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

DETECTION PROBES AND AFFINITY STANDARDS

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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 CuI ligand probes bicinchoninate, bathocuproine disulfonate, dithiothreitol (Dtt), and glutathione (GSH) is reviewed, and their CuI affinities are re-estimated and unified. Excess bicinchoninate or bathocuproine disulfonate reacts with CuI to yield distinct 1:2 chromatophoric complexes \([\text{CuI}]_{2}^{3+}\) with formation constants \(K_{D} = 10^{17.2}\) and \(10^{19.8}\) M\(^{-2}\), respectively. These constants do not depend on proton concentration for \(pH \geq 7.0\). Consequently, they are a pair of complementary and stable probes capable of detecting free Cu\(^{+}\) concentrations from 10\(^{-12}\) to 10\(^{-15}\) M. Dtt binds CuI with \(K_{D} = 10^{-15}\) M at pH 7, but it is air-sensitive, and its CuI affinity varies with pH. The CuI 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 CuI binding and the individual quantitative affinities \((K_{D} \text{ values})\) 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 CuI with sub-femtomolar affinities, consistent with tight control of labile Cu\(^{1+}\) concentrations in living cells.

The human metallo-chaperone protein Atox1 (known also as Hah1) delivers CuI to the trans-Golgi network (1, 2). Atox1, the version from the yeast Saccharomyces cerevisiae, was the first copper metallo-chaperone to be identified (3). They both feature the classic ferredoxin \(\beta\alpha\beta\alpha\beta\)-fold with a CXXC motif acting as a high affinity CuI-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) that may interact with and receive CuI directly from Atox1-type metallo-chaperones (6). It appears that, for some CuI-ATPases at least, metal-binding sites in the transmembrane domain may also independently receive CuI 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 CuI (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 \((K_{D} \sim 10^{-5}, 10^{-10}, 10^{-14}, \text{and} 10^{-18} \text{M})\) 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 CuI 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 CuI 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 CuI 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 Atox1, Atox1, and related proteins are re-estimated or recalculated accordingly, allowing a coherent system to be established for quantitative determination and comparison of CuI binding affinities.

References

1. This work was supported by Australian Research Council Grant A29930204 and National Health and Medical Research Council Grant 479502.
2. 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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* 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.
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EXPERIMENTAL PROCEDURES

Materials—Na2Bca, Na2Bcs, 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 [CuI(CH3CN)4]ClO4, 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 β-mercaptoethanol). This was followed by a Superdex-75 FPLC gel filtration column in KP1 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 CuI with higher affinity than wild type CopC, was also generated and purified as 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 titrated estimated via CuI reagent [CuI(Bca)2]3 418. The 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 \( \varepsilon_{412} = 13,600 \text{ M}^{-1} \text{ cm}^{-1} \) for the released chromophore 5-mercapto-2-nitrobenzoate (20); and (iii) CuI binding stoichiometries estimated via CuI reagent [CuI(Bca)2]3 418. 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 [CuII(Bcs)2(H2O)]2+—The determination was based on the Bjerrum method, i.e. the competition for ligand Bcs between CuII+ and protons was followed by pH titration with HClaq (24) as shown in Reactions 1 and 2.

\[
\begin{align*}
[Cu^{n}(Bcs)_{2}(H_{2}O)]^{2-} + H^{+} & \rightleftharpoons [Cu^{n}(Bcs)(H_{2}O)_{x}] + [HBcs]^{-} \\
[Cu^{n}(Bcs)(H_{2}O)_{x}] + H^{+} & \rightleftharpoons Cu^{2+}_{aq} + [HBcs]^{-}
\end{align*}
\]

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,

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

\[
\bar{n} = [Bcs]_{total} - [Bcs](1 + [H]/K_3/[CuII]_{total})
\]  
(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 CuII

FIGURE 1. Ribbon representation of the ferredoxin fold (βαβαβαβ) in CuI-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.
center under the conditions). $K_a$ is the acid dissociation constant of $[\text{HBcs}]^-$, 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,

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

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

$$[\text{Cu}^{\text{II}}(\text{Bcs})_2(\text{H}_2\text{O})]^{2-} + e^- \rightleftharpoons [\text{Cu}^{\text{I}}(\text{Bcs})_2]^{3-} + \text{H}_2\text{O} \tag{REACTION 3}$$

$$E'_{\text{red}} = E_0 - 0.059 \log (\beta_2 [\text{Cu}^{\text{II}}]/\beta_2([\text{Cu}^{\text{I}}])) \tag{Eq. 4}$$

**Formation Constant of $[\text{Cu}^{\text{I}}(\text{Bcs})_2]^{3-}$**—An estimate of $\beta_2$ ($= 10^{17.2} \text{M}^{-2}$) was obtained previously via indirect competition for $\text{Cu}^{\text{I}}$ between ligands Bca and Bcs mediated separately by three apoproteins that bind $\text{Cu}^{\text{I}}$ with different affinities (Atx1, nA-PcoC, and C42S-rubredoxin) (27). This value was consolidated in this work with another $\text{Cu}^{\text{I}}$-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$^{\text{I}}$ Binding Stoichiometries and Affinities of Atx1, Atox1, and Related Proteins**—The experiments were performed in an anaerobic glove box by reaction of apoproteins with $[\text{Cu}^{\text{II}}L_2]^{3-}$ ($L = \text{Bca or Bcs}$) in deoxygenated buffers, as described previously (11, 28). Briefly, apoprotein was titrated into solutions of $[\text{Cu}^{\text{I}}L_2]^{3-}$ of defined molar ratio $L: \text{Cu}^{\text{I}} \geq 2.5$ (to ensure the presence of the 1:2 complex $[\text{Cu}^{\text{I}}L_2]^{3-}$ with negligible contribution from the 1:1 complex $[\text{Cu}^{\text{II}}L]$). The reaction mixtures were diluted to a fixed volume to provide a series of solutions with constant total concentrations of $\text{Cu}^{\text{I}}$ and ligand L but varying concentrations of protein P. Transfer of $\text{Cu}^{\text{I}}$ from $[\text{Cu}^{\text{II}}L_2]^{3-}$ to P was established by the change in absorbance at 483 nm for $L = \text{Bcs}$ ($\varepsilon = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$) and at 562 nm for $L = \text{Bca}$ ($\varepsilon_1 = 7,900 \text{ M}^{-1} \text{ cm}^{-1}$) (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.

$$[\text{P} + \text{ML}_2] \rightleftharpoons [\text{M-P} + 2\text{L}] \tag{REACTION 4}$$

$$K_{D} = \frac{([\text{P}]_{\text{total}}/[\text{MP}]) - 1}{([\text{L}]_{\text{total}}/[\text{ML}_2] - 2)[\text{ML}_2]} \tag{Eq. 5}$$

To mimic cellular conditions, reactions in the presence of GSH (1 mM) were also conducted for competitive reactions between $[\text{Cu}^{\text{II}}L_2]^{3-}$ and P and compared with equivalent reactions without GSH.

**Cu$^{\text{I}}$ Binding Stoichiometry and Affinity of Dtt**—The method was the same as that for the proteins with minor modifications. Solutions of Dtt in H$_2$O 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 ($\sim 4$ mM) was included in the reaction mixture to scavenge trace dioxygen before addition of Dtt. Results for determination of the apparent dissociation constant $K_{D}^{\prime}$ 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 $\text{Cu}^{\text{I}}$ (Scheme 1). They are water-soluble and are proposed to buffer free $\text{Cu}^{\text{II}}$ concentrations over the respective ranges $10^{-12}$–$10^{-16}$ and $10^{-15}$–$10^{-19}$ M (29). Consequently, they are a pair of complementary $\text{Cu}^{\text{I}}$ 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 Bca and neocuproine (Scheme 1) provides steric hindrance at the coordination site, favoring $\text{Cu}^{\text{II}}$ binding (tetrahedral preference) over $\text{Cu}^{\text{I}}$ binding (square planar preference). Consequently, the reduction potentials of the redox couple $\text{Cu}^{\text{II}}/\text{Cu}^{\text{I}}$ of these
ligands are positive (0.64 V versus NHE; see Ref. 25), and the Cu
complex is stable to both disproportionation and reaction with
dioxyn. The affinities of neocuproine for the proton, Cu
and Cu
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. 2a) and so that for [HBcs]−
only was considered in Equations 1–3 for pH >2.7. Experimentally, a
solution of NaBcs of known concentration (4 mM; 10 ml) in
H
2O (0.1 M NaNO3) was titrated with a standard solution of
HClO
4 (98.6 mM) at 20 °C. The pKa
value was estimated from the
mid-point of the titration curve after 0.5 eq of HClO
4 had been
added, providing a value of pKa
= 5.7 (Fig. 2a), consistent with the
literature value pKa
= 5.9 (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. 2b),
allowing estimation of the Cu
formation constants log K
1 =
−5.6 and log β
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. 2b and in
Table 1 and supplemental Table S1. These data are in agree-
ment with those reported in Ref. 11.

The propensity of Cu
3+ to disproportionation prevents determi-
nation of the formation constants of [Cu
3(Bcs)
2]3− by the
Bjerrum method. However, the couple Cu
3+/Cu
in the pres-
ence of more than 2 eq of Bcs is chemically reversible (ΔE′p =
100 mV at ν = 20 mV s−1; I/lape = 1), allowing accurate esti-
mation 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 β
2 = 19.9 may be estimated for
[Cu
3(Bcs)
2]3− from the Nernst relationship (Equation 4). This
estimate is consistent with those (19.1–19.5) reported previ-
ously for the parent ligand neocuproine and consolidates the
previous value (log β
2 = 19.8) reported for the same ligand
(Table 1 and Scheme 1), but it is lower than a recent value of log β
2 = 22.1 at pH 6, which was estimated by competition for Cu
with cyanide (14). No experimental details were provided for the
latter estimate, and the competition is likely to be complicat-
ed by the formation of ternary complexes involving mono-
dentate cyanide ligand.

Ligand Probe Bca—The pKa
values of the carboxylate sub-
stituents in ligand Bca (Scheme 1) are expected to be compara-
ble with those of the pyridyl ring nitrogens, making it difficult to
estimate the affinities for Cu
2+ via the potentiometric approach
used above for Bcs. In addition, the Cu
2+/Cu
redox process is
irreversible (25), preventing derivation of β
2 for [Cu
3(Bca)
2]3−
from the Cu
2+ form via the Nernst equation. Consequently, the
formation constant β
2 for [Cu
3(Bca)
2]3− was determined previ-
ously by indirect competition for Cu
2+ between ligands Bca and
Bcs meditated separately by the three apoproteins Atx1, nAP-
coC, and C42S-rubredoxin (27) that bind Cu
with different affinities. A value of log β
2 = 17.2 for [Cu
3(Bca)
2]3− was esti-
mated on the basis of log β
2 = 19.8 for [Cu
3(Bcs)
2]3− as derived
above. However, the Cu
3 affinities of Atx1 and C42S-rubredox-
in (Km = 10−12.7 M) are a little too high for effective competi-
tion with the weaker affinity probe Bca and that of nAP-coC
(Km = 10−14.7 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
3+.

CopC is a copper-binding protein expressed to the periplas-
mic space in the copper resistance response of the bacterium
Pseudomonas syringae. It binds Cu
3+ 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
3+ affinity by 2 orders of magni-
tude to Km = 10−14.3 M in protein H48C-CopC, allowing effec-
tive competition with both Bca and Bcs to be induce in the same
reaction buffer (supplemental Fig. S2). A value of log β
2 = 17.3
for [Cu
3(Bca)
2]3− was derived, consolidating the previous esti-
mate of log β
2 = 17.2.

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

It should be noted that the value log β
2(Bca) = 17.2 derived is
dependent on accurate estimation of log β
2(Bcs), and so an inde-
pendent estimation would help in establishing this constant.
Such an estimate, log β
4(Bca) = 14.7, has been obtained by
isothermal titration calorimetry (ITC) by titration of a Cu
3+
solution with Bca (13). The two estimates differ by 2.5 orders of
magnitude. This estimation of weaker affinity may be rational-
ized from two experimental considerations as follows.

(i) The ITC binding isotherm showed a single exothermic
process upon titration of Bca into a Cu
3+ solution with a sharp
deredox end point as the ratio [Bca]/[Cu
3+ ] approached 2.0 (see supple-
mental Fig. S2 of Ref. 13), consistent with stoichiometric for-
mation of [Cu
3(Bcs)
2]3−, i.e. with effective saturation of the
metal ion. Consequently the affinity of Bca for Cu
3+ is too high
to be estimated by ITC under the conditions (34). This is con-
firmed by a complementary experiment in which the titration of
Cu
3+ into Bca solution is monitored by the absorbance at 562
nm (see Fig. 4b 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
3+ reagent employed was generated by reduction of
Cu
2+ with ascorbate in aqueous solution (200 mM NaCl),

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Ligand} & \text{Bcs} & \text{Neocuprine} & \text{Bca}^a & \text{Bq}^a \\
\hline
\text{pK}_{a1} & 5.7 & 5.7 & 5.88 & 5.79 \\
\text{pK}_{a2} & 0.95 & 0.95 & 0.94 & 1.0 \\
\text{Cu}^2+/\text{Cu}^+ & 19.9 & 19.8 & 19.1 & 19.5 \\
\text{Cu}^3+/\text{Cu}^2+ & 5.6 & 6.1 & 6.3 & 6.2 \\
\log K_{\text{Cu}^{2+}} & 11.7 & 11.7 & 11.7 & 11.7 \\
\log K_{\text{Cu}^{3+}} & 1.8 & 1.8 & 1.8 & 1.8 \\
\log K_{\text{Cu}^0} & 5.6 & 5.5 & 5.5 & 5.5 \\
\log \gamma & 11.8 & 11.7 & 11.7 & 11.7 \\
\log K_d & 11.8 & 11.7 & 11.7 & 11.7 \\
\gamma & 11.8 & 11.7 & 11.7 & 11.7 \\
\text{Reference} & \text{This work} & \text{10} & \text{26} & \text{This work} & \text{27} \\
\hline
\end{array}
\]
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which stabilizes Cu$^+$ as [CuI(Cl)$_n$]$_{(1-n)}$$. Titration with Bca then involves conversion of these species, rather than Cu$^{+aq}$, to [CuI(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 $\beta_2 \sim 1.4$ for [CuI(Bca)$_2$]$^{3-}$ was estimated by direct Cu$^+$ titration but without adequate documentation (14). However, as pointed out above, the Cu$^+$ 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 reducer ($E_m = -0.33$ 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 ($pK_a$ 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$^+$ 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$^+$-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$^+$ affinities of proteins (16, 40), its Cu$^+$ binding stoichiometry and affinity were re-investigated here to provide a unified scale.

Titration of [CuI(MeCN)$_4$]$^{2-}$ into a solution containing Bca (100 $\mu$M) and Dtt (50 $\mu$M) in Mops buffer (50 mM, pH 7.3) was followed by $A_{483}$, an absorbance maximum characteristic of the [CuI(Bca)$_2$]$^{3-}$ complex. The titration induced little initial absorbance change until the addition of $\sim 0.85$ eq of Cu$^+$ relative to Bca and then the absorbance increased steadily to a sharp end point at 1.5 eq of Cu$^+$ (Fig. 3a, 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 [CuI(Bca)$_2$]$^{3-}$ (Fig. 3a, trace i). These data lead to two conclusions. (i) Dtt competes overwhelmingly with Bca for Cu$^+$ under the conditions (see the first turning point). (ii) Dtt binds 2 eq of Cu$^+$ when Bca is nearly saturated with 0.5 eq of Cu$^+$ (see the second turning point). Accordingly, titration of Dtt into a [CuI(Bca)$_2$]$^{3-}$ solution (Cu$^+$, 40 $\mu$M; Bca, 100 $\mu$M; pH 7.3) led to linear transfer of Cu$^+$ from Bca to Dtt (Fig. 3b). At the point Dtt:Cu$^+$ = 0.5, $\sim 80$% of total Cu$^+$ had been extracted from Bca by Dtt, and each Dtt molecule binds, on average, 1.6 eq of Cu$^+$. 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$^+$ 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$^+$ at lower metal occupancy. Consequently, Bcs was used as a competing ligand to quantitatively probe this affinity.

Titration of Dtt into a [CuI(Bcs)$_2$]$^{3-}$ solution (Cu$^+$, 21 $\mu$M, Bcs, 50 $\mu$M) in Mops buffer (50 mM, pH 7.3) led to a steady decrease in $A_{483}$ indicative of transfer of Cu$^+$ from Bcs to Dtt (Fig. 4a, trace i). As the experiments with the Bca probe showed that Dtt can bind 2 eq of Cu$^+$, the fractional occupancy $Y$ is defined as the metal ratio of Cu$^+$ 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. 4b, trace i), consistent with little or no binding cooperativity between the two Cu$^+$-binding sites. The metal occupancy of Dtt in the Bcs solution is low (<0.6 Cu$^+$ per Dtt molecule). Consequently, the competition for Cu$^+$ between Bcs and Dtt can be modeled with the equivalent of Reaction 4 and the Cu$^+$ binding affinity estimated via curve-fitting of the experimental data to Equation 5 (Fig. 4a, 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$^+$ is pH-dependent.
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Table 2

| Ligand      | pH  | \( \log K_{D}' \) | [Bcs]_{total} | Ref.  |
|-------------|-----|------------------|---------------|-------|
| CopC-H48C   | 7.0 | -14.3 | 25 \( \mu \) | This work |
| Dtt         | 7.0 | -14.2 | 25 \( \mu \) | This work |
| Dtt         | 6.8 | -14.6 | 50             | This work |
| Dtt         | 7.3 | -15.3 | 50             | This work |
| Dtt         | 7.3 | -15.3 | 39             | This work |
| HMA4n       | 7.4 | -11.1 | 39             |       |
| Atox1       | 7.3 | -16.7 | 200            |       |
| Atox1       | 7.0 | -17.4 | 200 & 500      |       |
| Atox1       | -7.5| -13.8 | 16             |       |
| Atox1       | -7.5| -18.0 | 16             |       |
| WLN5–6      | 7.0 | -17.6 | 200 & 500      | This work |
| Atox1       | 7.0 | -17.7 | 300 & 500      | This work |
| HMA7n       | 8.0 | -18.2 | 1000           |       |
| Ccc2n       | 7.3 | -18.1 | 500            |       |
| HMA7n       | 8.0 | -18.8 | 1000           |       |

a Data were determined with [Bca]_{total} = 100 \( \mu \)M as competiting ligand.

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\(^{2+}\)-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\(^{2+}\) 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\(^{2+}\) complexes.

(ii) Dtt is also susceptible to aerobic 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\(^{2+}\)-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\(^{1+}\) by the Bcs ligand shown in Reaction 5.

\[
\text{O}_2 + 2 \text{Bcs}^{2-} \rightarrow [\text{Cu}(\text{Bcs})^2-]_{ox} + (\text{Dtt})_{ox}
\]

(iii) The conclusion 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\(^{1+}\) buffer that is able to protect protein thiols, it is unsatisfactory as a Cu\(^{1+}\) affinity reference as the affinity is sensitive to aerobic oxidation, to solution pH, and to the Cu\(^{1+}\)-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\(^{1+}\). Experiments with GSH similar to those with Dtt demonstrated that the Cu\(^{1+}\) affinity of GSH is much weaker than that of Dtt (the circles in Fig. 4a, 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\(^{1+}\), 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\(^{1+}\) is similar to that of Dtt and that it can compete for Cu\(^{1+}\) with the Atox1 protein (16). The current uncertainties associated with ESI-MS detection are addressed under the “Discussion.”

Cu\(^{1+}\) Binding Stoichiometries of Atox1, Atox1, and Related Proteins—As discussed in the Introduction, the reported Cu\(^{1+}\) affinities of these proteins are controversial. To resolve the issues, copper metallo-chaperones Atox1 (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\(^{1+}\) 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-
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Cu’ Binding Affinities of Atx1, Atox1, and Related Proteins—

Exploratory experiments with [Cu’(Bcs)]3– revealed that the amount of Cu’ removed by yeast Atx1 via Reaction 4 was less than 1 eq and that, under the condition of [Bcs]total = 300 μM, both the Cu’ occupancy on protein (as expressed by θ = [Cu’P]/ [P]total) and the change in Cu’ occupation on the ligand probe (as expressed by A/Aoriginal) varied sensitively with total protein concentration within the range θ = 0.2–0.8 (Fig. 6c; 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. 6c via Equation 5 based on β2 = 1019.8 M–2 for [Cu’(Bcs)]3– generated a consistent average KD = 2.1(2) × 10–18 M at pH 7.0 for Atx1 (supplemental Table S3). This value is slightly larger than a previous value (6.3 × 10–19 M) determined at pH 8.0 (11), indicating that the Cu’ affinities of these proteins may be pH-dependent. Equivalent experiments for Atox1 produced similar data with average KD = 3.9(1) × 10–18 M at pH 7.0 (29). WLN5–6 binds two Cu’ at two separate MBDs, but the experimental data with various Cu’ occupancy on the protein were fitted satisfactorily with a single KD of 4.0(4) × 10–18 M (Fig. 6d; supplemental Table S4), indicating that the Cu’ 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’ 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’-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’ specifically to form well defined complexes [Cu’(L)2]3– in the presence of excess ligand. The pKₐ values for the amine functions are in the range of 3–4 and 4–6, respectively (41). Consequently, the Cu’ binding affinities are not sensitive to pH variation at pH > 7 (0.9 < αₙ < 1.0 in Table 1). The affinities of Bca and Bcs for Cu’ 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. 4c of Ref. 29). Consequently, these two ligands in combination are able to buffer free Cu’ concentrations over
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the wide range from $10^{-12}$ to $10^{-19}$ M. They constitute a pair of complementary and versatile probes for Cu$^+$. 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$^+$ binding stoichiometry and affinity were re-investigated here to provide a unified scale. Conditions were found where Dtt competed strongly with Bca for Cu$^+$ (Fig. 3a). This allowed an average binding stoichiometry of Cu$^+$:Dtt 2.0 to be extracted when the probe ligand Bca was saturated relative to the control (Fig. 3a). The molecular nature of the Cu$^+$-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$^+$ 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$^+$ with the high affinity Cu$^+$ site ($K_D < 10^{-15}$ M) in these proteins. On the other hand, other (“adventitious”) weaker binding sites ($K_D > 10^{-9}$ 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$^+$ 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.

H48C-CopC(10$^{-14.3}$M) > Dtt(~10$^{-15}$) > HMA4n(10$^{-16.7}$)

> Atox1(10$^{-17.4}$) ~ WLD5–6(10$^{-17.4}$) ~ Atx1(10$^{-17.6}$)

> HMA7n(10$^{-18.2}$) (Eq. 6)

Notably, with $K_D$ values increasing from 10$^{-14.2}$ to 10$^{-18.2}$ M, the total Bcs concentrations required for effective competition increase accordingly from 25 to 500 μM (Table 2), as predicted from the calculation (see Fig. 4c of Ref. 29). This is to satisfy the conditions of an effective competition, i.e. both [CuP]/[P]total and A/Aoriginal vary within the sensitive range 0.2–0.8 with total protein and/or ligand concentrations.

The Cu$^+$ 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 ($K_D$ ~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$^+$ (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$^+$ buffer for Atox1-type proteins (see Fig. 6, a and b, and supplemental Table S1 in Ref. 29). Reconciliation of the fourth estimate ($K_D$ ~ 10$^{-14}$ M) is addressed below.

Returning to Equation 6, the average Cu$^+$ affinity of WLN5–6 is very similar to that of Atox1, consistent with the presence of a shallow thermodynamic gradient for Cu$^+$ 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$^+$ exchange experiments between Atox1 and WLN5 or between Atox1 and WLN6, which showed that these proteins competed for Cu$^+$ with similar affinities (13).

The endogenous reductant GSH is reported to bind to the [CuI(S-Cys)$_2$]$^-$ center in Atox1, expanding the coordination sphere from two to three (17). GSH has also been proposed to increase the Cu$^+$ affinity of Atx1 by acting as a bridging ligand in the formation of a Cu$^+$-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. 4a, trace i), as also observed previously for similar reactions (11, 42). These experiments demonstrate the following: (i) the affinity of GSH for Cu$^+$ is too low to compete when [Bcs]total >200 μM; (ii) GSH has little effect on the overall Cu$^+$ affinities, consistent with the observation that it is a weak co-ligand only (17).

Unification of the Affinities of Cu$^+$-binding Proteins—Recently, Banci et al. (16) estimated the relative affinities for Cu$^+$ 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$^+$ 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 (~10$^{-2}$ M). It both binds Cu$^+$ and protects protein cysteine thiol groups. These attributes would appear to confer significant advantages in estimating Cu$^+$ binding affinities via ESI-MS, especially for those proteins that employ cysteine as a Cu$^+$ 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 ≈ 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^1 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^1 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^1 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^1 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 for Cu^1 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^1 of important ligand probes and established a coherent system for quantitative comparison of the affinities of high affinity Cu^1-binding proteins on a unified scale. It confirms that Atox1-type proteins and protein domains bind Cu^1 with sub-femtomolar affinities, consistent with tight control of labile Cu^1 concentrations in living cells (43).

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