Kinetic Analysis of the Interaction of the Copper Chaperone Atox1 with the Metal Binding Sites of the Menkes Protein

Received for publication, December 6, 2002, and in revised form, April 1, 2003
Published, JBC Papers in Press, April 4, 2003, DOI 10.1074/jbc.M212437200

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Excess copper is effluxed from mammalian cells by the Menkes or Wilson P-type ATPases (MNK and WND, respectively). MNK and WND have six metal binding sites (MBSs) containing a CXCC motif within their N-terminal cytoplasmic region. Evidence suggests that copper is delivered to the ATPases by Atox1, one of three cytoplasmic copper chaperones. Attempts to monitor a direct Atox1-MNK interaction and to determine kinetic parameters have not been successful. Here we investigated interactions of Atox1 with wild-type and mutated pairs of the MBSs of MNK using two different methods: yeast two-hybrid analysis and real-time surface plasmon resonance (SPR). A copper-dependent interaction of Atox1 with the MBSs of MNK was observed by both approaches. Cys to Ser mutations of conserved CXCC motifs affected the binding of Atox1 underlining the essentiality of Cys residues for the copper-induced interaction. Although the yeast two-hybrid assay failed to show an interaction of Atox1 with MBS5/6, SPR analysis clearly demonstrated a copper-dependent binding with all six MBSs highlighting the power and sensitivity of SPR as compared with other, more indirect methods like the yeast two-hybrid system. Binding constants for copper-dependent chaperone-MBS interactions were determined to be 10^{-5}–10^{-6} M for all the MBSs representing relatively low affinity binding events. The interaction of Atox1 with pairs of the MBSs was non-cooperative. Therefore, a functional difference of the MBSs in the MNK N terminus cannot be attributed to cooperativity effects or varying affinities of the copper chaperone Atox1 with the MBSs.

The mechanisms regulating the concentration of essential yet potentially toxic copper ions has been the subject of many investigations in the recent past. Specific copper uptake, distribution, and delivery proteins have been identified, and intracellular copper trafficking pathways have been defined for yeast and mammalian cells (for a review, see Ref. 1). Two critical constituents are the homologous P-type copper transporting ATPases MNK1 (ATP7A) and WND (ATP7B) (2–7).

Both copper transporters are thought to fulfill a bifunctional role within the cell: under physiological conditions, the majority of MNK and WND proteins are localized to the trans-Golgi network, where they deliver copper onto secreted cuproenzymes (8). However, when cytoplasmic copper concentrations reach potentially toxic levels, MNK and WND redistribute from the trans-Golgi network to the plasma membrane and vesicular structures facilitating the efflux of excess copper (9–11). Cytoplasmic copper chaperones play another important part in the intracellular routing of copper by receiving and delivering the trace metal directly to copper-dependent enzymes (12). Atox1 was characterized as a copper chaperone in Saccharomyces cerevisiae (13). Subsequently, other Atox1 homologues were found including Atox1 of mammalian cells (14), CopZ of the bacteria Enterococcus hirae (15), CCH of the plant Arabidopsis thaliana (16), and Cuc1 of the nematode Caenorhabditis elegans (17). A unique feature of all these chaperones is an invariant MXCXXC metal binding motif, which also occurs six times in the N termini of MNK and WND. The solution structures of CopZ (18), Atox1 (19), the apo- and metal-bound form of the fourth metal binding site (MBS) of human MNK (20), and the metal-bound form of Atox1 (21) were recently resolved. They all displayed an overall structure of two α-helices and four β-strands folding an antiparallel β-sheet. In this open-sandwich structure, the two Cys residues of the CXCC motif were exposed on the surface of the protein, readily accessible for the docking of metal ions. The crystallization of Cu(Atox1), as a homodimer bridged by a copper with 3–4 cysteines from the two monomers provided an excellent model for heterodimeric complex formation between chaperone and target (21).

In yeast, Atox1 was localized to the cytosol, and its role in the copper trafficking pathway and in iron metabolism was described (22). A trigonal coordination chemistry between copper and Atox1 was proposed (23), and a copper-dependent interaction of the N terminus of the yeast MNK homologue Ccc2 and Atox1 was demonstrated by yeast two-hybrid analysis (23, 24). Complex formation and dissociation of Atox1 with Ccc2 was investigated through $^1$H and $^{15}$N chemical shift perturbation experiments and found to be in the order of milliseconds (25). A lysine-rich region resulting in a positively charged face of Atox1 was crucial for intracellular copper trafficking, suggesting an important interaction site between Atox1 and Ccc2 (24).

The functional significance of the six MBSs in WND and MNK is unclear, but there is growing evidence that there is a functional distinction between the sites. The prokaryotic and protein; MBS, metal binding site; SPR, surface plasmon resonance; wt, wild type; GST, glutathione S-transferase; RU, response units; BCS, bathocuproine disulphonate; TCEP, Tris-(2-carboxy-ethyl)phosphine.
lower eukaryotic orthologues have only 1–3 MBSs, suggesting that all six are not required for copper transport. Work from our laboratory has demonstrated that only one of MBS5 or MBS6 of MNK was necessary and sufficient for the protein to traffic to the plasma membrane in response to copper (26). This result showed that MBS5 and MBS6 have some specific function in the copper-induced trafficking process and suggested that the first four metal binding sites may have a separate function. The effect of mutating various combinations of MBSs on the ability of MNK and WND to deliver copper to the ceruloplasmin orthologue, Fet3p, in yeast has been assessed by function. The effect of mutating various combinations of MBSs result showed that MBS5 and MBS6 have some specific function. A recent report (27) found that progressive mutations of the MBSs from CXXC to SXXS from the N-terminal end caused a complete loss of copper delivery to Fet3p after only the first two MBSs had been mutated. A zipper-like mechanism for copper binding to MBS1–4 would explain why Forbes et al. (28) found that only MBS6 of WND was necessary for complete restoration of Fet3p activity. It also was proposed that these results may indicate that the various MBSs perform distinct functions in MNK as compared with WND.

Using both yeast and mammalian cell assays, a copper-dependent interaction of Atox1 with the MBSs in the WND N terminus has been demonstrated (29). Two cysteines in the CXXC motif of Atox1 were necessary for an interaction with WND and a single MBS of WND was sufficient to promote this interaction. Interestingly, MBS5 and MBS6 of WND failed to interact with Atox1. In the same report, an interaction of the complete MNK N terminus with Atox1 was demonstrated, although a functional difference between individual MBSs of MNK had not been investigated.

Yeast two-hybrid analysis is based on an interaction-dependent reconstitution of a transcription factor in the yeast nucleus and hence is an indirect method to measure protein-protein interactions. Yeast two-hybrid screens are subject to artifacts and may miss many interactions or produce false positive results. Therefore, a recently established real-time metallochaperone-target interaction method (30) was applied here, and the data were compared with yeast two-hybrid analysis. As demonstrated for the MNK homologue WND (29), using the yeast two-hybrid assay, we found a copper-dependent interaction of Atox1 with the first four MBSs of MNK but not with MBS5 and MBS6, suggesting a preferential binding of the chaperone with the N-terminal MBSs. However, using surface plasmon resonance (SPR), we detected a copper-dependent interaction of Atox1 with the first four MBSs of MNK but not with MBS5 and MBS6. This suggested a functional interaction of the chaperone with all six MBSs. We quantitatively demonstrated for the MNK homologue WND (29), using the yeast two-hybrid system (Clontech), that only MBS6 of WND was necessary for complete reconstitution of a transcription factor in the yeast nucleus.

**EXPERIMENTAL PROCEDURES**

**Construction of Vectors Used for Yeast Two-hybrid Analysis**—The yeast two-hybrid plasmid pGAD GH was obtained from Clontech, and the construction of pLexA/pBTM116 has been described (31). Atox1 cDNA was reverse-transcribed from human fibroblast RNA using the primers 5′-CATGCGGAAAGCCAGGATCTC-3′ and 5′-CTCAAGGCGGATGGAACAGAC-3′ and cloned into Bluescript II-KS (Stratagene, La Jolla, CA) using a T-vector protocol (32). Atox1 was then excised by BarnHI/EcoRI and cloned into the same sites of pGAD GH, leading to plasmid pLexA152. A 1.8-kb MNK fragment encoding the full-length MNK N terminus was amplified from pCMB99 (26) using the primers 5′-GGACTGTGACGAGGGAATAC-3′ and 5′-TCAACTTGATATCGAGCTCCAC-3′ and cloned as a BamHI/PstI fragment into pLexA, resulting in the plasmid pCMB151. To generate the LexA-MBS1/2 fusion protein, a 0.9-kb BamHI/NsiI fragment was excised from the plasmid pCMB151 and cloned into the BamHI/PstI sites of pLexA, resulting in pCMB226. The regions encoding MBS2/4, MBS3/4, and MBS3/4m34 were amplified by PCR from pCMB151, pCMB99m1–3, and pCMB99m1–6, respectively (26), using the primers 5′-GAGGTAGTTGATCCGAGGGAATAC-3′ and 5′-AAGACGGCTCGAGTGTCTTTATT- TCTCG-3′. These fragments were then subcloned as BamHI/PstI fragments into pLexA, thus generating the constructs pCMB222 (pLexA-MBS2/4), pCMB223 (pLexA-MBS3/4m34), and pCMB224 (pLexA-MBS3/ 4m34). MBS5/6 was amplified from pCMB142 (26), isolated as a BamHI/PstI fragment, and subcloned into the BamHI/PstI sites of pLexA, generating the construct pCMB225. All the amplified sequences were verified by manual or automated sequence analysis using the Thermo Sequenase cycle sequencing kit or the dyeoxy chain termination kit (both from Amersham Biosciences) and the protocol provided by the manufacturer.

**Western Blots**—Protein extracts from transformed yeast L40 cells were prepared by the trichloroacetic acid method as described in the yeast protocols handbook provided by Clontech. 30 μg of total protein extract were fractionated by SDS-PAGE and transferred onto nitrocellulose (Schleicher & Schuell). Atox1-Gal4 (see Fig. 2A) and LexA-MBS5/6 (see Fig. 2B) were detected using monoclonal anti-Ga1 and anti-LexA antibodies, respectively (both purchased from Clontech), followed by a secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amrad Biotech). The detection of peroxidase activity was carried out using the chemiluminescent peroxidase substrate (Roche Molecular Biochemicals). All the protocols were performed essentially as described previously (35).

**Peptide Synthesis**—For solid phase synthesis of the 69 amino acids containing Atox1, we employed the Fmoc (N-(9-fluorenylmethoxycarbonyl) strategy (36, 37) in a fully automated synthesizer (Applied Biosystems 433). Peptide chain assembly was performed using in situ activation of amino acid building blocks by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate. The purified material was analyzed by high pressure liquid chromatography and laser desorption mass spectrometry (Vision 2000, Finnigan MAT). Purified peptides were dissolved in double distilled water at a concentration of 700 μM and stored at −70 °C until use.

**Construction of Vectors for Overexpression of MBSs**—pGEX-4T-2 was obtained from Amersham Biosciences. A 0.7-kb fragment encoding MBS5 was amplified from pCMB226 using the primers: BSf1/BSr1 5′-TATAAGGATCGGTTGTAATCTCTCACC-3′ and MBS1/2rev 5′-AATATTCGTCGACTTCTTGTGCTACTCAACAGCCAC-3′. A 0.48-kb fragment encoding MBS6/5 was amplified from pCMB225 using the primers: BSf2/BSr2 5′-TATAAGGATCGGTTGTAATCTCTCACC-3′ and MBS3/4rev 5′-AATATTCGTCGACTTCTTGTGCTACTCAACAGCCAC-3′. All the amplified fragments were digested with SalI/BamHI and cloned into pGEX-4T-2 yielding in plasmids pGST-MBS1/2, pGST-MBS3/4, pGST-MBS4/3m3 and pGST-MBS3/4m34. All the amplified sequences were verified by automated sequence analysis using the dyeoxy chain termination kit (Amersham Biosciences) and the protocol provided by the manufacturer.

**Expression and Purification of MBS Fragments**—The plasmids were transformed and expressed in Escherichia coli strain BL21(pREP4). Cells were grown at 37 °C in LB medium with 100 μg/ml ampicillin, harvested 2 h after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside, and lysed in 50 mM Tris-Cl (pH 8.0), 10 μg/ml lysozyme, and 2 mM dithioretilol by sonicating the cells 20 times with an ultrasonic tip set to 50% power. After the addition of 1% Triton X-100, the lysate was centrifuged at 35,000 × g for 30 min, and the proteins were harvested (nieldified using 50% GSH-Sepharose-4B beads) in phosphate-buffered saline + 5 mM dithiobetitol as described by the manufacturer. The purified MBSs were released from both GST and GSH-Sepharose in a single reaction step by 10 NIH units/ml of thrombin (Sigma) and subsequently analyzed by SDS-PAGE.
and silver staining (38). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad).

**Surface Plasmon Resonance—**Surface plasmon resonance experiments were performed using a Biacore Upgrade biosensor system (Biacore Inc., Uppsala, Sweden). Purified mutant or wt MBS3/4, MBS5/6, GST-MBS1/2, or GST were diluted to 50 μg/ml in 10 mM NaOAc, pH 4.6, and covalently coupled to the sensor surface by the standard N-hydroxysuccinimide/N-ethyl-N’-(dimethyl-amino)propyl-carbodiimide activation chemistry according to the manufacturer’s instructions. Fusion proteins were injected until 800–3000 response units (RU) of wt and mutared MBS3/4, MBS5/6, GST-MBS1/2, and GST were coupled. These values corresponded to 0.8–4.8 ng/mm² immobilized protein on the chip surface. Remaining free N-hydroxysuccinimide groups were saturated by a 6-min 1 M ethanolamine injection. The interaction analysis was performed at 37 °C in a flow buffer containing 2 mM ascorbate and 50 mM NaCl, pH 7.4, at a flow rate of 5 μl/min. The sequence of the Atox1-MBS interaction experiments was as depicted in Fig. 4: metal ions were first removed from the system by a 2-min wash with 1 mM EDTA, 0.1 mM bathocuproine disulfonate (BCS) in 50 mM NaCl, pH 7.4. The immobilized proteins on the chip were then reduced by 20 mM Tris-(2-carboxy-ethyl) phosphine (TCEP) in 10 mM sodium citrate, pH 3.0 for 4 min (39). When required, the chip was preloaded with copper using 0.01 mM CuCl₂ in flow buffer. Subsequently, Atox1 was injected for 7 min. As described under “Results,” the interaction event was monitored in real-time (see Fig. 4, inset), and the sensor chip was finally regenerated with 2 mM NaCl. This washing step reset the RUs to the initial, baseline values.

**Surface Plasmon Resonance Analysis—**To estimate the apparent association and dissociation rate constants, we used linear transformation of the biosensor curves. The primary data were analyzed using the BIAevaluation 3.1 Software (Biacore Inc.), applying a Langmuir binding model (stochiometry of 1:1) to calculate $k_a$ (association rate constant in $M^{-1} s^{-1}$), $k_d$ (dissociation rate constant, $s^{-1}$), and $k_{on}$ (equilibrium constant) for the interaction of Atox1 with mutared or wt MBS fragments. For each concentration, $k_a$ and $k_d$ were obtained simultaneously from the equation $K_D = k_{on}/k_{off}$. The goodness of the fit between the fitted curve and the experimental data was expressed as $x^2$ according to the manufacturer’s protocol. For good fitting to ideal data, $x^2$ is of the same order of magnitude as the noise in RU, typically $<10$.

**RESULTS**

Our initial experiments investigated the interaction of Atox1 with fragments of MNK by yeast two-hybrid analysis. The assay was performed with LexA fusions containing various combinations of only two MBSs. As can be seen in Fig. 1 (bars 1), Atox1 interacted strongly with MBS1/2 in a copper-dependent manner as 2 μM of the copper(I) chelator BCS in the growth medium almost abolished the interaction (1, white bar). In the present experiment, the presence of BCS did reduce the activity of β-galactosidase by ~40% in the positive control (+), the dimerization of the early endosomal autoantigen EEA1 (31); negative control (−), interaction of the DNA-binding domain LexA with Gal4-Atox1; 1, interaction of Atox1 with MBS1 + MBS2; 2, interaction of Atox1 with MBS3 + MBS4; 3, interaction of Atox1 with MBS3 + MBS4 with MBS3 mutated from C to A; 4, interaction of Atox1 with MBS3 + MBS6. Black bars, activities measured under normal growth conditions; striped bars, cells grown in 200 μM CuCl₂; white bars, cells grown in 2 mM BCS. The diagram on the left of the graph depicts the MNK constructs used in the experiment. Open circle, wt MBSs; shaded circle, MBS mutated from CXXC to SXXS. The striped boxes represent LexA DNA-binding domains and the numbering of the MBSs is indicated.

**Fig. 1. Interaction of Atox1 with wt and mutant MBSs of MNK.** Yeast L40 cells were co-transformed with pLexA- and pGADGH-based plasmids, and β-galactosidase activity was measured as described under “Experimental Procedures.” For each transformation, two independent clones were assayed. The mean β-galactosidase activity of a triplet experiment of one representative clone is shown. Positive control (+), dimerization of the early endosomal autoantigen EEA1 (31); negative control (−), interaction of the DNA-binding domain LexA with Gal4-Atox1; 1, interaction of Atox1 with MBS1 + MBS2; 2, interaction of Atox1 with MBS3 + MBS4; 3, interaction of Atox1 with MBS3 + MBS4 with MBS3 mutated from CXXC to SXXS; 4, interaction of Atox1 with MBS3 + MBS6. Black bars, activities measured under normal growth conditions; striped bars, cells grown in 200 μM CuCl₂; white bars, cells grown in 2 mM BCS. The diagram on the left of the graph depicts the MNK constructs used in the experiment. Open circle, wt MBSs; shaded circle, MBS mutated from CXXC to SXXS. The striped boxes represent LexA DNA-binding domains and the numbering of the MBSs is indicated.

**Fig. 2. Western blot analysis of yeast cells co-expressing Gal4-Atox1 and LexA-MBSs.** Whole cell protein extracts were prepared from Gal4-Atox1 and LexA-MBS co-expressing strains as described under “Experimental Procedures.” Proteins (30 μg) were separated by 12.5% (A) or 7.5% (B) SDS-PAGE. Gal4-Atox1 and LexA-MBS fusion proteins were detected by immunolabeling using a monoclonal anti-Gal4 (A) and a monoclonal anti-LexA antibody (B), respectively. Negative control (−), yeast L40 cells; lanes 1–4, L40 co-expressing Gal4-Atox1 and MBS1–2 (lane 1), MBS3–4 (lane 2), MBS3–4m3 (lane 3), and MBS5–6 (lane 4). Specific bands of molecular masses of ~65, ~55, ~55, and ~50 kDa, corresponding to LexA-MBS1/2, LexA-MBS3/4, LexA-MBS3/4m3, and LexA-MBS5/6, respectively, were detected.

The negative copper effect observed with Atox1 and the MBSs of Ccc2 (23).

To investigate kinetics of the interaction of Atox1 with the MBSs of the Menkes protein, we used real-time SPR analysis. This method has been widely used in several studies to analyze relatively stable receptor-ligand interactions (40), and in a recent study of transient, copper-dependent chaperone-acceptor interaction (30). SPR is based on a change in the refractive index of a light beam upon binding of a molecule in solution to a molecule immobilized on a dextran-coated chip surface. The data are expressed in RUs and are directly proportional to the mass of protein that is bound to the immobilized protein. A response of 1000 units corresponds to a change in surface
protein concentration of ~1 ng/mm². In expectation of a transient and therefore low signal, we used GST-tagged MNK fragments purified over a GSH-Sepharose resin. The GST tag was removed by thrombin, and the purified fragments were analyzed on an SDS-polyacrylamide gel (Fig. 3). GST-MBS1/2 could be cleaved only in phosphate-buffered saline containing 0.005% SDS. Digestion of this slightly denatured protein with thrombin resulted in the release of two fragments of molecular mass of 26 kDa (data not shown). Because of near identical sizes and similar pI-values of GST and MBS1/2, we were unable to effectively isolate MBS1/2 and therefore used GST-MBS1/2 and GST as a control in the SPR experiments. All of the fragments had the expected molecular weights (Fig. 3). The 69-amino-acid, water-soluble chaperone Atox1 was chemically synthesized and analyzed as described under “Experimental Procedures.”

The purified MNK fragments were covalently coupled to the dextran surface of the sensor chip as outlined by the manufacturer. When Atox1 was immobilized on the chip, no interaction with any of the MBSs could be detected. This was also observed when the bacterial copper chaperone CopZ was coupled onto the chip (30) and was assigned to steric hindrance of the interaction caused by preferential covalent binding of the chaperone to the chip via its basic amino acids (see also “Discussion”). Between 3.0 and 4.8 ng of MNK fragments corresponding to 90 fmol/mm² GST-MBS1/2, 160 fmol/mm² wt, and mutant MBS3/4 and 200 fmol/mm² MBS5/6 were immobilized on the dextran-coated surface. For the analysis of the interaction, we used a regeneration/binding/washing procedure as shown in Fig. 4.

The presence of TCEP, which readily reduces oxidized thiols in proteins (39), and a flow buffer containing 2 mM ascorbate turned out to be crucial. When either TCEP or ascorbic acid was removed from the system, the RUs continuously increased over time in a linear fashion and, after an extensive regeneration wash, did not drop to the baseline, indicating non-specific accumulation of Atox1 on the chip surface (data not shown). When required, the MBSs were copper-loaded by a short (1 min) burst of 10 μM copper sulfate in flow buffer.

We first investigated interactions of the immobilized fragments GST, GST-MBS1/2, MBS3/4, and MBS5/6 with 10 μg/ml (1.35 μM) and 100 μg/ml (13.5 μM) Atox1 (Fig. 5, A–D). There was no specific interaction detected when Atox1 was injected onto the GST surface (Fig. 5A, I–IV). The slight increase in response units with 100 μg/ml Atox1 appeared to be non-specific because the relative response units dropped to zero when the injection cycle of the chaperone was finished. When 100 μg/ml Atox1 was injected onto a copper-loaded GST-MBS1/2-coated chip surface, we observed an increase of ~100 RUs, indicating a binding to MBS1/2 (Fig. 5B, IV). This interaction was copper-dependent (Fig. 5B, compare I and III (–copper) with II and IV (+copper)) and specific to Atox1 as 200 μg/ml (2.3 μM) of the amyloid precursor protein, a copper-binding protein suggested to be involved in copper homeostasis in the brain (41), only showed marginal interaction (Fig. 5B, gray curve). This subtle interaction may be due to the presence of a heavy metal binding site in amyloid precursor protein (42).

Similar to MBS1/2, Atox1 interacted with MBS3/4 and MBS5/6 in a copper-dependent fashion (Fig. 5, C and D, compare f and III (–copper) with II and IV (+copper)), and there was no interaction observed with 150 μg/ml (2.17 μM) bovine serum albumin (Fig. 5, C and D, curves in gray), a protein containing heavy metal binding sites (43), which was shown previously to refuse the acceptance of copper from the copper-loaded yeast homologue Atx1 (44).

Mutations in the MBSs have been demonstrated to affect both copper binding (45) and Atox1-MBS interactions, as has been analyzed by yeast two-hybrid (Ref. 29 and this report). We therefore used purified MBS3/4 fragments with CXXC to SXXX mutations in MBS3 (MBS3/4m3) and Cys to Ser mutations in both MBS3 and MBS4 (MBS3/4m34) to investigate real-time interaction with Atox1 (Fig. 6). In line with previously published data (29) and yeast two-hybrid data from this report, we did not detect a significant interaction between Atox1 and the copper-loaded double-mutant MBS3/4m34 (Fig. 6B). There was some interaction observed between Atox1 and the single site mutant MBS3/4m3, indicating that Atox1 interacted with the single, copper-loaded MBS4 (Fig. 6A).

Association (k_on) and dissociation (k_off) rate constants were determined by linear transformation of the sensorgrams according to the Langmuir binding model, which assumes that the MBS-Atox1 interaction is pseudo-first order. k_on and k_off
were determined from linear regression analysis at varying concentrations of the ligand (10, 20, 40, 60, 100, and 150 μg/ml Atox1). When the MBSs were not preloaded with copper, we were not able to calculate association and dissociation rate constants. Therefore, kinetic parameters of copper-dependent interactions only, which were obtained by curve fitting using the BIAevaluation software version 3.0, are listed in Table I.

Atox1 did not bind to a GST surface preloaded with copper (Fig. 5A), and therefore association and dissociation rates were not determined (indicated as NA (not analyzable) in Table I). Importantly, this indicated that GST alone did not contribute significantly to the binding of GST-MBS1/2 to Atox1. However, the association rate of Atox1 with GST-MBS1/2 was somewhat lower (0.12 × 10^3 M⁻¹ sec⁻¹) than with MBS3/4 and MBS5/6 (1.64 × 10^3 M⁻¹ sec⁻¹ and 0.89 × 10^3 M⁻¹ sec⁻¹, respectively), which may be assigned to the bulky GST in the GST-MBS1/2 fragment. Dissociation occurred in a similar fashion for the two MNK fragments MBS3/4m3 and MBS5/6 (around 4 × 10⁻³ sec⁻¹) and was 2-fold lower for GST-MBS1/2 and MBS3/4 (around 2 × 10⁻³ sec⁻¹). These differences in the association and dissociation rate constants led to varying binding constants, indicating different binding affinities. However, due to the relatively weak and transient nature of the copper-dependent interactions, variations were observed in different experiments. Thus, the subtle differences in the equilibrium constants observed in these SPR experiments were considered to be non-significant.

CXXC to SXXS mutations in MBS3 did not significantly affect the association and dissociation rate constant of Atox1, indicating that both MBS3 and MBS4 may have similar affinities for the copper chaperone. CXXC to SXXS mutations in both MBS3 and MBS4 completely abolished the binding of Atox1 to the fragment MBS3/4m34 (Fig. 6B and in Table I, indicated as NA, not analyzable), which underlined the essentiality of the Cys residues for the chaperone-MBS interaction.

**DISCUSSION**

The role of the six metal binding sites in the N-terminal region of MNK and WND has not been clarified. Previous work from our laboratory has established that only one of the MBS5 or MBS6 is necessary and sufficient for MNK to traffic from the trans-Golgi network to the plasma membrane in response to copper (26). If this is the case, why do MNK and WND have six MBSs, especially in view of the fact that the bacterial and yeast Cu-ATPases have only one and two, respectively? One hypothesis is that the additional MBSs in the human ATPases are involved in sequestering copper from the intracellular chaperone Atox1. A functional disparity of the human MBSs was supported by physicochemical and structural features of the MBSs (46): (i) The individual MBSs differ in their pIs. MBS1, MBS4, and MBS5 have predicted isoelectric points between 7.3 and 9.3. (ii) Other domains possess linkers of typically greater than 30 residues. To reveal the characteristic binding properties of the N-terminal MBSs of MNK to the chaperone Atox1, we here applied two independent techniques, yeast two-hybrid and SPR.

Our results from both assays demonstrated a copper-dependent interaction of Atox1 with MBS1–4 of MNK. The β-galacto-
Kinetic Analysis of Atox1 with the Menkes Protein N Terminus

High salt concentrations, varying pH, or instabilities caused by intermolecular interactions in the yeast nucleus may have a dramatic effect on the formation of a fully functional transcription factor and therefore may lead to false positive or false negative results. Data from yeast two-hybrid analysis should therefore be taken with caution and were here verified by a real-time protein-protein interaction method.

For over 10 years, SPR analysis has been used for the investigation of high affinity receptor-ligand interactions (40), and more recently, in several lower affinity binding studies (30, 47, 48). Relatively low affinity interactions ($k_{on} = 10^3 M^{-1} s^{-1}$) were observed when the MBSs were immobilized on the chip surface and the copper chaperone was injected, and not vice versa, when Atox1 was immobilized on the chip and the MBSs were injected. Clustered basic sites are exposed on the surface of copper chaperones, whereas the MBSs of the ATPases contain clusters of acidic residues (24, 49). These regions were predicted to be involved in direct electrostatic protein-protein interactions. Preferential immobilization of Atox1 to the chip surface by these basic regions may explain why an interaction was detected only when the MBSs but not the chaperone were covalently linked to the dextran layer of the chip surface. The validity of this “reversed approach” is underlined by an investigation on the complex formation and dissociation of the yeast homologue Atx1 with Ccc2, which was reversible, rapid, and with a half-life for the complex in the order of milliseconds (25). Another in vitro binding study also demonstrated that purified Cu(I)-Atx1 donated its metal ion cargo to the N-terminal MBS in a direct and reversible manner (44).

The kinetic data calculated from our experiments proposed similar affinities for all the MBSs with Atox1 (Table I) with binding constants in the range of $1 \times 10^{-8}$ to $10^{-6}$ M for MBS3–6 and $19 \times 10^{-6}$ M for MBS1/2. However, the apparent lower affinity of MBS1/2 with Atox1 may be attributed to steric hindrance of the interaction due to the bulky GST attached at the N terminus.

The hyperbolic graphs suggested a negative Atox1 cooperativity with all the MBSs and therefore a functional identity in terms of copper-dependent binding of the chaperone. The negative cooperativity is strengthened by the association and dissociation rate constants obtained with MBS3/4m3: the affinity of this single site mutant was not significantly altered as compared with the wild type fragment.

The non-cooperative interaction of the chaperone with all the MBSs may contradict the model of copper acceptance and delivery, as suggested by Huffman et al. (46). In this report, the structures of the MBSs were generated by homology modeling utilizing the coordinates of silver-loaded MBS4 (20) as a template. As the MBS5 and MBS6 of the human copper ATPase are linked by only 2 or 3 amino acids, Huffman et al. (46) proposed that domains 5 and 6 may act in concert and as copper acceptors that receive the cargo from other cytosolic domains of the P-type ATPases. However, our results were generated with pairs of MBSs and did not include the full-length N terminus. We therefore cannot entirely rule out the possibility that Atox1 transfers copper to the N-terminal MBSs and from there is then directly passed onto MBS5 and MBS6. To elucidate this model, SPR experiments involving the full-length N terminus and mutants thereof are under investigation.

In conclusion, we here showed by real-time surface plasmon resonance that all the metal binding sites of the Menkes protein were involved in binding of copper-loaded Atox1. The interaction of Atox1 with the MBSs was transient, of non-cooperative nature, and indicated similar binding constants for all the MBSs in the order of $10^{-8} \times 10^{-6}$ M. Therefore, a functional difference of the MBSs in the MNK N terminus may not be

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**TABLE I**

Kinetic parameters of copper-dependent Atox1-MBS interactions

| Protein     | $k_{on}$ ($\times 10^3$) | $k_{off}$ ($\times 10^3$) | $K_D$ ($\times 10^6$) | Figure |
|-------------|--------------------------|--------------------------|-----------------------|--------|
| GST         | NA                       | NA                       | NA                    | 5A     |
| GST-MBS1/2  | 0.12                     | 3.69                     | 19.00                 | 5B     |
| MBS3/4      | 1.64                     | 1.57                     | 0.96                  | 5C     |
| MBS3/4, m3  | 2.07                     | 4.25                     | 2.05                  | 6A     |
| MBS3/4, m34 | NA                       | NA                       | NA                    | 6B     |
| MBS5/6      | 0.88                     | 3.98                     | 4.47                  | 5D     |

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sidase activity in the yeast two-hybrid assay was minimal for MBS5/6, suggesting that Atox1 did not bind to these two MBSs. This result is in accordance with previously published two-hybrid data on the WND N terminus (29) and in apparent contradiction to the results obtained by SPR (Fig. 5D). Yeast two-hybrid analysis is based on an interaction-dependent reconstitution of a transcription factor in the yeast nucleus and thus is an indirect method for protein-protein interactions.
induced by varying affinities of the metallochaperone Atox1 to the MBSs of MNK.

Acknowledgments—We thank Michael Petris for critical reading of the manuscript. We are indebted to Judy Callaghan for the yeast strain L40, the yeast two-hybrid vectors, and the EEA1 positive control.

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J. Biol. Chem. 2003, 278:20821-20827.
doi: 10.1074/jbc.M212437200 originally published online April 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212437200

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