry (Harding, S. E., and Chowdhry, B. Z., eds) pp. 263–285, Oxford University Press, Oxford
36. Pierce, M. K., Raman, C. S., and Nall, B. T. (1999) Methods 19, 213–221
37. 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
38. Hirase, J., Ohkita, T., Hirata, H., and Kudani, Y. (1982) Arch. Biochem. Biophys. 218, 179–186
39. Syvertsen, C., Gaustad, R., Schroder, K., and Ljones, T. (1986) J. Inorg. Biochem. 26, 63–76
40. Hamer, D. H. (1986) Annu. Rev. Biochem. 55, 913–951
41. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culetta, V. C., and O'Halloran, T. V. (1999) Science 284, 805–808
42. Frausto da Silva, J. J. R., and Williams, R. J. P. (2001) in The Biological Chemistry of the Elements, 2nd Ed., p. 431, Oxford University Press, Oxford
43. Jensen, P. Y., Bonander, N., Møller, L. B., and Farver, O. (1999) Biochim. Biophys. Acta 1434, 103–113
44. Walker, J. M., Piševčovski, R., and Lutsenko, S. (2002) J. Biol. Chem. 277, 27953–27959
45. Huffman, D. L., and O'Halloran, T. V. (2000) J. Biol. Chem. 275, 18611–18614
46. Xian, Z., and Wedd, A. G. (2002) J. Chem. Soc. Chem. Commun. 588–589
47. Portnoy, M. E., Schmidt, P. J., Rogers, R. S., and Culotta, V. C. (2001) Mol. Genet. Genomics 263, 873–882
48. Portnoy, M. E., Rosenzweig, A. C., Rae, T., Huffman, D. L., O'Halloran, T. V., and Culotta, V. C. (1999) J. Biol. Chem. 274, 15041–15045
49. Huffman, D. L., and O'Halloran, T. V. (2001) Annu. Rev. Biochem. 70, 677–701
50. Tao, T. Y., Liu, F., Klomp, L., Wijmenga, C., and Gitlin, J. D. (2003) J. Biol. Chem. 278, 41593–41596