

Ratiometric two-photon microscopy reveals attomolar copper buffering in normal and Menkes mutant cells

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Copper is controlled by a sophisticated network of transport and storage proteins within mammalian cells, yet its uptake and efflux occur with rapid kinetics. Present as Cu(I) within the reducing intracellular environment, the nature of this labile copper pool remains elusive. While glutathione is involved in copper homeostasis and has been assumed to buffer intracellular copper, we demonstrate with a ratiometric fluorescent indicator, *crisp-17*, that cytosolic Cu(I) levels are buffered to the vicinity of 1 aM, where negligible complexation by glutathione is expected. Enabled by our phosphine sulfide-stabilized phosphine (PSP) ligand design strategy, *crisp-17* offers a Cu(I) dissociation constant of 8 aM, thus exceeding the binding affinities of previous synthetic Cu(I) probes by four to six orders of magnitude. Two-photon excitation microscopy with *crisp-17* revealed rapid, reversible increases in intracellular Cu(I) availability upon addition of the ionophoric complex CuGTSM or the thiol-selective oxidant 2,2'-dithiodipyridine (DTDP). While the latter effect was dramatically enhanced in 3T3 cells grown in the presence of supplemental copper and in cultured Menkes mutant fibroblasts exhibiting impaired copper efflux, basal Cu(I) availability in these cells showed little difference from controls, despite large increases in total copper content. Intracellular copper is thus tightly buffered by endogenous thiol ligands with significantly higher affinity than glutathione. The dual utility of *crisp-17* to detect normal intracellular buffered Cu(I) levels as well as to probe the depth of the labile copper pool in conjunction with DTDP provides a promising strategy to characterize perturbations of cellular copper homeostasis.

copper homeostasis | fluorescent probes | two-photon microscopy | glutathione | Menkes disease

Copper is an essential trace nutrient for most forms of life and plays critical roles in fundamental biological processes, including respiration, iron acquisition, and connective tissue formation (1). In mammalian cells, a complex network of membrane transporters, cytosolic metallochaperones, and storage proteins is responsible for acquiring, distributing, and regulating cellular copper levels (2). Within the reducing intracellular environment, copper is transported in its monovalent oxidation state (3), and increasing evidence suggests that cells maintain a ligand-bound but kinetically labile pool of Cu(I), which may serve as a buffer against fluctuations in external copper availability. Radiotracer studies with lymphoid cells revealed both rapid cellular uptake and efflux of copper, providing direct experimental evidence that a significant portion of the acquired cellular copper remains mobile and can dynamically exchange with the extracellular medium (4). While free aquacopper(I) is rapidly oxidized by molecular oxygen or precipitates as cuprous oxide at neutral pH, labile cellular copper is stabilized through coordination to proteins and low-molecular-weight biomolecules (5). For example, copper metallochaperones, which are responsible for trafficking Cu(I) to specific subcellular targets, stabilize Cu(I) through coordination to the sulfhydryl group of cysteine residues (6). Despite high complex stabilities with dissociation constants in the attomolar range or

below (7), the exchange of Cu(I) between copper-transporting proteins is characterized by rapid kinetics, likely mediated through an associative exchange mechanism involving direct protein–protein interactions (8).

While modern microanalytical imaging techniques such as synchrotron X-ray fluorescence microscopy or secondary ion mass spectrometry are capable of visualizing the subcellular distribution of copper with submicron resolution (9), such methods cannot be employed for dynamic monitoring of live biological specimens and yield only total copper levels regardless of the kinetic availability. To this end, synthetic fluorescent probes that selectively respond to Cu(I) rank among the most promising tools for exploring the dynamics of labile copper in live cells (10–12). Provided that they possess suitable binding affinities and remain functional within the intracellular environment, such probes may engage in a dynamic exchange equilibrium with endogenous ligands to report on the thermodynamic and kinetic availability of labile Cu(I).

We recently devised a water-soluble Cu(I)-selective turn-on fluorescent probe, CTAP-3, which offers a detection limit in the subpart-per-trillion range and retains a high fluorescence contrast ratio even in the presence of lipid bilayers (13). Microinjection experiments of the brightly fluorescent probe–Cu(I) complex revealed an unexpected rapid dissipation of the fluorescence signal (see below), suggesting competitive chelation of Cu(I) by endogenous ligands. Although the identity of the ligands responsible for buffering labile cellular Cu(I) has not been fully established, glutathione (GSH) emerged early on as a candidate

Significance

Impaired copper regulation has been implicated in a range of human diseases, yet the current mechanistic understanding of intracellular copper homeostasis remains incomplete. While Cu(I)-selective synthetic fluorescent probes could shed light on copper homeostasis within living cells by dynamically reporting intracellular Cu(I) availability, the affinity range of previously employed probes was insufficient to achieve significant metallation in the presence of glutathione (GSH) at normal physiological concentrations. Employing an air-stable phosphine-based ligand design, we devised a ratiometric indicator capable of competing for Cu(I) at attomolar buffered levels. Contrary to prior assumptions, our ratiometric imaging data indicate that intracellular Cu(I) is not buffered directly by GSH but by thiol ligands of still higher affinity.

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based on chromatographic fractionation studies of cell lysates harvested under copper-supplemented growth conditions (14). More recent studies suggest that GSH plays an important role in the regulation of cellular copper uptake and homeostasis (15). For example, depletion of cellular GSH in HEK293 cells attenuated copper uptake, while exposure to elevated copper concentrations increased cellular GSH levels (16).

Despite the interplay between cellular copper levels and GSH, the mechanistic role of GSH in copper homeostasis remains unclear. On the basis of combined spectroscopic and potentiometric studies, we recently discovered that the major species formed by coordination of Cu(I) to GSH is an adamantane-like tetranuclear cluster (17). Concluding from the derived thermodynamic model, the millimolar levels of GSH normally present within cells limit aquacopper(I) concentrations to the subfemtomolar regime, three orders of magnitude lower than previously estimated (17). GSH can thus readily remove Cu(I) from its complex with CTAP-3, which has a dissociation constant of 50 pM. Although a sizable number of Cu(I)-selective synthetic fluorescent probes have been reported over the past decade (10–12), all of them coordinate Cu(I) via structurally similar chelators comprising amine and thioether donors, yielding a rather narrow range of affinities from the picomolar to high femtomolar range, which is insufficient to measure Cu(I) in the presence of normal cytosolic GSH levels. The rapid depletion of CTAP-3-bound Cu(I) by endogenous ligands underscores the need for probes with stronger Cu(I) affinities.

To overcome the limitations of conventional thioether-based Cu(I) chelators, we recently devised an alternative ligand design strategy featuring phosphine sulfide-stabilized phosphine (PSP) donor motifs (18). The electron-withdrawing phosphine sulfide substituents stabilize the central phosphine donor against oxidation while at the same time acting as auxiliary donors for Cu(I) coordination (Fig. 1). For example, the ligand PSP-1 offers an apparent dissociation constant of 0.8 fM and suffered no detectable oxidation upon continuous mixing with air for 15 d at 37 °C. PSP-2, which combines two PSP motifs in an EDTA-like architecture, is also air-stable and provides an even tighter K_d of 10 zM ($\log K = 20.04 \pm 0.06$), yet showed no evident interaction with Zn(II), Fe(II), or strong acid even at millimolar concentrations.

To harness the advantages of the PSP ligand design strategy within a Cu(I)-responsive fluorescent probe, we now report crisp-17 (Fig. 1), in which the electron-withdrawing PSP motif is incorporated into a push–pull fluorophore architecture (19) to yield an emission-ratiometric indicator suitable for two-photon excitation microscopy. Employed in live mammalian cells, crisp-17 allows visualization of base levels of labile Cu(I) as well as rapid dynamic changes evoked by treatment with the membrane-permeant thiosemicarbazone Cu(II) complex CuGTSM or the thiol-selective oxidant 2,2'-dithiodipyridine (DTDP). The experimental results reveal remarkably tight buffering in the low attomolar

concentration range without evidence for significant participation of GSH under normal physiological conditions.

Results and Discussion

Cu(I) Is Rapidly Removed from CTAP-3 by Endogenous Ligands. To passively diffuse across the plasma membrane and reach cellular targets, fluorescent probes must be sufficiently lipophilic, a requirement that is often accompanied by poor water solubility, aggregation in aqueous solution, and accumulation in membranes or lipid droplets. These effects can produce large changes in fluorescence independently from the target analyte, making reliable interpretation of cellular staining patterns difficult if not impossible (20). Solubilized by two anionic sulfonate groups, CTAP-3 circumvents these effects to provide a high-contrast response to Cu(I) even in the presence of lipid bilayers (13). To probe to what extent endogenous ligands might remove Cu(I) from CTAP-3, we microinjected the preformed [Cu(I)–CTAP-3] complex (0.8 mM in 0.1 M KCl) into live BSC-1 cells (*SI Appendix, Fig. S1*). As the injection volume is estimated to be on the order of 10% compared with the total cell volume, the actual intracellular concentration is expected to be an order of magnitude lower. Even upon repeated injections, the fluorescence emission rapidly dissipated each time without evidence of saturation. To test whether this behavior could be explained by simple photobleaching, we doubled the time interval between each image acquisition. An overlay of the normalized decay profiles revealed a constant half-life near 15 s regardless of the frame rate (*SI Appendix, Fig. S1C*), confirming that the fluorescence decay corresponds to rapid demetallation of injected [Cu(I)–CTAP-3] by an endogenous ligand pool with large Cu(I) binding capacity. Our recently determined stability constants for the GSH–Cu(I) equilibrium system imply that normal cellular GSH levels alone could account for these results, regardless of the participation of other biomolecules (17). Imaging the availability of endogenous Cu(I) therefore requires a fluorescent probe with sufficient affinity to operate within and below the buffer window set by GSH, and we reasoned that our recently reported PSP ligand design strategy could be employed to meet this challenge.

Design and Synthesis of Crisp-17, an Emission-Ratiometric Cu(I)-Responsive Probe with Attomolar Dissociation Constant. Given the electron-withdrawing power of phosphine sulfide substituents, the PSP ligand motif can be readily integrated into a push–pull fluorophore architecture. Metal ion coordination would reinforce photoinduced intramolecular charge transfer (ICT), thus yielding a red shift of the absorption and emission spectra suitable for ratiometric imaging. As an added benefit, fluorophores with a large degree of ICT offer increased sensitivity for two-photon excitation microscopy, which minimizes background fluorescence, photobleaching, and phototoxicity. Aiming for a Cu(I) affinity intermediate between the tripodal monophosphine PSP-1 and diphosphine PSP-2, we replaced one of the methylene bridges of PSP-1 with a 2,3-disubstituted thiophene ring, which was combined with a thienofused julolidine heterocycle to provide strong charge donation.

The target probe crisp-17 (copper ratiometric indicator utilizing stabilized phosphines with stability constant $\log K = 17$; see below) was convergently assembled via building blocks **3**, **4**, and **6** (Fig. 2). Expedient access to the tetracyclic donor **3** was achieved via triple tandem cyclization of acetal **2** in refluxing 1-bromo-3-chloropropane. In this process, the hydrogen halides produced during formation of the julolidine ring system serve as acid catalysts for closure of the benzothiophene ring, while Hünig's base suppresses imine formation by delaying cleavage of the acetal until N-alkylation is complete. Final attachment of the phosphine sulfide-substituted phosphine moiety posed unexpected challenges: While PSP-1 and PSP-2 were obtained by substitution of phosphorus(III) electrophiles with lithiated trimethylphosphine sulfide, this approach proved incompatible with the dimethylphosphine sulfide group due to rapid proton exchange and subsequent intramolecular substitution. Taking advantage of the lower kinetic basicity of organomagnesium versus organolithium reagents, we

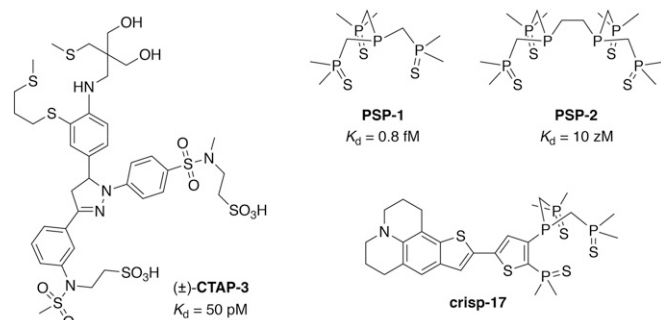


Fig. 1. Structure of the lipid-compatible fluorescent indicator CTAP-3 and Cu(I)-selective reagents utilizing phosphine sulfide-stabilized phosphine (PSP) coordination motifs, including ligands PSP-1 and PSP-2, and the Cu(I)-indicator crisp-17.



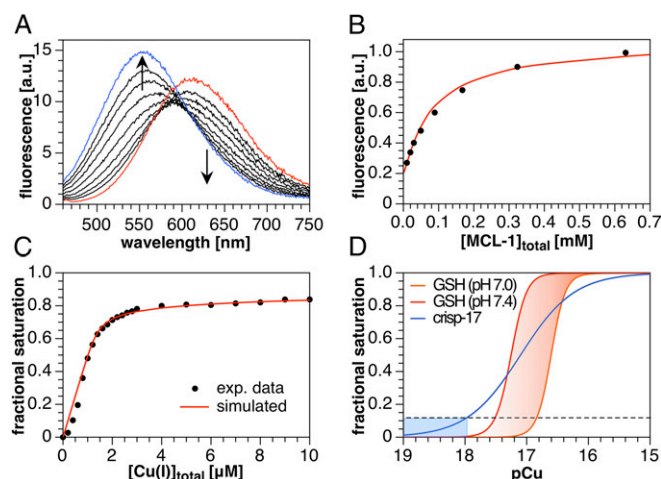


Fig. 4. Fluorimetric Cu(I) competition titrations of crisp-17 against MCL-1 and GSH (4:1 DMPC–DMPG, 100 μ M total lipids, 10 mM Pipes, pH 7.0, 0.1 M KCl, 25 $^{\circ}$ C, λ_{ex} = 450 nm). (A) crisp-17 (1 μ M) was equilibrated with 10 μ M [Cu(I)MCL-1]PF₆ (red trace) at pH 7.0 and subsequently titrated with MCL-1 (10–640 μ M, black traces). Full reversibility was confirmed by adding 10 μ M PSP-2 (blue trace). Nonlinear least-squares fitting yielded $\log K$ of 17.0 ± 0.1 . (B) Fit quality evaluated at 530 nm. (C) Apparent fractional saturation of crisp-17, calculated as $(I - I_{\text{free}})/(I_{\text{bound}} - I_{\text{free}})$, where I represents integrated fluorescence intensity from 500 to 550 nm, upon titration with Cu(I) (as [Cu(I)MCL-2]PF₆) in the presence of 4 mM GSH at pH 7.0. Least-squares fitting (red line) yielded $\log K = 17.1 \pm 0.1$. (D) Calculated fraction of crisp-17 bound to Cu(I) and of total Cu(I) bound to GSH versus thermodynamically available Cu(I) ($p\text{Cu} = -\log[\text{Cu}^{+}_{\text{aq}}]$). The dashed line and blue shading demarcate the Cu(I) availability corresponding to 10% fractional saturation of the probe. The red-shaded area indicates the GSH Cu(I) buffer window within the cytosolic pH range.

benefit of ratiometric probes, which, unlike simple turn-on probes, compensate for uneven probe distributions.

To elicit a dynamic change in labile Cu(I), cells were exposed to CuGTSM, a lipophilic Cu(II) thiosemicarbazone complex that

undergoes reduction ($E_{1/2} = -0.44$ V vs. AgCl/Ag) upon cellular uptake followed by rapid dissociation of Cu(I) from the ligand (22, 23). In agreement with this mechanism, the cytoplasmic intensity ratio dramatically increased from 0.72 ± 0.04 ($n = 20$) to 1.00 ± 0.07 (Fig. 5B). Although higher ratios appear in cell nuclei, their significance is uncertain as the emission intensities in both channels are five to eight times lower compared with the cytoplasmic region. Similar distributions were observed with other lipophilic probes (10, 19) and presumably indicate a partition equilibrium that disfavors the polar nuclear environment. Addition of the membrane-permeant high-affinity Cu(I) chelator PSP-2 (50 μ M) resulted in complete reversal of the initial change to reach an intensity ratio around 0.66 ± 0.05 , slightly below the basal value ($n = 20$; $P = 0.0008$). In contrast to CuGTSM, the related Cu(II) thiosemicarbazone complex CuATSM yielded only a small increase of the intensity ratio (SI Appendix, Fig. S6), consistent with its more negative reduction potential ($E_{1/2} = -0.60$ V vs. AgCl/Ag) (24), while a control experiment with crisp-17ctrl showed no ratio change upon exposure to CuGTSM (SI Appendix, Fig. S7). Although CuGTSM is toxic to cells (25, 26), an MTT assay revealed no toxicity for crisp-17 at up to 50 times the concentration employed for microscopy (SI Appendix, Fig. S8).

In a second set of experiments, we tested the ability of crisp-17 to respond to changes in endogenous copper availability. Mouse 3T3 fibroblasts incubated with crisp-17 showed a small, statistically nonsignificant increase of the fluorescence ratio from 0.65 ± 0.04 to 0.68 ± 0.05 (Fig. 5C; $n = 20$, $P = 0.082$) upon addition of the thiol-selective oxidant DTDP (27). Growing cells in medium supplemented with 50 μ M CuCl₂ for 1 wk gave a negligible increase in the average ratio to 0.67 ± 0.03 (Fig. 5D) and no reduction in cell viability (SI Appendix, Fig. S8), despite the fact that the average total cellular copper levels increased more than twofold (SI Appendix, Fig. S11). However, treatment of these cells with DTDP produced a dramatic increase in fluorescence ratio up to 1.9 with an average of 1.12 ± 0.17 ($n = 20$) within the region of interest (ROI), consistent with dissociation of a sizable pool of Cu(I) from sulfhydryl coordination sites. A gradual regression of the ratio is also apparent, suggesting recapture of Cu(I) by endogenous ligands. Indeed, when

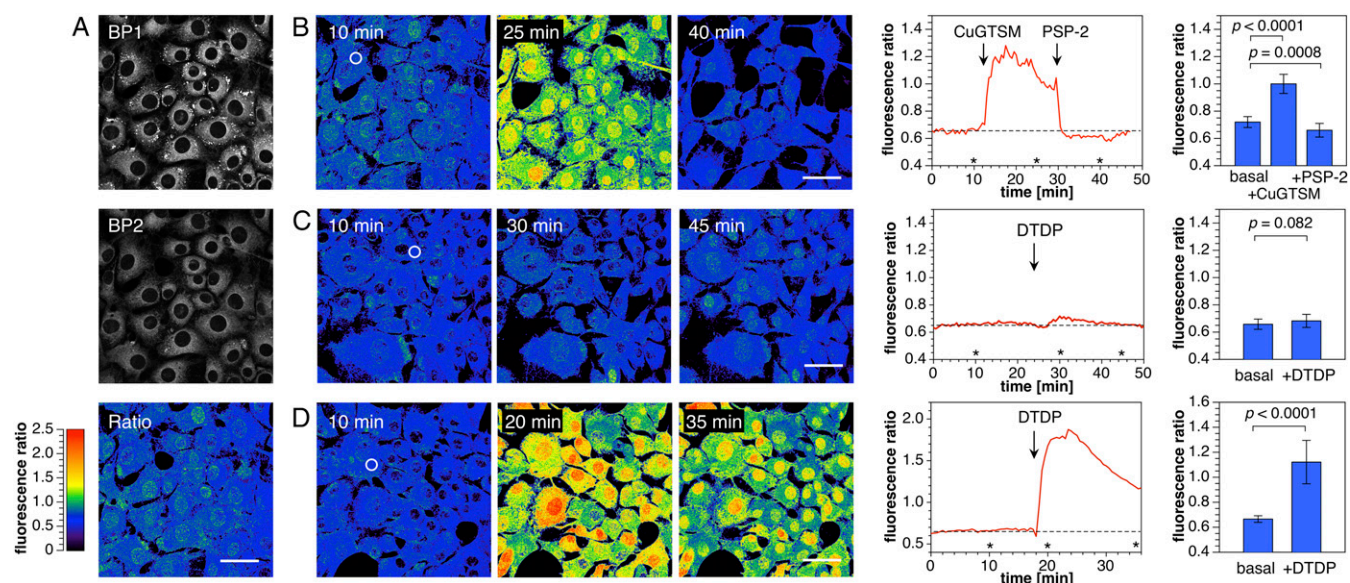


Fig. 5. Two-photon ratiometric imaging of labile copper dynamics in live mouse fibroblasts stained with crisp-17 (1 μ M). (A, Top) Fluorescence intensity images acquired with emission channels of 479–536 nm (BP1) and 611–750 nm (BP2) with two-photon excitation at 880 nm. (A, Bottom) Ratio image with ratio = BP2/BP1. (B, Left) Ratio images before and after addition of 10 μ M CuGTSM. (Scale bars, 50 μ m.) (B, Middle) Time course of the average ratio change for the region of interest (ROI) indicated with a white circle in the Left. The asterisks indicate time points for the respective ratio images shown to the Left. (B, Right) Mean fluorescence ratio of the cytoplasmic region averaged over 20 cells (P values calculated for $n = 20$ using a two-tailed test). (C) Experiment as described for B but with addition of 1 mM 2,2'-dithiodipyridine (DTDP) in lieu of CuGTSM. (D) Experiment as described for C but employing cells grown in medium supplemented with 50 μ M CuCl₂.

by controlling the redox state of Cu(I)-binding cysteine residues (34, 35). The activation of mammalian Cu/Zn superoxide dismutase (SOD1) in the absence of its metallochaperone CCS provides direct evidence for such a role. When cellular levels of oxidized GSH were increased through deletion of GSH reductase, the CCS-independent activation of SOD1 was abolished despite the fact that total GSH levels remained unaltered, thus suggesting delivery of copper by a yet-unidentified redox-sensitive carrier (36). While we do not exclude that direct buffering of Cu(I) by GSH may come to play under acutely toxic conditions, such as ionophore-mediated rapid influx of Cu(II) or partial oxidation of cellular thiols by DTDP, the ability of GSH–Cu(I) complexes to catalyze superoxide formation in vitro (37) implies that cellular Cu(I) levels should be maintained below the threshold for GSH cluster assembly to avoid toxicity in an oxygenated atmosphere. In contrast, the tetranuclear Cu(I)-cluster formed in the β -domain of metallothioneins is redox-inert in air (29), thus rendering metallothionein a safe Cu(I)-storage site, consistent with its up-regulation in ATP7A-null fibroblasts. Nevertheless, copper-supplemented 3T3 cells maintain tight control over Cu(I) availability without up-regulating metallothionein, thus suggesting the involvement of a more complex polydisperse buffer, potentially including yet-to-be-discovered thiol ligands. In light of this uncertainty, we prefer not to speculate as to the identity of the ligands involved.

As the sensing window of crisp-17 extends below the threshold of Cu(I) complexation by GSH, the probe should be well suited for monitoring transient changes of Cu(I), for example to screen potential copper-based metallodrugs or PET imaging agents for intracellular stability. Moreover, the remarkable redox stability of crisp-17 toward disulfides also enables studies of oxidatively mediated Cu(I) release, which would presumably be incompatible with the cysteine-based binding sites of genetically encoded Cu(I) probes (38). Given that the PSP ligand design strategy underpinning crisp-17 is capable of delivering still lower Cu(I) dissociation constants, we anticipate that this approach can be further tuned for maximal sensitivity toward endogenous changes in intracellular Cu(I) availability to study the interplay between cellular copper levels and other aspects of biology.

Materials and Methods

Detailed descriptions of all experimental methods are provided in *SI Appendix*, including the synthesis and characterization of crisp-17 and crisp-17ctrl, photophysical characterization, binding selectivity evaluation, binding affinity measurements, ratiometric two-photon imaging studies, and toxicity evaluation.

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