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# Unification of the Copper(I) Binding Affinities of the Metallo-chaperones Atx1, Atox1, and Related Proteins

## *DETECTION PROBES AND AFFINITY STANDARDS*[*] [S]

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**Literature estimates of metal-protein affinities are widely scattered for many systems, as highlighted by the class of metallo-chaperone proteins, which includes human Atox1. The discrepancies may be attributed to unreliable detection probes and/or inconsistent affinity standards. In this study, application of the four $Cu^{I}$ ligand probes bicinchoninate, bathocuproine disulfonate, dithiothreitol (Dtt), and glutathione (GSH) is reviewed, and their $Cu^{I}$ affinities are re-estimated and unified. Excess bicinchoninate or bathocuproine disulfonate reacts with $Cu^{I}$ to yield distinct 1:2 chromatophoric complexes $[Cu^{I}L_2]^{3-}$ with formation constants $\beta_2 = 10^{17.2}$ and $10^{19.8}$ $M^{-2}$, respectively. These constants do not depend on proton concentration for pH ≥7.0. Consequently, they are a pair of complementary and stable probes capable of detecting free $Cu^{+}$ concentrations from $10^{-12}$ to $10^{-19}$ M. Dtt binds $Cu^{I}$ with $K_D \sim 10^{-15}$ M at pH 7, but it is air-sensitive, and its $Cu^{I}$ affinity varies with pH. The $Cu^{I}$ binding properties of Atox1 and related proteins (including the fifth and sixth domains at the N terminus of the Wilson protein ATP7B) were assessed with these probes. The results demonstrate the following: (i) their use permits the stoichiometry of high affinity $Cu^{I}$ binding and the individual quantitative affinities ($K_D$ values) to be determined reliably via noncompetitive and competitive reactions, respectively; (ii) the scattered literature values are unified by using reliable probes on a unified scale; and (iii) Atox1-type proteins bind $Cu^{I}$ with sub-femtomolar affinities, consistent with tight control of labile $Cu^{+}$ concentrations in living cells.**

The human metallo-chaperone protein Atox1 (known also as Hah1) delivers $Cu^{I}$ to the *trans*-Golgi network (1, 2). Atx1, the version from the yeast *Saccharomyces cerevisiae*, was the first copper metallo-chaperone to be identified (3). They both feature the classic ferredoxin $\beta\alpha\beta\beta\alpha\beta$-fold with a *CXXC* motif acting as a high affinity $Cu^{I}$-binding site (Fig. 1) (4, 5). Homologues are found in cyanobacteria (Atx1), in *Enterococcus hirae* (CopZ), in *Bacillus subtilis* (CopZ), and in many other organisms (6).

The human $P_{1B}$-type ATPase ATP7A accepts copper from Atox1 and transports it into the lumen of the *trans*-Golgi network (2). ATP7B performs a related role in liver cells. The inherited disorders Menkes and Wilson diseases are associated with defects in ATP7A and ATP7B, respectively (7). Equivalent metal transporters exist in other organisms such as Ccc2 from *S. cerevisiae* (3) and heavy metal ATPases 5–8 (HMA5–8) in the simple plant *Arabidopsis thaliana* (8). Their N termini contain between one and six metal-binding domains (MBDs)[2] that may interact with and receive $Cu^{I}$ directly from Atox1-type metallo-chaperones (6). It appears that, for some $Cu^{I}$-ATPases at least, metal-binding sites in the transmembrane domain may also independently receive $Cu^{I}$ from copper chaperones (9). The overall molecular structure and binding site of each MBD is similar to that of Atox1 (10).

Accurate estimation of affinities for $Cu^{I}$ (as expressed by the dissociation constant $K_D$) is essential for a quantitative understanding of reactivity and mechanisms of action. Yet reported $K_D$ values are scattered widely as highlighted by those of Atox1-type proteins, which differ by more than 10 orders of magnitude ($K_D \sim 10^{-5}$, $10^{-10}$, $10^{-14}$, and $10^{-18}$ M) even though the structures and metal-binding sites of these proteins essentially superimpose (11–16). The various values were determined via different experimental approaches with different ligand probes and affinity standards. The affinities of some of the probes and standards remain in dispute. In addition, the intrinsic instability of free $Cu^{+}$ in aqueous solution and the tendency to aerial oxidation of cysteine ligands impose special conditions on these experiments. These aspects are complicated further by reports that thiol ligands such as endogenous glutathione (GSH) may expand the $Cu^{I}$ coordination sphere in these proteins or lead to polymeric forms (14, 17, 18).

In an attempt to resolve these fundamental issues for this iconic set of proteins, this study surveys the literature values for the $Cu^{I}$ affinities of the four probe ligands bicinchoninate (Bca), bathocuproine disulfonate (Bcs), dithiothreitol (Dtt), and glutathione (GSH) (Scheme 1). By direct experimental comparison, their relative affinities are unified with the single standard Bcs whose absolute formation constant is also documented. Then the affinities of Atx1, Atox1, and related proteins are re-estimated or recalculated accordingly, allowing a coherent system to be established for quantitative determination and comparison of $Cu^{I}$ binding affinities.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and supplemental Figs. S1 and S2.

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[2] The abbreviations used are: MBD, metal-binding domain; Bca, bicinchoninic acid anion; Bcs, bathocuproine disulfonate; ESI, electrospray ionization; ITC, isothermal titration calorimetry; NHE, normal hydrogen electrode.

## EXPERIMENTAL PROCEDURES

*Materials*—$Na_2Bca$, $Na_2Bcs$, and GSH were purchased from Sigma and Dtt from Astral Scientific and were used as received. Concentrations of Bca and Bcs solutions were standardized routinely as described previously (19). Stock solutions of Dtt and GSH were both prepared in deoxygenated Milli-Q water and stored in an anaerobic glove box. Their concentrations based on quantitative dissolution were confirmed and calibrated with the Ellman assay (20). The copper standards were either purchased from Aldrich as an atomic absorption standard solution or prepared directly from the salt $[Cu^I(CH_3CN)_4]ClO_4$, prepared, and purified according to the literature (21).

*Protein Isolation and Quantification*—Atox1 was expressed from an expression plasmid pET20b-Atox1 transformed in *Escherichia coli* BL21(DE3)pLysS cells, and the expressed Atox1 protein was purified by gradient elution (0–0.3 M NaCl) from a cation-exchange CM-52 column equilibrated in an acetate buffer (20 mM; pH 5.4; 5 mM $\beta$-mercaptoethanol). This was followed by a Superdex-75 FPLC gel filtration column in $KP_i$ buffer (20 mM; pH 7.0; 150 mM NaCl, 0.5 mM tris(2-carboxyethyl)phosphine). Atx1 and the N-terminal MBDs 5 and 6 of human Wilson protein (WLN5–6) were expressed and isolated as detailed previously (11, 22). A CopC protein variant CopC-H48C, which binds $Cu^I$ with higher affinity than wild type CopC, was also generated and purified as reported previously (23). The purity of the proteins was confirmed as >95% by SDS-PAGE and the identity by ESI-MS. As reported previously (17), two components (7401.7 and 7270.5 Da) were detected in the Atox1 preparation, corresponding to molecules with and without the first methionine residue, respectively. All proteins were isolated in apo-forms with no detectable copper or zinc content. Prior to the copper binding studies, apoproteins were reduced fully by incubation overnight with Dtt (5 mM) in a glove box under dinitrogen and separated from the reductant via a Bio-Gel P-6 DG gel desalting column (Bio-Rad) in the glove box.

Protein concentrations were estimated by three independent approaches as follows: (i) solution absorbance at 280 nm with reported extinction coefficients; (ii) thiol content analyzed with Ellman reagent, 5,5-dithiobis(2-nitrobenzoic acid), employing $\epsilon_{418} = 13{,}600\ M^{-1}\ cm^{-1}$ for the released chromophore 5-mercapto-2-nitrobenzoate (20); and (iii) $Cu^I$ binding stoichiometries estimated via $Cu^I$ reagent $[Cu^I(Bca)_2]^{3-}$. The protein concentrations estimated by the approaches ii and iii matched each other exactly (see below), whereas the concentrations estimated by approach i fluctuated slightly (<20% variation), especially for those proteins with low extinction coefficients at 280 nm. Such small fluctuation is likely caused by the contribution to the absorbance of other minor adventitious components, and consequently the protein concentrations estimated via the approaches ii and iii were used for the final calculations. Fully reduced apo-Atx1, apo-Atox1, apo-WLN5–6, and apo-CopC-H48C contain 2, 3, 5, and 1 eq of cysteine thiol, respectively. Although Atx1, Atox1, and CopC-H48C react with 5,5-dithiobis(2-nitrobenzoic acid) quantitatively within several minutes, reaction of 5,5-dithiobis(2-nitrobenzoic acid) with WLN5–6 takes >10 min to complete. However, addition of urea (to ~3 M) accelerates the reaction considerably.

*Formation Constant of $[Cu^{II}(Bcs)_2(H_2O)]^{2-}$*—The determination was based on the Bjerrum method, *i.e.* the competition for ligand Bcs between $Cu^{2+}$ and protons was followed by pH titration with $HCl_{aq}$ (24) as shown in Reactions 1 and 2,

$$[Cu^{II}(Bcs)_2(H_2O)]^{2-} + H^+ \rightleftharpoons [Cu^{II}(Bcs)(H_2O)_x] + [HBcs]^- \quad \text{REACTION 1}$$

$$[Cu^{II}(Bcs)(H_2O)_x] + H^+ \rightleftharpoons Cu^{2+}{}_{aq} + [HBcs]^- \quad \text{REACTION 2}$$

Formation constants $K_1$ and $\beta_2$ of the 1:1 and 1:2 complexes, respectively, were derived by a graphical solution of Equation 1 (supplemental Fig. S1). For clarity, ionic charges are omitted and $[HBcs]^-$ is represented as "Bcs" in all equations,

$$\frac{\bar{n}}{(1-\bar{n})[Bcs]} = K_1 + \frac{(2-\bar{n})[Bcs]}{(1-\bar{n})}\beta_2 \qquad \text{(Eq. 1)}$$

$$\bar{n} = [Bcs]_{total} - [Bcs](1 + [H]/K_a)/[Cu^{II}]_{total} \qquad \text{(Eq. 2)}$$

where $\bar{n}$ is the Bjerrum formation function as defined by Equation 2 (*i.e.* the average number of Bcs ligands bound per $Cu^{II}$

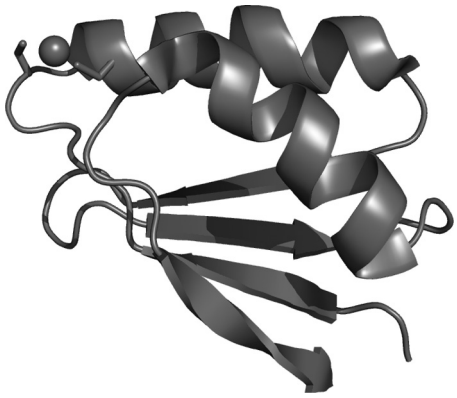
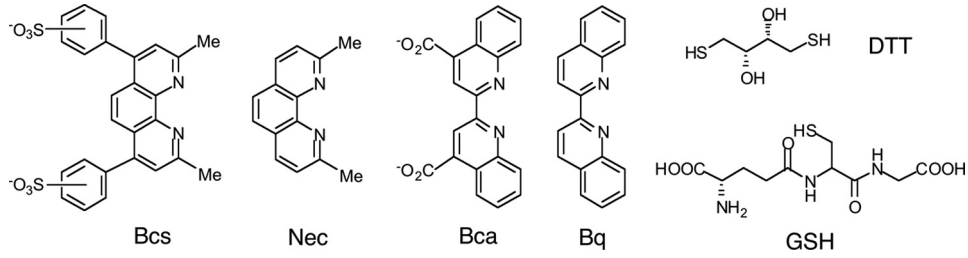


FIGURE 1. **Ribbon representation of the ferredoxin fold ($\beta\alpha\beta\beta\alpha\beta$) in $Cu^I$-Atox1 (Protein Data Bank code 1FD8).** The copper atom is represented as a *sphere*, and the two cysteinyl ligands are shown as *sticks*.




SCHEME 1. **Molecular structures of metal ligands.**

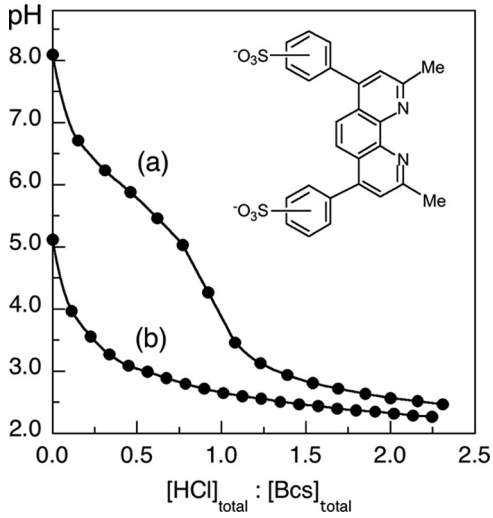



FIGURE 2. **Determination of formation constants in the $H^+$-$Cu^{2+}$-Bcs system.** *a*, titration of $Na_2Bcs$ solution (4.0 mM; 0.1 M $NaNO_3$) with $HCl_{aq}$ (~100 mM) at 20 °C; *b*, as for *a* but in the presence of $CuCl_2$ (2.0 mM).

center under the conditions). $K_a$ is the acid dissociation constant of $[HBcs]^-$, and [H] is the proton concentration determined by a pH meter. The free ligand concentration [Bcs] was determined from a mass balance of proton concentration according to Equation 3,

$$[Bcs] = ([HCl]_{total} - [H] + K_w/[H])/[H]/K_a \qquad \text{(Eq. 3)}$$

where $K_w/[H]$ is the proton concentration derived from the self-ionization of water. Results are presented in Fig. 2, Table 1, and supplemental Table S1.

*Formation Constant of $[Cu^I(Bcs)_2]^{3-}$*—The redox couple $Cu^{II}/Cu^I$ is chemically reversible in the presence of more than 2 eq of Bcs (Reaction 3) with a well defined reversible potential $E'_m = 0.64$ V *versus* NHE (see supplemental Fig. S6 of Ref. 25). Consequently, an estimation of the formation constant of $[Cu^I(Bcs)_2]^{3-}$ may be made via the Nernst relationship (Equation 4) from an $E_0$ value of 0.164 V (26) and the formation constant of $[Cu^{II}(Bcs)_2(H_2O)]^{2-}$. Experiments were performed as detailed previously (25) and results are presented in Table 1.

$$[Cu^{II}(Bcs)_2(H_2O)]^{2-} + e^- \rightleftharpoons [Cu^{I}(Bcs)_2]^{3-} + H_2O$$

REACTION 3

$$E'_m = E_0 - 0.059 \log (\beta_2(Cu^{II})/\beta_2(Cu^{I})) \qquad \text{(Eq. 4)}$$

*Formation Constant of $[Cu^I(Bca)_2]^{3-}$*—An estimate of $\beta_2$ ($=10^{17.2}$ $M^{-2}$) was obtained previously via indirect competition for $Cu^I$ between ligands Bca and Bcs mediated separately by three apoproteins that bind $Cu^I$ with different affinities (Atx1, nA-PcoC, and C42S-rubredoxin) (27). This value was consolidated in this work with another $Cu^I$-binding protein CopC-H48C that competes more effectively with *both* Bca and Bcs under the same conditions (Table 1). The experimental details follow those for Atx1-type proteins as detailed below.

*$Cu^I$ Binding Stoichiometries and Affinities of Atx1, Atox1, and Related Proteins*—The experiments were performed in an anaerobic glove box by reaction of apoproteins with $[Cu^IL_2]^{3-}$ (L = Bca or Bcs) in deoxygenated buffers, as described previously (11, 28). Briefly, apoprotein was titrated into solutions of $[Cu^IL_2]^{3-}$ of defined molar ratio $L:Cu^I \geq 2.5$ (to ensure the presence of the 1:2 complex $[Cu^IL_2]^{3-}$ with negligible contribution from the 1:1 complex $[Cu^IL]^-$). The reaction mixtures were diluted to a fixed volume to provide a series of solutions with constant total concentrations of $Cu^I$ and ligand L but varying concentrations of protein P. Transfer of $Cu^I$ from $[Cu^IL_2]^{3-}$ to P was established by the change in absorbance at 483 nm for L = Bcs ($\epsilon$ = 13,000 $M^{-1}$ $cm^{-1}$) and at 562 nm for L = Bca ($\epsilon$ 7,900 $M^{-1}$ $cm^{-1}$) (11, 27). Systematic variation of ligands L and their concentrations defined conditions that favored either competitive or noncompetitive reactions. The metal binding stoichiometry was derived from the noncompetitive reactions, whereas the dissociation constants were estimated from competitive reactions, according to Reaction 4 and Equation 5 (29). Results are presented in Table 2.

$$P + ML_2 \rightleftharpoons M\text{-}P + 2L$$

REACTION 4

$$K_D\beta_2 = \frac{([P]_{total}/[MP]) - 1}{\{([L]_{total}/[ML_2]) - 2\}^2[ML_2]} \qquad \text{(Eq. 5)}$$

To mimic cellular conditions, reactions in the presence of GSH (1 mM) were also conducted for competitive reactions between $[Cu^IL_2]^{3-}$ and P and compared with equivalent reactions without GSH.

*$Cu^I$ Binding Stoichiometry and Affinity of Dtt*—The method was the same as that for the proteins with minor modifications. Solutions of Dtt in $H_2O$ at concentrations >1 mM are stable for at least 1 week under anaerobic conditions. However, they are air-sensitive in buffered solutions at pH ≥7 and can be oxidized significantly by trace dioxygen (see *e.g.* Fig. 5). Therefore, the effective concentrations of Dtt were verified prior to each experiment by the Ellman assay. Dithionite (~4 mM) was included in the reaction mixture to scavenge trace dioxygen before addition of Dtt. Results for determination of the apparent dissociation constant $K_D'$ at pH 6.8 and 7.3 are given in Tables 2 and supplemental Table S3.

## RESULTS

The two bidentate di-anions Bca and Bcs are classic chromophoric ligands for $Cu^I$ (Scheme 1). They are water-soluble and are proposed to buffer free $Cu^+$ concentrations over the respective ranges $10^{-12}$–$10^{-16}$ and $10^{-15}$–$10^{-19}$ M (29). Consequently, they are a pair of complementary $Cu^I$ probes with versatile applications in quantitative study of copper chemistry in biology (29). However, the overall formation constant $\beta_2$ for both ligands are in dispute (see below), and those for Bcs were reported as either unpublished or undocumented data only in Refs. 11, 14. It is important to provide proper documentation and confirmation of these two formation constants if the probes are to form the foundation of a reliable and unified scale.

*Ligand Probe Bcs*—The presence of methyl groups at the 2- and 9-positions of the 1,10-phenanthroline ligands Bcs and neocuproine (Scheme 1) provides steric hindrance at the coordination site, favoring $Cu^I$ binding (tetrahedral preference) over $Cu^{II}$ binding (square planar preference). Consequently, the reduction potentials of the redox couple $Cu^{II}/Cu^I$ of these

**TABLE 1**
**Acid dissociation constants and absolute formation constants for ligands**
Ionic strength was 0.1 M.

| Ligand | Bcs | | Neocuproine | | Bca[a] | | Bq[b] |
|---|---|---|---|---|---|---|---|
| $pK_a$ | 5.7 | 5.7 | 5.88 | 5.79 | | | 3.1 |
| $\alpha_{H\text{-}L}$[c] | 0.95 | 0.95 | 0.93 | 0.94 | | | 1.0 |
| $Cu^I$, log $\beta_2$ | 19.9 | 19.8 | 19.1 | 19.5 | 17.3 | 17.2 | ~16.5 |
| $Cu^{II}$, log $K_{a1}$ | ~5.6 | 6.1 | ~6.1 | 6.2 | | | 4.27 |
| log $K_{a2}$ | ~6.2 | 5.5 | ~5.6 | 5.5 | | | 3.46 |
| log $\beta_2$ | 11.8 | 11.7 | ~11.7 | 11.7 | | | 7.73 |
| Reference | This work | 11 | 30 | 26 | This work | 27 | 30 |

[a] Estimates by indirect ligand competition based on $\beta_2 = 10^{19.8}$ M$^{-2}$ for $[Cu^I(Bcs)_2]^{3-}$.
[b] Bq is 2,2-biquinolyl. Data were obtained in 50% dioxane/water.
[c] Acid coefficient at pH 7 calculated via the following: $\alpha_{H\text{-}L} = 1/(1 + 10^{(pK_a - pH)})$, where $K_a$ is the acid dissociation constant of ligand L.

ligands are positive (0.64 V *versus* NHE; see Ref. 25), and the $Cu^I$ complex is stable to both disproportionation and reaction with dioxygen. The affinities of neocuproine for the proton, $Cu^I$, and $Cu^{II}$ are provided in Refs. 26, 30 (Table 1), but as discussed above, those for Bcs are poorly documented.

In potentiometric titrations, the acid dissociation constant of $H_2Bcs$ is not detected (Fig. 2*a*) and so that for $[HBcs]^-$ only was considered in Equations 1–3 for pH >2.7. Experimentally, a solution of $Na_2Bcs$ of known concentration (~4 mM; 10 ml) in $H_2O$ (0.1 M $NaNO_3$) was titrated with a standard solution of $HCl_{aq}$ (98.6 mM) at 20 °C. The $pK_a$ value was estimated from the mid-point of the titration curve after 0.5 eq of $HCl_{aq}$ had been added, providing a value of $pK_a = 5.7$ (Fig. 2*a*), consistent with the literature value $pK_a = 5.8$ (31). Equivalent titrations in the presence of $CuCl_2$ (~0.50 eq) induced competition between $Cu^{2+}$ and $H^+$ for Bcs according to Reactions 1 and 2 (Fig. 2*b*), allowing estimation of the $Cu^{II}$ formation constants log $K_1$ = ~5.6 and log $\beta_2$ = 11.8 by the Bjerrum method (24). The data were processed according to Equations 2 and 3 and a graphical solution of Equation 1. Results are presented in Fig. 2*b* and in Table 1 and supplemental Table S1. These data are in agreement with those reported in Ref. 11.

The propensity of $Cu^+_{aq}$ to disproportionation prevents determination of the formation constants of $[Cu^I(Bcs)_2]^{3-}$ by the Bjerrum method. However, the couple $Cu^{II}/Cu^I$ in the presence of more than 2 eq of Bcs is chemically reversible ($\Delta E_p$ = 100 mV at $\nu$ = 20 mV s$^{-1}$; $I_c/I_a$ = ~1), allowing accurate estimation of the reversible potential $E'_m$ = 0.64 V *versus* NHE (25). This is consistent with a previously determined value of 0.62 V *versus* NHE (32). The reversible redox chemistry is attributed to Reaction 3 and log $\beta_2$ = 19.9 may be estimated for $[Cu^I(Bcs)_2]^{3-}$ from the Nernst relationship (Equation 4). This estimate is consistent with those (19.1–19.5) reported previously for the parent ligand neocuproine and consolidates the previous value (log $\beta_2$ = 19.8) reported for the same ligand (Table 1 and Scheme 1), but it is lower than a recent value of log $\beta_2$ = 22.1 at pH 6, which was estimated by competition for $Cu^I$ with cyanide (14). No experimental details were provided for the latter estimate, and the competition is likely to be complicated by the formation of ternary complexes involving monodentate cyanide ligand.

*Ligand Probe Bca*—The $pK_a$ values of the carboxylate substituents in ligand Bca (Scheme 1) are expected to be comparable with those of the pyridyl ring nitrogens, making it difficult to estimate the affinities for $Cu^{II}$ via the potentiometric approach used above for Bcs. In addition, the $Cu^{II}/Cu^I$ redox process is irreversible (25), preventing derivation of $\beta_2$ for $[Cu^I(Bca)_2]^{3-}$ from the $Cu^{II}$ form via the Nernst equation. Consequently, the formation constant $\beta_2$ for $[Cu^I(Bca)_2]^{3-}$ was determined previously by indirect competition for $Cu^I$ between ligands Bca and Bcs mediated separately by the three apoproteins Atx1, nA-PcoC, and C42S-rubredoxin (27) that bind $Cu^I$ with different affinities. A value of log $\beta_2$ = 17.2 for $[Cu^I(Bca)_2]^{3-}$ was estimated on the basis of log $\beta_2$ = 19.8 for $[Cu^I(Bcs)_2]^{3-}$ as derived above. However, the $Cu^I$ affinities of Atx1 and C42S-rubredoxin ($K_D$ ~$10^{-18}$ M) are a little too high for effective competition with the weaker affinity probe Bca and that of nA-PcoC ($K_D$, $10^{-12.7}$ M) is a little too low for effective competition with higher affinity probe Bcs. A protein with the intermediate affinity necessary for simultaneous competition with both Bca and Bcs was created in this study to reliably establish the relative affinities of Bca and Bcs for $Cu^I$.

CopC is a copper-binding protein expressed to the periplasmic space in the copper resistance response of the bacterium *Pseudomonas syringae.* It binds $Cu^I$ with an affinity of $10^{-12.2}$ M at a site involving His-48 as a metal ligand (23, 29). Mutation of His-48 to Cys increases the $Cu^I$ affinity by 2 orders of magnitude to $K_D = 10^{-14.3}$ M in protein H48C-CopC, allowing effective competition with *both* Bca and Bcs to be induce in the same reaction buffer (supplemental Fig. S2). A value of log $\beta_2$ = 17.3 for $[Cu^I(Bca)_2]^{3-}$ was derived, consolidating the previous estimate of log $\beta_2$ = 17.2.

These estimates are supported by independent work on H61A-CsoR, a protein variant of the $Cu^I$ sensor protein CsoR from *Mycobacterium tuberculosis.* Competition with either Bca and Bcs under the same conditions provided the same value, $K_D$, ~$10^{-14.7}$ M, within experimental error (33). In addition, the value of log $\beta_2$ = 17.2 for Bca is comparable with that estimated for the parent ligand 2,2-biquinolyl in mixed dioxane/water media (log $\beta_2$ = 16.5; Scheme 1; Table 1).

It should be noted that the value log $\beta_2$(Bca) = 17.2 derived is dependent on accurate estimation of log $\beta_2$(Bcs), and so an independent estimation would help in establishing this constant. Such an estimate, log $\beta_2$(Bca) = 14.7, has been obtained by isothermal titration calorimetry (ITC) by titration of a $Cu^+$ solution with Bca (13). The two estimates differ by 2.5 orders of magnitude. This estimation of weaker affinity may be rationalized from two experimental considerations as follows.

(i) The ITC binding isotherm showed a single exothermic process upon titration of Bca into a $Cu^+$ solution with a sharp end point as the ratio [Bca]/[$Cu^+$] approached 2.0 (see supplemental Fig. S2 of Ref. 13), consistent with stoichiometric formation of $[Cu^I(Bcs)_2]^{3-}$, *i.e.* with effective saturation of the metal ion. Consequently the affinity of Bca for $Cu^I$ is too high to be estimated by ITC under the conditions (34). This is confirmed by a complementary experiment in which the titration of $Cu^+$ into Bca solution is monitored by the absorbance at 562 nm (see Fig. 4*b* of Ref. 29). A suitable competing ligand is required to decrease the metal ion fractional saturation to <90% for reliable estimation (35–37).

(ii) The $Cu^+$ reagent employed was generated by reduction of $Cu^{2+}$ with ascorbate in aqueous solution (200 mM NaCl),

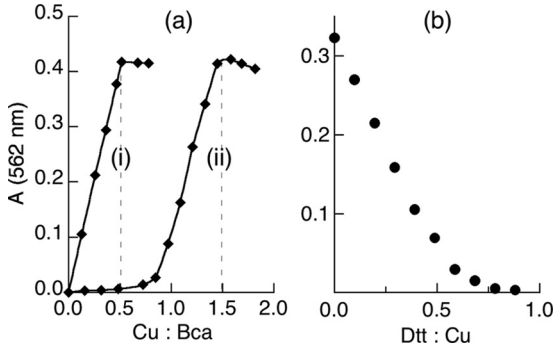



FIGURE 3. **Determination of the stoichiometry of $Cu^I$ binding to Dtt.** *a,* titration of $[Cu^I(MeCN)_4]^+$ into a solution of Bca (100 $\mu$M) in Mops buffer (50 mM, pH 7.3) under the following conditions: *Trace i* is without Dtt; *trace ii* is with Dtt (50 $\mu$M). *b,* Titration of Dtt into a $[Cu^I(Bca)_2]^{3-}$ solution with total concentrations of $Cu^I$ = 40 $\mu$M and Bca = 100 $\mu$M.

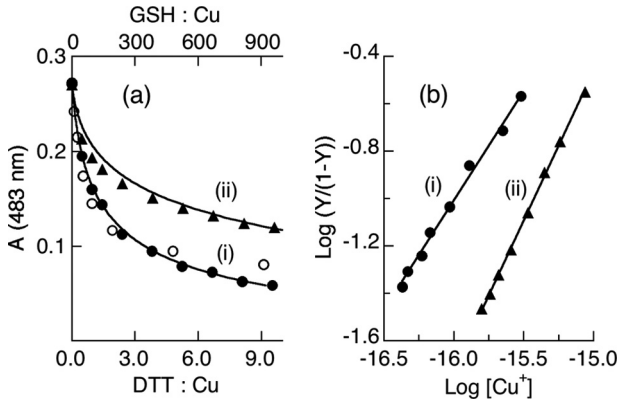



FIGURE 4. **Determination of the affinity of $Cu^I$ binding to Dtt.** *a,* variation of $A_{483}$ (proportional to the concentration of $[Cu^I(Bcs)_2]^{3-}$) with increasing concentrations of Dtt (10–240 $\mu$M) or GSH (0.2–19 mM) under the condition of fixed concentrations of $[Cu^I]_{total}$ (21 $\mu$M) and $[Bcs]_{total}$ (50 $\mu$M) in Mops buffer (50 mM) containing sodium dithionite (4 mM). The traces shown were the best fits of the experimental data to Equation 5 at pH 7.3 (*trace i*) and pH 6.8 (*trace ii*). The *open circles* in *trace i* are for equivalent experiments in which Dtt was replaced by GSH at ~100-fold higher concentration. *b,* Hill plots of the experimental data in *a*, producing Hill coefficients 0.9 and 1.3 for the data at pH 7.3 (*trace i*) and pH 6.8 (*trace ii*). *Y* is defined as the fractional occupancy of the total $Cu^I$ binding.

which stabilizes $Cu^+$ as $[Cu^ICl_n]^{(1-n)-}$. Titration with Bca then involves conversion of these species, rather than $Cu^+_{aq}$, to $[Cu^I(Bca)_2]^{3-}$. This difference is expected to diminish the observed heat release leading to an underestimate (35). The value of log $\beta_2$(Bca) derived from the ITC experiments represents a lower limit of the affinity.

Another report of $\beta_2$ ~11.4 for $[Cu^I(Bca)_2]^{3-}$ was estimated by direct $Cu^+$ titration but without adequate documentation (14). However, as pointed out above, the $Cu^I$ affinity of Bca is too high to be estimated by direct $Cu^+$ titration.

*Ligands Dtt and GSH*—Dtt (Cleland's reagent; Scheme 1) is a strong reductant ($E'_m$ = −0.33 V *versus* NHE at pH 7). When present at sufficiently high concentration, it can scavenge $O_2$ and has been used widely to protect protein cysteine thiols against aerial oxidation (38). It is most effective at pH values above 7 as only the negatively charged thiolate form is reactive (p$K_a$ 9.0 and 10.0) (39). The reducing power is driven partially by the formation upon oxidation of a stable six-membered ring with an internal disulfide bond.

Dtt binds $Cu^I$ to produce complexes whose stoichiometries depend on the reaction conditions (39). The absolute dissociation constant log $K_D$ = −15.3 for the 1:1 complex was estimated via potentiometric titration of $Cu^I$-Dtt samples prepared *in situ* via Dtt reduction of $Cu^{2+}$ (39). As Dtt has been used previously as both a competing ligand and an affinity standard for estimation of the $Cu^I$ affinities of proteins (16, 40), its $Cu^I$ binding stoichiometry and affinity were re-investigated here to provide a unified scale.

Titration of $[Cu^I(MeCN)_4]^+$ into a solution containing Bca (100 $\mu$M) and Dtt (50 $\mu$M) in Mops buffer (50 mM, pH 7.3) was followed by $A_{562}$, an absorbance maximum characteristic of the $[Cu^I(Bca)_2]^{3-}$ complex. The titration induced little initial absorbance change until the addition of ~0.85 eq of $Cu^+$ relative to Bca and then the absorbance increased steadily to a sharp end point at 1.5 eq of $Cu^+$ (Fig. 3*a, trace ii*). A control titration without Dtt increased the absorbance linearly with an end point at 0.5 eq of $Cu^+$, as expected for formation of $[Cu^I(Bca)_2]^{3-}$ (Fig. 3*a, trace i*). These data lead to two conclusions. (i) Dtt competes overwhelmingly with Bca for $Cu^I$ under the conditions (see the first turning point). (ii) Dtt binds 2 eq of $Cu^I$ when Bca is nearly saturated with 0.5 eq of $Cu^I$ (see the second turning point). Accordingly, titration of Dtt into a $[Cu^I(Bca)_2]^{3-}$ solution ($Cu^I$, 40 $\mu$M; Bca, 100 $\mu$M; pH 7.3) led to linear transfer of $Cu^I$ from Bca to Dtt (Fig. 3*b*). At the point Dtt:Cu = 0.5, ~80% of total $Cu^I$ had been extracted from Bca by Dtt, and each Dtt molecule binds, on average, 1.6 eq of $Cu^I$. The free $Cu^+$ concentration at this titration point can be calculated to be $10^{-13.8}$ M from the known affinity of Bca. Consequently, the second eq of $Cu^I$ binds to Dtt with $K_D \leq 10^{-13.8}$ M (*i.e.* with higher affinity). Therefore, to act as a metal buffer, the metal occupancy on the ligand Dtt must be lower, and so Bca is not an effective probe of the affinity of Dtt for $Cu^I$ at lower metal occupancy. Consequently, Bcs was used as a competing ligand to quantitatively probe this affinity.

Titration of Dtt into a $[Cu^I(Bcs)_2]^{3-}$ solution ($Cu^I$, 21 $\mu$M, Bcs, 50 $\mu$M) in Mops buffer (50 mM, pH 7.3) induced a steady decrease in $A_{483}$, indicative of transfer of $Cu^I$ from Bcs to Dtt (Fig. 4*a, trace i*). As the experiments with the Bca probe showed that Dtt can bind 2 eq of $Cu^I$, the fractional occupancy $Y$ is defined as the mole ratio of $Cu^I$ removed from Bcs to twice the total Dtt added. A Hill plot of log($Y$/(1 − $Y$)) *versus* log[$Cu^+$] generates a straight line with slope (Hill coefficient) of 0.9 (Fig. 4*b, trace i*), consistent with little or no binding cooperativity between the two $Cu^I$-binding sites. The metal occupancy of Dtt in the Bcs solution is low (<0.6 $Cu^I$ per Dtt molecule). Consequently, the competition for $Cu^I$ between Bcs and Dtt can be modeled with the equivalent of Reaction 4 and the $Cu^I$ binding affinity estimated via curve-fitting of the experimental data to Equation 5 (Fig. 4*a, trace i*; and supplemental Table S2). An apparent $K_D' = 10^{-15.3}$ M at pH 7.3 is derived. To evaluate the impact of pH on the binding affinity, the same experiments were performed at the lower pH of 6.8 (Fig. 4, *a* and *b, trace ii*). The Hill coefficient is a little higher (1.3), but the data can be fitted satisfactorily with Equation 5, producing an apparent $K_D$ value of $10^{-14.6}$ M at pH 6.8. This is five times larger than the value determined experimentally at pH 7.3 (Table 2). It is apparent that the affinity of Dtt for $Cu^I$ is pH-dependent.

**TABLE 2**
**Apparent dissociation constants $K_D'$ of ligands for $Cu^I$ determined via competition with probe Bcs**
Unified estimates are shown in boldface type.

| Ligand | pH | log $K_D'$ | $[Bcs]_{total}$ | Ref. |
|---|---|---|---|---|
| | | | $\mu M$ | |
| CopC-H48C | 7.0 | **−14.3** | 25 | This work |
| | 7.0 | **−14.2**[a] | | This work |
| Dtt[b] | 6.8 | **−14.6** | 50 | This work |
| | 7.3 | **−15.3** | 50 | This work |
| | | −15.3[c] | | 39 |
| | 7.4 | −11.1[d] | | 39 |
| HMA4n | 7.3 | **−16.7** | 200 | 28 |
| Atox1 | 7.0 | **−17.4** | 200 & 500 | 29 |
| | ~7.5 | −13.8[e] | | 16 |
| | ~7.5 | −18.0[f] | | 16 |
| WLN5–6 | 7.0 | **−17.6** | 200 & 500 | This work |
| Atx1 | 7.0 | **−17.7** | 300 & 500 | This work |
| | 8.0 | **−18.2** | 1000 | 11 |
| HMA7n | 7.3 | **−18.1** | 500 | 28 |
| Ccc2n | 8.0 | **−18.8** | 1000 | 11 |

[a] Data were determined with $[Bca]_{total} = 100\ \mu M$ as competing ligand.
[b] Affinity for $Cu^I$ is highly pH-dependent.
[c] Absolute $K_D$ value was determined via potentiometric titration assuming formation of the 1:1 complex $Cu^I$-Dtt.
[d] Apparent $K_D'$ value at pH 7.4 was calculated from the absolute $K_D$ value ($=10^{-15.3}$ M) via the calculation in Footnote 3 assuming formation of a 1:1 complex $Cu^I$-Dtt, where $pK_{a1}$ 10.0 and $pK_{a2}$ 9.0 represent the two proton ionization constants of Dtt (39).
[e] Apparent $K_D'$ value estimated via ESI-MS in $NH_4Ac$ (pH ~7.5) relative to apparent $K_D' = 10^{-11.1}$ M at pH 7.4 for $Cu^I$-Dtt as calculated in Footnote 3.
[f] Apparent $K_D'$ value estimated via ESI-MS in $NH_4Ac$ (pH ~7.5) after correction of the apparent $K_D' = 10^{-15.3}$ M at pH 7.3 for $Cu^I$-Dtt, which was estimated via competition with Bcs. However, Dtt is very air-sensitive (see Fig. 5) and will be oxidized partially under the conditions of ESI-MS. Consequently, the apparent Cu(I) $K_D'$ value determined via competition with Dtt with ESI-MS as a detection probe will be overestimated, depending on the extent of Dtt oxidation.

Assuming the presence of a 1:1 complex, an absolute dissociation constant $K_D = 10^{-15.3}$ M was estimated previously via potentiometric titration (39). Coincidentally, this is the same value as that derived above for the conditional dissociation constant $K_D'$ at pH 7.3. The experimental sample solutions were prepared *in situ* via Dtt reduction of $Cu^{2+}$ in air. A standard calculation of the apparent pH dependence of $K_D$ using the equation in Footnote 3 (which assumes formation of a 1:1 complex $Cu^I$-Dtt) estimated an apparent dissociation constant $K_D' = 10^{-11.1}$ M at pH 7.4. The equation predicts that the $K_D'$ value will change by 2 orders of magnitude per pH unit change around pH 7.

Note that this calculated $K_D' = 10^{-11.1}$ M at pH 7.4 is 4 orders of magnitude larger (weaker affinity) than that estimated experimentally here by ligand competition as $K_D' = 10^{-15.3}$ M at pH 7.3. These discrepancies prompted scrutiny of the application of potentiometric titration to Dtt and the calculation to the pH variation. Several pitfalls became apparent as follows.

(i) Free $Cu^+$ is susceptible to disproportionation and oxidation in aqueous solution, and so potentiometric titrations in air are liable to underestimate the absolute formation constants of $Cu^I$ complexes.

(ii) Dtt is also susceptible to aerial oxidation in a pH buffer, even at pH 7 and significant oxidation would also lead to underestimation of $K_D$. The experiment in Fig. 5 demonstrates that brief exposure of a solution of $Cu^I$-Dtt to air can lead to complete oxidation of Dtt ligand (200 $\mu M$) in minutes. In this experiment, Dtt oxidation is coupled to trapping of $Cu^I$ by the Bcs ligand shown in Reaction 5.

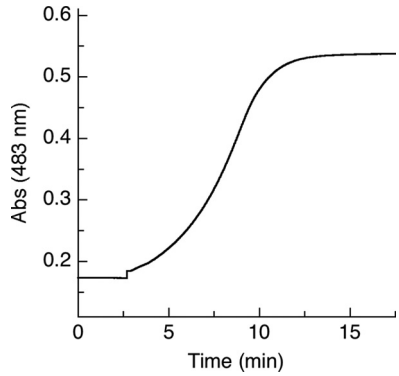



FIGURE 5. **Time course of aerial oxidation of Dtt.** The reaction was monitored by Reaction 5 in Mops buffer (50 mM, pH 7.3). The initial solution compositions are as follows: $[Cu^I]_{total}$, 40 $\mu M$; $[Bcs]_{total}$, 100 $\mu M$; $[Dtt]_{total}$, 200 $\mu M$. The oxidation was initiated by a brief bubbling of air at $t = 2.5$ min.

$$\text{“}Cu^I\text{-Dtt”} + 2Bcs^{2-} \xrightarrow{O_2} [Cu^I(Bcs)_2]^{3-} + (Dtt)_{ox}$$

REACTION 5

(iii) The equation used to calculate the pH dependence of $K_D$ assumed the presence of a complex with Cu:Dtt = 1:1.[3] However, the present experiments indicate an average value of ~2:1.

The conclusions from the ligand competition experiments in Fig. 4 are that $K_D' = 10^{-15.3}$ M at pH 7.3 and that it is pH-dependent. It is apparent that although Dtt has advantages as a volatile $Cu^I$ buffer that is able to protect protein thiols, it is unsatisfactory as a $Cu^I$ affinity reference as the affinity is sensitive to aerial oxidation, to solution pH, and to the $Cu^I$:Dtt ratio present in solution.

GSH (Scheme 1) is the low molar mass thiol present in the cytosol (0.5–10 mM). It functions as a redox buffer and may also act as a metal buffer for soft metal ions such as $Cu^I$. Experiments with GSH similar to those with Dtt demonstrated that the $Cu^I$ affinity of GSH is much weaker than that of Dtt (the *circles* in Fig. 4*a, trace i,* are for GSH at 2 orders of magnitude higher concentration than that used for Dtt). At a higher Bcs concentration (>200 $\mu M$) where Atox1-type proteins compete strongly for $Cu^I$, GSH cannot compete (even at millimolar concentration; supplemental Tables S3 and S4). This result contrasts with that from gas phase experiments that concluded that the affinity of GSH for $Cu^I$ is similar to that of Dtt and that it can compete for $Cu^I$ with the Atox1 protein (16). The current uncertainties associated with ESI-MS detection are addressed under the “Discussion.”

*$Cu^I$ Binding Stoichiometries of Atx1, Atox1, and Related Proteins*—As discussed in the Introduction, the reported $Cu^I$ affinities of these proteins are controversial. To resolve the issues, copper metallo-chaperones Atx1 (yeast), Atox1 (human), and the N-terminal metal-binding domains 5–6 of Wilson disease protein (WLN5–6) were expressed and isolated, and their $Cu^I$ binding properties were re-investigated with the Bca and Bcs probes. The results were compared with two related protein domains HMA4n and HMA7n, the N-ter-

[3] Ref. 39 used the equation $K_D' = K_D\ (1 + 10^{(pK_{a1}-pH)} + 10^{(pK_{a1}+pK_{a2}-2pH)})$, derived assuming the presence of a 1:1 complex $Cu^I$-Dtt.

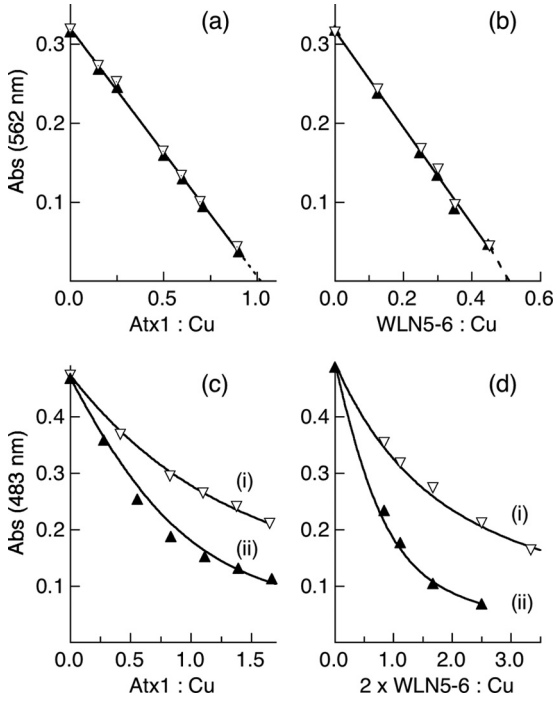



FIGURE 6. **$Cu^I$ binding stoichiometries and affinities of yeast Atx1 and human WLN5–6.** *a* and *b*, determination of $Cu^I$ binding stoichiometry of Atx1 (*a*) and WLN5–6 (*b*) under the copper limiting condition imposed by $[Cu^I(Bca)_2]^{3-}$ as a noncompetitive probe. Conditions are as follows: $[Cu^I]_{total}$ = 40 $\mu$M; $[Atx1]_{total}$ = 6–36 $\mu$M or $[WLN5-6]_{total}$ = 5–18 $\mu$M; $[Bca]_{total}$ = 100 $\mu$M (*filled triangles*) or 500 $\mu$M (*empty triangles*) in $KP_i$ buffer (25 mM, pH 7.0; NaCl 100 mM). The trace shown is a linear best fit of all data points providing an intercept of 1.0 for Atx1 and of 0.51 for WLN5–6 at $A_{562}$ = 0. *c* and *d*, determination of dissociation constant $K_D(Cu^I)$ of Atx1 (*c*) and WLN5–6 (*d*) under copper limiting conditions imposed by $[Cu^I(Bcs)_2]^{3-}$ as a competitive probe. Conditions are as follows: $[Cu^I]_{total}$ ~36 $\mu$M; $[Atx1]_{total}$ = 10–60 $\mu$M or $[WLN5-6]_{total}$ = 15–60 $\mu$M; $[Bcs]_{total}$ = 500 $\mu$M in *trace i*, 300 $\mu$M in *c, trace ii,* and 200 $\mu$M in *d, trace ii*; 25 mM buffer $KP_i$, pH 7.0; 100 mM NaCl. Addition of GSH (1.0 mM) into each Bcs solution did not alter the equilibrium position significantly. The traces shown were the best fits to Equation 5 of each set of experimental data.

minal MBDs of the metal transporters HMA4 and HMA7 of *A. thaliana* that transport $Zn^{II}$ and $Cu^I$, respectively (28).

Prior to the $Cu^I$ binding experiments, care was taken to ensure that the Cys thiols of each protein were fully reduced and that common reductants such as Dtt and tris(2-carboxyethyl)phosphine were removed completely. These reductants also bind $Cu^I$ with high affinities (see above). The present experiments were performed under anaerobic conditions. After reduction with Dtt overnight and recovery of proteins via a desalting column in a glove box with $[O_2]$ <2 ppm, the concentrations of reduced proteins Atx1, Atox1, and WLN5–6 were estimated via solution absorbance and the reduced cysteine content. The concentrations estimated via the two approaches agreed with <20% difference, but those estimated via cysteine content gave more consistent $Cu^I$ binding stoichiometries and affinities and thus were adapted in this study.

Addition of Atx1 to a solution of $[Cu^I(Bca)_2]^{3-}$ (40 $\mu$M; $[Bca]_{total}$, 100 $\mu$M) led to quantitative extraction of 1 eq of $Cu^I$ from $[Cu^I(Bca)_2]^{3-}$ (Fig. 6*a*). The $Cu^I$ occupancy on protein remained essentially unchanged upon increasing $[Bca]_{total}$ from 100 to 500 $\mu$M or upon variation of protein concentration within the range 6–36 $\mu$M. Similar data were obtained from equivalent experiments with Atox1 (29) and WLN5–6 proteins except that WLN5–6 contains two separate Atox1-type domains and extracted 2 eq of $Cu^I$ from $[Cu^I(Bca)_2]^{3-}$ (Fig. 6*b*) (29). These results are consistent with our previous observations for the two related protein domains HMA4n and HMA7n that exhibit similar ferredoxin-type folds and related metal-binding sites (28). These data confirmed that Bca at concentrations ≤500 $\mu$M cannot compete for $Cu^I$ with these proteins and that each protein or domain binds 1 eq of $Cu^I$ under the $Cu^I$ limiting conditions defined by that probe (Reaction 6; Fig. 6, *a* and *b*). Other weaker binding sites (adventitious) cannot compete under the above conditions.

$$Atx1 + [Cu^I(Bca)_2]^{3-} \rightarrow Cu^I\text{-}Atx1 + 2Bca^{2-}$$

REACTION 6

*$Cu^I$ Binding Affinities of Atx1, Atox1, and Related Proteins*—Exploratory experiments with $[Cu^I(Bcs)_2]^{3-}$ revealed that the amount of $Cu^I$ removed by yeast Atx1 via Reaction 4 was less than 1 eq and that, under the condition of $[Bcs]_{total}$ ≥300 $\mu$M, both the $Cu^I$ occupancy on protein (as expressed by $\theta = [Cu^IP]/[P]_{total}$) and the change in $Cu^I$ occupation on the ligand probe (as expressed by $A/A_{original}$) varied sensitively with total protein concentration within the range $\theta$ = 0.2–0.8 (Fig. 6*c*; supplemental Table S3). This confirmed an *effective* competition for $Cu^+$ between Atx1 and Bcs under the defined conditions. Accordingly, analysis of each data set in Fig. 6*c* via Equation 5 based on $\beta_2 = 10^{19.8}$ $M^{-2}$ for $[Cu^I(Bcs)_2]^{3-}$ generated a *consistent* average $K_D = 2.1(2) \times 10^{-18}$ M at pH 7.0 for Atx1 (supplemental Table S3). This value is slightly larger than a previous value ($6.3 \times 10^{-19}$ M) determined at pH 8.0 (11), indicating that the $Cu^I$ affinities of these proteins may be pH-dependent. Equivalent experiments for Atox1 produced similar data with average $K_D = 3.9(1) \times 10^{-18}$ M at pH 7.0 (29). WLN5–6 binds two $Cu^I$ at two separate MBDs, but the experimental data with various $Cu^I$ occupancy on the protein were fitted satisfactorily with a single $K_D$ of 4.0(4) $\times 10^{-18}$ M (Fig. 6*d*; supplemental Table S4), indicating that the $Cu^I$ affinities of WLN5 and WLN6 are similar and are essentially the same as that of Atox1 (Table 2).

## DISCUSSION

This work unifies the affinities for $Cu^I$ of the ligand probes Bca and Dtt with that of Bcs (Tables 1 and 2). This, in turn, allows rationalization of literature estimates for high affinity $Cu^I$-binding proteins and establishes a coherent system for quantitative comparisons.

*Ligand Probes*—Both ligands Bca and Bcs are stable, soluble in water, and bind $Cu^I$ specifically to form well defined complexes $[Cu^IL_2]^{3-}$ in the presence of excess ligand. The $pK_a$ values for the amine functions are in the range of 3–4 and 4–6, respectively (41). Consequently, the $Cu^I$ binding affinities are not sensitive to pH variation at pH >7 ($0.9 < \alpha_{H\text{-}L} < 1.0$ in Table 1). The affinities of Bca and Bcs for $Cu^I$ differ by 2.6 orders of magnitude (Table 1), and so the free $Cu^+$ concentration that can be buffered also differs by 2.6 orders of magnitude at the same ligand concentration. Importantly, the buffered $Cu^+$ concentration also changes sensitively with ligand concentration (see Fig. 4*c* of Ref. 29). Consequently, these two ligands in combination are able to buffer free $Cu^+$ concentrations over

the wide range from $10^{-12}$ to $10^{-19}$ M. They constitute a pair of complementary and versatile probes for $Cu^I$.

Dtt has been used as both a competing ligand and an affinity standard in work employing ESI-MS as an indirect detector of solution phase equilibria (16, 40). Its $Cu^I$ binding stoichiometry and affinity were re-investigated here to provide a unified scale. Conditions were found where Dtt competed strongly with Bca for $Cu^I$ (Fig. 3*a*). This allowed an average binding stoichiometry of $Cu^I$:Dtt 2.0 to be extracted when the probe ligand Bca was saturated relative to the control (Fig. 3*a*). The molecular nature of the $Cu^I$-Dtt complexes present remains undefined, although they are almost certainly polymeric. Use of the stronger ligand Bcs permitted estimation of the apparent average dissociation constant $K_D'$ (Dtt) = $10^{-14.6}$ M (pH 6.8) and $10^{-15.3}$ M (pH 7.3) (Fig. 4; Table 2). Unfortunately, the ligand is highly air-sensitive. This is the key to its widespread application in protection of protein thiols but is highly disadvantageous in the present application. In addition, its $Cu^I$ affinity is pH-dependent. Overall, its use as an affinity standard is highly problematic.

*Affinities of Atx1, Atox1, and Related Proteins*—On the one hand, ligand Bca at low concentrations cannot compete for $Cu^I$ with the high affinity $Cu^I$ site ($K_D < 10^{-15}$ M) in these proteins. On the other hand, other ("adventitious") weaker binding sites ($K_D > \sim 10^{-9}$ M) in these proteins cannot compete with Bca under the same conditions. Bca proves to be an excellent probe that can define, via Reaction 6, the $Cu^I$ binding stoichiometry of the high affinity site in these proteins (*e.g.* Fig. 6, *a* and *b*; and supplemental Table S1 in Ref. 29).

Ligand Bcs does compete effectively with Atox1-type proteins for $Cu^+$ under various defined conditions. The affinity data derived for the proteins are summarized in Table 2 and provide the following order of decreasing $K_D$ values (increasing affinity) within the pH range 7.0–7.3 shown in Equation 6.

$$\text{H48C-CopC}(10^{-14.3}\text{M}) > \text{Dtt}(\sim 10^{-15}) > \text{HMA4n}(10^{-16.7}) > \text{Atox1}(10^{-17.4}) \sim \text{WLD5–6}(10^{-17.4}) \sim \text{Atx1}(10^{-17.6}) > \text{HMA7n}(10^{-18.2}) \quad \text{(Eq. 6)}$$

Notably, with $K_D$ values increasing from $10^{-14.3}$ to $10^{-18.2}$ M, the total Bcs concentrations required for effective competition increase accordingly from 25 to 500 $\mu$M (Table 2), as predicted from the calculation (see Fig. 4*c* of Ref. 29). This is to satisfy the conditions of an effective competition, *i.e.* both $[CuP]/[P]_{total}$ and $A/A_{original}$ vary within the sensitive range 0.2–0.8 with total protein and/or ligand concentrations.

The $Cu^I$ affinities of Atox1-type proteins in Equation 6 fall within a narrow range of $K_D$ $10^{-17}$–$10^{-18}$ M. Some have been determined previously in several studies, but the reported affinities are scattered widely over more than 10 orders of magnitude ($K_D \sim 10^{-5}$, $10^{-10}$, $10^{-14}$, and $10^{-18}$ M) (11–13, 16, 29). Historically, the estimate of $10^{-18}$ M for yeast Atx1 introduced Bcs as a ligand probe for $Cu^I$ (11). The approach is now consolidated with many protein examples (see Equation 6; Table 2). The low affinities implied by the first two values (12, 13) appear to have arisen from the following: (i) lack of competition and the difficulties of achieving the anaerobic conditions required for quantitative study of these proteins with the ITC technique, and (ii) the use of Bca whose affinity is too weak for it to act as an effective $Cu^I$ buffer for Atox1-type proteins (see Fig. 6, *a* and *b*, and supplemental Table S1 in Ref. 29). Reconciliation of the fourth estimate ($K_D \sim 10^{-14}$ M) is addressed below.

Returning to Equation 6, the average $Cu^I$ affinity of WLN5–6 is very similar to that of Atox1, consistent with the presence of a shallow thermodynamic gradient for $Cu^I$ transfer from Atox1 to ATP7A and ATP7B proteins in humans or from Atx1 to Ccc2 in yeast (11, 13, 16, 42). This is also consistent with previous direct $Cu^I$ exchange experiments between Atox1 and WLN5 or between Atox1 and WLN6, which showed that these proteins competed for $Cu^I$ with similar affinities (13).

The endogenous reductant GSH is reported to bind to the $[Cu^I(S\text{-}Cys)_2]^-$ center in Atox1, expanding the coordination sphere from two to three (17). GSH has also been proposed to increase the $Cu^I$ affinity of Atx1 by acting as a bridging ligand in the formation of a $Cu^I$-bridged Atx1 dimer (14). However, addition of GSH (even up to a concentration of 1 mM) into reactions designed to induce competition with Atx1, Atox1, or WLN5–6 did not affect the equilibrium positions (supplemental Tables S3 and S4; *cf.* Fig. 4*a, trace i*), as also observed previously for similar reactions (11, 42). These experiments demonstrate the following: (i) the affinity of GSH for $Cu^I$ is too low to compete when $[Bcs]_{total} > 200$ $\mu$M; (ii) GSH has little effect on the overall $Cu^I$ affinities, consistent with the observation that it is a weak co-ligand only (17).

*Unification of the Affinities of Cu$^I$-binding Proteins*—Recently, Banci *et al.* (16) estimated the relative affinities for $Cu^I$ of a representative set of intracellular copper proteins involved in copper trafficking and redox catalysis. The strength of the work lies in the application of a single detection technique (ESI-MS) to assess the thermodynamic fitness for $Cu^I$ transfer to and from proteins in defined metabolic pathways. The proteins were suspended in volatile ammonium acetate solution (pH ~7.5) with ESI-MS as the detection probe and volatile Dtt as both metal buffer and affinity standard (16). The difficulties associated with air sensitivity of each of the components $Cu^+$, Dtt, and the proteins were partially circumvented by performing the experiments in the presence of high concentrations of Dtt. As Dtt is in its uncharged form at neutral pH, it is compatible with ESI-MS experiments, even at high concentrations ($\sim 10^{-2}$ M). It both binds $Cu^I$ and protects protein cysteine thiol groups. These attributes would appear to confer significant advantages in estimating $Cu^I$ binding affinities via ESI-MS, especially for those proteins that employ cysteine as a $Cu^I$ ligand.

However, multiple uncertainties remain in the current application of ESI-MS techniques to estimation of metal-protein affinities. The prime issue is whether the relative concentrations of species reported by the gas phase data are those present at equilibrium in solution. There are a number of considerations due to the time-dependent chemistry of the transfer of ions from the solution phase to the gas phase that include the following: (i) changes in relative and absolute concentrations during loss of solvent, ligand probe, and buffer molecules as part of the droplet evaporation and coulombic fission stages of ion transfer; (ii) significant changes in pH during the above processes as "volatile buffers," such as ammonium acetate, do

not act as proton buffers around pH 7 ($pK_a(NH_4^+/NH_3) = 9.3$; $pK_a(MeCO_2H/MeCO_2^-) = 4.7$); and (iii) use of volatile Dtt as an affinity standard and competing ligand. This practice is compromised by both the pH dependence of its affinity and its oxidative instability that will alter effective concentrations during ion transfer. In addition, it is necessary to confirm that the M-P ion *only* is observed from solution samples with M:P $\geq$ 1:1 in the absence of competing ligand (*i.e.* no apoprotein must be detected under these conditions).

The uncertainties that can arise from ESI-MS studies are illustrated by the conclusion that the $Cu^I$ affinities of GSH and Dtt are similar (16), although this work concludes that the affinity of GSH is much lower than that of Dtt. The former suggestion seemed reasonable, based on an indirect assessment of the relative $Cu^I$ affinities of GSH and Dtt mediated via proteins in the gas phase. However, Dtt is much more air-sensitive than GSH, making it difficult to maintain constant relative concentrations during ion transfer. In addition, GSH is intrinsically anionic at pH ~7, a fact that will affect the efficiency of its transfer into the gas phase relative to that of neutral species.

Nevertheless, the relative affinities estimated in this way using excess Dtt as the affinity reference (16) appear to match those determined previously by direct $Cu^I$ exchange between proteins or via ligand competition (11, 13, 42). Apparently, a large excess of Dtt offers some tolerance to pH fluctuation and air oxidation. However, the estimated $K_D$ values are 3 to 4 orders of magnitude larger (*i.e.* the affinities are equivalently weaker) than those determined in this study with Bcs as both probe and affinity reference (Table 2). As demonstrated above, the affinity of $Cu^I$ for Dtt, as reported in Ref. 39 and employed in Ref. 16, was underestimated by 3 to 4 orders of magnitude. Consequently, using the value of $K_D'$ at pH 7.3 estimated above via the Bcs probe, the affinities of Atox1 and related proteins determined via ESI-MS with Dtt as metal buffer and affinity reference are unified with those determined in the present work via the Bcs probe.

In summary, this work has standardized the affinities for $Cu^I$ of important ligand probes and established a coherent system for quantitative comparison of the affinities of high affinity $Cu^I$-binding proteins on a unified scale. It confirms that Atox1-type proteins and protein domains bind $Cu^I$ with sub-femtomolar affinities, consistent with tight control of labile $Cu^+$ concentrations in living cells (43).

## REFERENCES


1. Hamza, I., Schaefer, M., Klomp, L. W., and Gitlin, J. D. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 13363–13368
2. Kim, B. E., Nevitt, T., and Thiele, D. J. (2008) *Nat. Chem. Biol.* **4,** 176–185
3. Lin, S. J., Pufahl, R. A., Dancis, A., O'Halloran, T. V., and Culotta, V. C. (1997) *J. Biol. Chem.* **272,** 9215–9220
4. Wernimont, A. K., Huffman, D. L., Lamb, A. L., O'Halloran, T. V., and Rosenzweig, A. C. (2000) *Nat. Struct. Biol.* **7,** 766–771
5. Arnesano, F., Banci, L., Bertini, I., Huffman, D. L., and O'Halloran, T. V. (2001) *Biochemistry* **40,** 1528–1539
6. Banci, L., Bertini, I., McGreevy, K. S., and Rosato, A. (2010) *Nat. Prod. Rep.* **27,** 695–710
7. Llanos, R. M., and Mercer, J. F. (2002) *DNA Cell Biol.* **21,** 259–270
8. Williams, L. E., and Mills, R. F. (2005) *Trends Plant Sci.* **10,** 491–502
9. González-Guerrero, M., and Argüello, J. M. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 5992–5997
10. Kaplan, J. H., and Lutsenko, S. (2009) *J. Biol. Chem.* **284,** 25461–25465
11. Xiao, Z., Loughlin, F., George, G. N., Howlett, G. J., and Wedd, A. G. (2004) *J. Am. Chem. Soc.* **126,** 3081–3090
12. Wernimont, A. K., Yatsunyk, L. A., and Rosenzweig, A. C. (2004) *J. Biol. Chem.* **279,** 12269–12276
13. Yatsunyk, L. A., and Rosenzweig, A. C. (2007) *J. Biol. Chem.* **282,** 8622–8631
14. Miras, R., Morin, I., Jacquin, O., Cuillel, M., Guillain, F., and Mintz, E. (2008) *J. Biol. Inorg. Chem.* **13,** 195–205
15. Zhou, L., Singleton, C., and Le Brun, N. E. (2008) *Biochem. J.* **413,** 459–465
16. Banci, L., Bertini, I., Ciofi-Baffoni, S., Kozyreva, T., Zovo, K., and Palumaa, P. (2010) *Nature* **465,** 645–648
17. Ralle, M., Lutsenko, S., and Blackburn, N. J. (2003) *J. Biol. Chem.* **278,** 23163–23170
18. Hearnshaw, S., West, C., Singleton, C., Zhou, L., Kihlken, M. A., Strange, R. W., Le Brun, N. E., and Hemmings, A. M. (2009) *Biochemistry* **48,** 9324–9326
19. Djoko, K. Y., Xiao, Z., Huffman, D. L., and Wedd, A. G. (2007) *Inorg. Chem.* **46,** 4560–4568
20. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
21. Kubas, G. J. (1990) *Inorg. Syntheses* **28,** 68–70
22. Achila, D., Banci, L., Bertini, I., Bunce, J., Ciofi-Baffoni, S., and Huffman, D. L. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 5729–5734
23. Zhang, L., Koay, M., Maher, M. J., Xiao, Z., and Wedd, A. G. (2006) *J. Am. Chem. Soc.* **128,** 5834–5850
24. Albert, A., and Serjeant, E. P. (1984) *The Determination of Ionisation Constants: A Laboratory Manual*, pp. 176–191, Cambridge University Press, London
25. Djoko, K. Y., Chong, L. X., Wedd, A. G., and Xiao, Z. (2010) *J. Am. Chem. Soc.* **132,** 2005–2015
26. Hawkins, C. J., and Perrin, D. D. (1963) *J. Chem. Soc.,* 2996–3002
27. Xiao, Z., Donnelly, P. S., Zimmermann, M., and Wedd, A. G. (2008) *Inorg. Chem.* **47,** 4338–4347
28. Zimmermann, M., Clarke, O., Gulbis, J. M., Keizer, D. W., Jarvis, R. S., Cobbett, C. S., Hinds, M. G., Xiao, Z., and Wedd, A. G. (2009) *Biochemistry* **48,** 11640–11654
29. Xiao, Z., and Wedd, A. G. (2010) *Nat. Prod. Rep.* **27,** 768–789
30. James, B. R., and Williams, R. J. (1961) *J. Chem. Soc.,* 2007–2019
31. Blair, D., and Diehl, H. (1961) *Talanta* **7,** 163–174
32. Lappin, A. G., Youngblood, M. P., and Margerum, D. W. (1980) *Inorg. Chem.* **19,** 407–413
33. Ma, Z., Cowart, D. M., Ward, B. P., Arnold, R. J., DiMarchi, R. D., Zhang, L., George, G. N., Scott, R. A., and Giedroc, D. P. (2009) *J. Am. Chem. Soc.* **131,** 18044–18045
34. Freire, E., Mayorga, O. L., and Straume, M. (1990) *Anal. Chem.* **62,** 950A–959A
35. Hong, L., Bush, W. D., Hatcher, L. Q., and Simon, J. (2008) *J. Phys. Chem. B* **112,** 604–611
36. Deranleau, D. A. (1969) *J. Am. Chem. Soc.* **91,** 4044–4049
37. Deranleau, D. A. (1969) *J. Am. Chem. Soc.* **91,** 4050–4054
38. Cleland, W. W. (1964) *Biochemistry* **3,** 480–482
39. Krzel, A., Lesniak, W., Jezowska-Bojczuk, M., Mlynarz, P., Brasuñ, J., Kozlowski, H., and Bal, W. (2001) *J. Inorg. Biochem.* **84,** 77–88
40. Palumaa, P., Kangur, L., Voronova, A., and Sillard, R. (2004) *Biochem. J.* **382,** 307–314
41. Peard, W. J., and Pflaum, R. T. (1958) *J. Am. Chem. Soc.* **80,** 1593–1596
42. Huffman, D. L., and O'Halloran, T. V. (2000) *J. Biol. Chem.* **275,** 18611–18614
43. Rae, T. D., Schmidt, P. J., Pufahl, R. A., Culotta, V. C., and O'Halloran, T. V. (1999) *Science* **284,** 805–808