Structure-Function Analyses of the ATX1 Metallochaperone*

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Saccharomyces cerevisiae Atx1p represents a member of the family of metallochaperone molecules that escort copper to distinct intracellular targets. Atx1p specifically delivers copper to the Ccc2p copper transporter in the Golgi. Additionally, when overproduced, Atx1p substitutes for superoxide dismutase 1 in preventing oxidative damage; however the mechanistic overlap between these functions is unresolved. The crystal structure of Atx1p has been solved recently. By examining a surface electrostatic potential distribution, multiple conserved lysines are revealed on one face of Atx1p. An additional conserved lysine (Lys65) lies in close proximity to the metal binding site. Through site-directed mutagenesis, residues in the metal binding region including Lys65 were found to be necessary for both copper delivery to Ccc2p and for Atx1p antioxidant activity. Copper trafficking to Ccc2p also relied on the lysine-rich face of Atx1p. Surprisingly however, elimination of these lysines did not inhibit the antioxidant activity of Atx1p. We provide evidence that Atx1p does not suppress oxidative damage by a metallochaperone mechanism but may directly consume superoxide. Purified Cu-Atx1p acts noncatalytically with superoxide anion in vitro. We conclude that the copper-trafficking and antioxidant functions of Atx1p arise from chemically and structurally distinct attributes of this metallochaperone.

Copper is required by all organisms as a cofactor for specific enzymes that participate in oxygen chemistry. In eukaryotes, copper metalloenzymes are found in multiple cellular locations including the cytosol, mitochondria, and cell surface (1). Until recently, it was unknown how copper could be widely distributed in the cell for the activation of many copper enzymes. A new class of small proteins termed copper chaperones, or metallochaperones, deliver copper to specific intracellular targets (for review, see Ref. 2). All of the copper chaperones isolated to date were first identified in the baker’s yeast Saccharomyces cerevisiae, and functional homologues have been noted in Arabidopsis thaliana, Caenorhabditis elegans, mice, and humans (3–7). Three copper metallochaperones have been characterized thus far: (i) yeast Lys7p (human CCS), which delivers copper to superoxide dismutase 1 (Sod1p) in the cytosol; (ii) yeast and human Cox17p, which direct copper to the mitochondria for activation of cytochrome oxidase (5, 9); and (iii) yeast Atx1p (human HAH1 or ATOX1), which specifically carries copper to the secretory pathway for incorporation into copper enzymes destined for the cell surface or extracellular milieu (4, 10, 11).

The target of copper delivery by Atx1p is a P-type copper transporting ATPase confined to a late Golgi compartment (10–12). In humans, this intracellular copper pump is encoded by the Wilson and Menkes disease genes (13), and a functional homologue (Ccc2p) has been characterized in yeast (14, 15). Via the action of Ccc2p, copper is incorporated into a multi-copper oxidase Fet3p, which translocates to the plasma membrane and works in conjunction with the iron permease to mediate high affinity iron uptake (14, 16). Atx1p was not originally identified as a metallochaperone for Ccc2p, but rather as an antioxidant molecule (hence the name Atx1) capable of suppressing oxidative damage in yeast lacking SOD1 (17). Currently, nothing is known about the mechanism by which ATX1 acts as antioxidant.

Atx1p is a prototype for a structural family of metal binding proteins and domains. Included in this family are MerP, a bacterial carrier for mercury ions (18), and the metal binding domains of the Ccc2p and Wilson and Menkes copper transporters (11). Homology among these various domains extends throughout the 8-kDa polypeptide segment, including an invariant metal binding motif, MXXXC. The NMR structures for MerP (18) and an Atx1-like domain of the Menkes transporter (19) have been obtained, and very recently, the crystal structure of Atx1p has been solved (36). A common structural unit has been revealed, consisting of a βαββαβ fold and a metal binding site on the polypeptide surface (18, 19, 36).

Using the crystal structure as a guide, we have employed a structure-function approach to probe the biological roles of Atx1p. Our studies have revealed that the antioxidant and metallochaperone functions of Atx1p require overlapping but distinct regions of the polypeptide. Furthermore, we provide evidence that the ATX1 antioxidant function does not occur by a metallochaperone mechanism, but Atx1p itself may be acting as a scavenger of free radicals.

EXPERIMENTAL PROCEDURES

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Mutagenesis of Yeast ATX1

Uptake were conducted in quadruplicate samples precisely as described (23).

Plasmids—pMIP025 was generated by mobilizing the ATX1 fragment from pRSA-1 (17) through digestion with BamHI and Xhol and by inserting this fragment into the same sites of the URA3 2-μm plasmid, pRS426 (24). The majority of the mutant ATX1 alleles (K24,28E; K24,28A; K61,62E; K65E; K24,28,61,62E) were created by standard 4-primer polymerase chain reaction mutagenesis (25) using outer primers engineered with BamHI and Xhol restriction sites to facilitate ligation of the polymerase chain reaction product into the pRS413 (CEN) and pRS423 (2-μm) vectors (24). The remaining mutants (K61,62A; K65A; K65F) were generated using the QuickChange™ site-directed mutagenesis kit from Stratagene (per instructions of the manufacturer) and the ATX1 expression vectors p413-A1 (CEN) and pRS-A1 (2-μm) as templates. All mutants were confirmed by dyeoxy automated sequencing (Johns Hopkins University CORE sequencing facility). The Atx1p-Gal4 DNA binding domain fusions (for 2-hybrid analysis) were created as described previously (11), using the mutant alleles of ATX1 as template for polymerase chain reaction.

Biochemical Analyses—Two-hybrid studies were conducted essentially as described using the MATCHMAKER System (CLONTECH) (11) and the yeast strain MP101. For Western blot analysis, yeast cells were grown overnight in selecting synthetic dextrose medium to confluence, and protein extracts were prepared as described previously (10). Extracts were subjected to SDS-polyacrylamide gel electrophoresis using 14% precast Tris-glycine gels (Novex) followed by immunoblot analysis using a rabbit anti-Atx1p antibody diluted 1:1,000. The secondary antibody consisted of anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:10,000. Detection employed the ECL kit (Amer sham Pharmacia Biotech), according to the specifications of the manufacturer.

The in vitro SOD activity studies utilized a purified Cu(I) Atx1p prepared according to previously described protocols (11), with the exception that Cu(I)CH3CNPF6 was used to load the protein rather than the combination of CuSO4 and dithiothreitol. Superoxide scavenging activity was measured by the standard cytochrome c xanthine oxidase assay (26) in buffers supplemented with 1.0 mm EDTA.

RESULTS

Structural Features of Atx1p—The x-ray structure of Atx1p was recently solved at 1.02-Å resolution (36). Using the refined coordinates, a surface electrostatic potential distribution was generated with the program GRASP (27) (Fig. 1A). Examination of this distribution reveals a region comprising multiple lysine residues that generate a positively charged face on the protein surface (Fig. 1A). These basic residues are highly conserved between the yeast and human metallochaperones (Fig. 1B) and include: Lys24 and Lys28 located on α-helix 1 and loop 2, Lys61 and Lys62 located on α-helix 2, and Lys65 which is found in close proximity to the metal ion coordinated by cysteine at positions 15 and 18 (Fig. 1A). All these regions were targeted for mutagenesis, and substitutions engineered at these sites are summarized in Fig. 1B.

The Effects of ATX1 Mutations on the Delivery of Copper to Ccc2p—atx1Δ strains are defective for iron uptake because of lack of copper incorporation into Fet3p (10). Therefore, iron uptake provides a simple assay for monitoring Atx1p activity and can be used to obtain a qualitative estimate for how well copper is delivered from Atx1p to Ccc2p. Low copy CEN plasmids expressing mutant alleles of ATX1 (Fig. 1B) were used to transform an atx1Δ strain and were tested for the ability to support high affinity uptake of 55Fe. The majority of these Atx1p mutant variants accumulated to near wild-type levels when expressed in the atx1Δ strain (Fig. 24).

The lysine patches represented by K24,28 and K61,62 of Atx1p were found to be critical for the delivery of copper ions to Ccc2p. Altering K24,28 to acidic glutamates (K24,28E) resulted in an Atx1p molecule that was severely crippled in supporting iron uptake (Fig. 2B). Additionally, changing K61,62 to negatively charged glutamates and neutral alanines also reduced iron uptake, but to a lesser extent than was observed with mutations at K24,28 (Fig. 2B). Changing the overall charge of the Atx1p basic region from positive to negative in the K24,28,61,62E Atx1p may in fact represent an overestimation of mutant protein activity because iron starvation is known to induce Fet3p synthesis (28).

We recently observed that the two cysteines of the Atx1p metal binding site (MTCX) are essential for copper delivery to Ccc2p (29). We additionally tested the role of Lys65 adjacent to this metal binding site. As seen in Fig. 2B, the K65E allele of Atx1p was severely crippled in this assay. Surprisingly, variants K65A and K65F each exhibited nearly wild type levels of activity (Fig. 2B) even though these alleles were expressed to very low degrees. Thus, a negative charge adjacent to the metal binding site of Atx1p prohibits copper delivery to Ccc2p, whereas a basic, neutral, or hydrophobic residue at this position appears well tolerated.

As a second assay for Atx1p activity, we monitored physical interaction between Atx1p and Ccc2p by use of the 2-hybrid system. Consistent with previous results (11), a 2-hybrid signal can be detected in cells expressing Gal4 fusions to wild type Atx1p and to the metal binding domains of Ccc2p (Fig. 3, A and B). This interaction requires a functional metal binding site of Atx1p because a C15,18S mutation abolished the signal (Fig. 3A). Lys65F adjacent to the metal site, as well as lysines in the basic face of Atx1p, likewise seemed important for physical interaction with Ccc2p (Fig. 3B). It seemed surprising that K61,62E, which exhibited 50% activity in the iron uptake assay, was unscored for interaction with Ccc2p. As would be expected for a transient reaction, the 2-hybrid signal obtained with Atx1p is normally very weak (11), and any reduction in Atx1p recognition of Ccc2p is likely to lower the signal to levels beyond our detection.
The Effects of ATX1 Mutations on the Antioxidant Role of Atx1p—To define the regions of Atx1p needed for antioxidant protection, the various Atx1p mutants were expressed from a multi-copy 2-µm yeast vector in a strain lacking both Atx1p and Sod1p. As seen in Fig. 4, all mutants tested accumulated to the same high level obtained with wild type Atx1p.

Fig. 4. The effect ATX1 mutations on suppression of oxidative damage. The atx1Δ sod1Δ strain SL106 was transformed with the multi-copy plasmid pRS423 (VECTOR) or with the same vector expressing either wild type or the indicated mutant alleles of Atx1p. 1 × 10⁶ cells were spotted onto medium lacking lysine and were incubated either aerobically (+O₂) or in anaerobic culture jars (−O₂). The bottom panel shows Western blot analysis expression of the Atx1p variants as described in Fig. 2A.

The antioxidant activity of each mutant was monitored by assaying for the ability to overcome the aerobic lysine auxotrophy of sod1Δ cells. In the presence of oxygen (but not under anaerobic conditions), sod1Δ strains fail to grow on medium lacking lysine as a result of oxidative damage to component(s) of the lysine biosynthetic pathway (30, 31). This defect is suppressed by overexpressing wild type ATX1 (Fig. 4). Full suppression of oxidative damage was also achieved with Atx1p mutants targeting lysines 24 and 28. Moreover, the K24,28,61,62E variant of Atx1p, which was completely inactive for copper activation of Ccc2p and Fet3p (Fig. 2B), fully suppressed the aerobic lysine auxotrophy of the sod1Δ strain (Fig. 4). It was curious that the K61,62E allele failed to suppress oxidative damage, but the significance of this result is difficult to reconcile because these substitutions have no deleterious effect when present in combination with K24,28E (Fig. 4). In any case, changing the overall charge of the lysine-rich face from basic to acidic (in the case of K24,28,61,62E) resulted in a molecule that was still wild type for suppressing oxidative damage.

In comparison to results obtained with the lysine patch mutants, mutations directed at the metal binding region of Atx1p greatly inhibited its antioxidant activity. We previously found that the C15,18S mutant lacking the copper binding cysteine ligands fails to suppress the sod1Δ aerobic lysine auxotrophy (29). Mutations that targeted Lys 65 positioned near the metal site also affected antioxidant function. Alleles K61E and K65F were completely inactive, and the K65A mutant was only partially functional in this assay (Fig. 4).

How Does ATX1 Function As an Antioxidant?—We addressed whether ATX1 suppresses oxidative damage by acting as a metallochaperone for a molecule other than Ccc2p. By definition, copper chaperones are only required under conditions of limiting cellular copper (9, 10, 32, 37). Therefore, if Atx1p suppresses oxidative damage by acting as a metallochaperone, it should be possible to mimic this activity by high copper. However, we noted that treatment with high copper did not substitute for multi-copy ATX1 in suppressing sod1Δ defects (Fig. 5A). By contrast, the same treatment with copper quite effectively substituted for Atx1p in delivering copper to Ccc2p, as monitored by high affinity iron uptake (Fig. 5B). In fact, high copper treatment resulted in decreased physical interaction between Atx1p and Ccc2p, as revealed by 2-hybrid analysis (Fig. 5C), emphasizing the notion that copper chaperones are not required when copper is plentiful. Therefore, while Atx1p delivers copper to Ccc2p and Fet3p only under metal limiting conditions, the antioxidant behavior of Atx1p cannot
be explained by a similar metallochaperone mechanism.

We addressed whether Atx1p itself may serve as an antioxidant. By the standard cytochrome c/xanthine oxidase assay for SOD activity (26), a purified Cu-Atx1p did exhibit the ability to consume superoxide, as demonstrated by the inhibition of cytochrome c reduction toward the reaction onset (Fig. 6, slope A). The activity obtained with 720 nM Cu-Atx1p was equivalent to that seen with 0.84 ± 0.09 nM Sod1 dimer, or 1.7 ± 0.18 nM Sod1 monomer; hence, the reaction of superoxide with Cu-Atx1 is approximately 430-fold less efficient than with Sod1. Moreover, with Atx1p, the rate of cytochrome c reduction toward the later portion of the assay, fit to slope B in Fig. 6, returned to the rate of the control sample, indicating that purified Atx1p does not act catalytically as a dismutase in vitro. Despite this limited activity, our calculations indicate that when Atx1p is over-produced in vitro, the corresponding superoxide consumption capacity may be sufficient to substitute for Sod1p (see "Discussion").

DISCUSSION

The x-ray structure of Atx1p reveals an intriguing patch of lysine residues along one face of the molecule, as well as an additional lysine (Lys$^{65}$) adjacent to the MXXXC metal binding site. We find that sequences in the metal coordination site, including the two copper binding cysteines and the neighboring Lys$^{65}$, are necessary for both Atx1p activation of Ccc2p/Fet3p and for its antioxidant role. In comparison, the basic face of Atx1p (residues K24, 28, 61, 62) is important only for copper distribution to Ccc2p but not for the antioxidant function of Atx1p, supporting the proposal that this region may be uniquely important for interactions between Atx1p and Ccc2p (36). A structurally characterized metal binding domain of the Menkes protein (19) contains an acidic face, and a number of these acidic residues are conserved in Ccc2p (36). The mutagenesis data are consistent with the hypothesis that docking of Atx1p with Ccc2p involves electrostatic interactions between the basic face of the metallochaperone and the corresponding acidic face in its target protein (36).

Previously, Gitlin and co-workers reported that lysines 57 and 60 of the HAH1 human homologue to Atx1p (corresponding to lysines 62 and 65 of Atx1p) are more critical for the antioxidant function of this metallochaperone than for delivery of copper to Ccc2p (33). Although these conclusions appear to be at odds with our findings, the results of the two studies are in fact, compatible. With the crystal structure of Atx1p now in hand, allele K57,60G of HAH1 is predicted to impinge on both the metal binding region (Lys$^{60}$) and on the basic patch (Lys$^{57}$) of the metallochaperone and should, therefore, affect both activities of the metallochaperone. Accordingly, Gitlin and co-workers observed a partial reduction in copper delivery to Fet3p and complete inhibition of antioxidant activity with this mixed allele (33).

What is the significance of the essential basic residue near the metal binding site of Atx1p? This lysine is conserved among all known members of the Atx1p metallochaperone family, including those from plants (3), nematodes (6), and mammals (4, 7). In comparison, a hydrophobic aromatic residue is present at the corresponding position in 5 of 6 Atx1-like domains in the Wilson and Menkes proteins (19) and in both of the metal binding domains of Ccc2p (11). In the case of Atx1p, Lys$^{65}$ may affect the kinetics or thermodynamics of copper-protein interaction through hydrogen bonding to a coordinated cysteine sulfur atom. Lys$^{65}$ is predicted to partially neutralize the negative charge that results from a coordination of two (or more) cysteinate anions to Cu(I) and would thereby stabilize the copper-chaperone complex. A working hypothesis is that allosteric conformation changes at Lys$^{65}$ may occur in the docking of the chaperone with its partner Ccc2p (11).

The studies presented here provide several lines of evidence that the antioxidant activity of Atx1p is distinct from its ability to act as a metallochaperone. First, the basic surface residues that are critical for copper delivery to Ccc2p are not needed for the antioxidant activity of Atx1p. Second, unlike other metallochaperone functions (9, 10, 32, 37), high levels of copper cannot substitute for the antioxidant activity of Atx1p, indicat-

![Fig. 5. The effects of increasing copper availability on Atx1p activity.](Image)

![Fig. 6. Superoxide scavenging by Cu(I)-Atx1p in vitro.](Image)
ing that Atx1p does not suppress oxidative damage by delivering copper to another molecule.

Finally, we provide evidence that Atx1p itself acts as a scavenger of superoxide anion, albeit a poor one compared with Sod1p. Yet the level of activity observed with purified Atx1p in vitro may be sufficient to suppress sod1Δ deficiency in vivo based on the following calculations: approximately 430 molecules of Atx1p are needed to neutralize an equivalent amount of superoxide as 1 molecule of Sod1p. We observed that Atx1p molecules per cell (34), it is conceivable that the superoxide consumption by multi-copy Atx1p is sufficient to substitute for Sod1p in this regard. This antioxidant activity appears to be physiologic and not simply an artifact of ATX1 overexpression because complete deletion of Atx1p causes paraquat sensitivity (17). We conclude that this small copper binding molecule has evolved with dual and separable functions that aid in the handling of copper in aerobic environments.

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1 This calculation is based on reports that yeast cells harbor ~4 × 10^4 active monomers Sod1p/cell (37), and that ≤2% of the total Sod1p of a yeast cell yields wild type levels of protection against oxidative damage (34). The lower limit of Sod1p that is required for antioxidant protection has not been determined but is expected to be far less than the upper limit of 8 × 10^2 molecules/cell.