Comparative in Vivo Stability of Copper-64-Labeled Cross-Bridged and Conventional Tetraazamacrocyclic Complexes

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The increased use of copper radioisotopes in radiopharmaceutical applications has created a need for bifunctional chelators (BFCs) that form stable radiocopper complexes and allow covalent attachment to biological molecules. The chelators most commonly utilized for labeling copper radionuclides to biomolecules are analogues of 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA); however, recent reports have communicated the instability of the radio-Cu(II)-TETA complexes in vivo. A class of bicyclic tetraazamacrocycles, the ethylene "cross-bridged" cyclam (CB-cyclam) derivatives, form highly kinetically stable complexes with Cu(II) and therefore may be less susceptible to transchelation than their nonbridged analogues in vivo. Herein we report results on the relative biological stabilities and identification of the resulting radiolabeled metabolites of a series of ^{64}Cu-labeled macrocyclic complexes. Metabolism studies in normal rat liver have revealed that the ^{64}Cu complex of 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (^{64}Cu-CB-TE2A) resulted in significantly lower values of protein-associated ^{64}Cu than ^{64}Cu-TETA [13 ± 6% vs 75 ± 9% at 4 h]. A similar trend was observed for the corresponding cyclen derivatives, with the ^{64}Cu complex of 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (^{64}Cu-CB-DO2A) undergoing less transchelation than the ^{64}Cu complex of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (^{64}Cu-DOTA) [61 ± 14% vs 90.3 ± 0.5% protein associated ^{64}Cu at 4 h]. These data indicate that the structurally reinforcing cross-bridge enhances in vivo stability by reducing metal loss to protein in both the cyclam and cyclen cross-bridged ^{64}Cu complexes and that ^{64}Cu-CB-TE2A is superior to ^{64}Cu-CB-DO2A in that regard. These findings further suggest that a bifunctional chelator derivative of CB-TE2A is a highly desirable alternative for labeling copper radionuclides to biological molecules for diagnostic imaging and targeted radiotherapy.

Introduction

Copper-64 has favorable properties as a radionuclide for use in both positron emission tomography (PET) imaging and targeted radiotherapy due to its half-life ($t_{1/2}$ = 12.7 h), decay characteristics (β^+ (19%); β^- (39%)), and the ability for large-scale production with high specific activity on a biomedical cyclotron.[1−7] Increased use of ^{64}Cu and other copper radioisotopes in nuclear medicine applications has created a need for copper chelators with high stability against metal loss. Development of optimal chelators for copper is of considerable importance when designing systems for the in vivo delivery of copper radioisotopes.[8−12]

Macrocyclic chelators possess relatively high in vitro stability compared to acyclic chelators such as EDTA and DTPA and have been traditionally used as bifunctional chelators (BFCs) to bind ^{67}Cu(II) ($t_{1/2}$ = 62 h; β^-(100%)) to antibodies.[13−16] The relative in vitro stability of radiometal-labeled BFC−mAb conjugates has been shown to parallel the in vitro stability of the metal-labeled BFCs themselves.[15] In addition, our group has reported that the biological clearance properties of radiometal-labeled BFCs are often indicative of the in vivo behavior of their corresponding peptide/protein conjugates.[11,17,18] As a result, the approach presented here involves measurement of the relative in vivo stability of a series of four ^{64}Cu-labeled BFCs (Figure 1) as a means for predicting the in vivo organ clearance behavior of the corresponding biomolecule conjugates.

TETA has been extensively used as a BFC for copper radionuclides in clinical imaging and therapy studies involving both antibodies and peptides.[19−22] Despite its widespread use, TETA is not an optimal BFC for biomedical applications, as Anderson and co-workers have demonstrated the dissociation of ^{64}Cu from TETA-D-Phe^1-octreotide (TETA-OC) in rat liver and subsequent binding to superoxide dismutase (SOD).[23] The current study addresses this problem through investigation of a novel class of cross-bridged macrocyclic chelators under the expectation that they may provide enhanced in vivo stability.

The syntheses of several novel "cross-bridged" tetraamine ligands having nonadjacent nitrogens connected by an ethylene (CH_2CH_2) bridge have been reported by Weisman and Wong.[24−26] These ligands were designed

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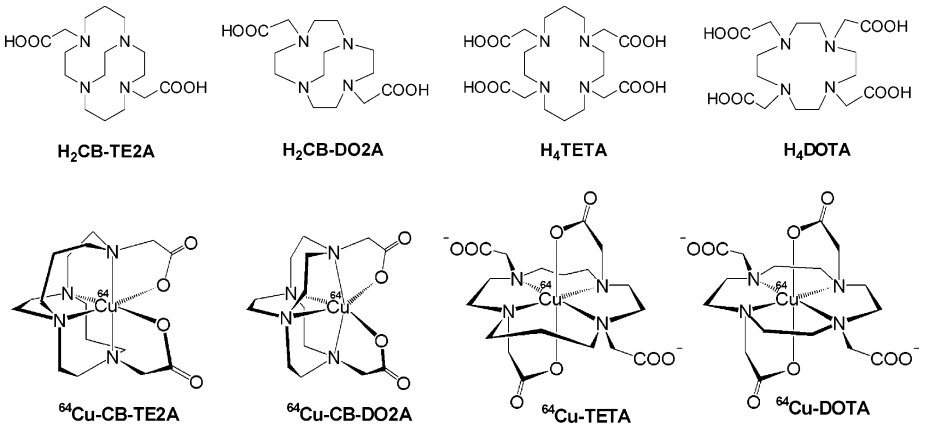

Figure 1. Structural comparison of H_2CB-TE2A, H_2CB-DO2A, H_4TETA, and H_4DOTA and the corresponding ^{64}Cu-labeled complexes.

to form complexes with small metal ions such as Cu(II) but are also remarkably efficient proton sponges due to their ability to adopt low-energy conformations having all four nitrogen lone pairs convergent upon a cleft. An extended series of related Cu(II) complexes of these ligands has been synthesized, including those featuring carboxymethyl arms that serve to fully envelop the six-coordinate cation and neutralize its dicationic charge.[26] Additional Cu(II) complexes of ethylene cross-bridged ligands have been reported by Busch and colleagues.[27−30] Springborg and Micheloni have investigated Cu(II) complexes of related bicyclic ligands having other bridging units. Recently, Springborg has thoroughly reviewed the coordination chemistry of bicyclic tetraaza ligands.[31]

The liver is the critical organ involved in regulation of copper homeostasis. Hepatocytes are not only primarily responsible for hepatic uptake of copper, but are also able to regulate excretion of copper into the bile depending on cytoplasmic concentrations of copper.[32] One of the key copper-binding proteins is Cu/Zn superoxide dismutase (SOD1), a 32-kDa enzyme that is distributed widely in the cytosol of eukaryotic cells and is especially abundant in the liver, kidney, adrenal, and red blood cells. Each subunit of this homodimeric enzyme contains one copper and one zinc atom believed to be bridged by an imidazole group.[33] The function of SOD is to provide a defense mechanism against the potential toxicity of oxygen radicals by catalyzing the disproportionation of the superoxide ion to hydrogen peroxide and oxygen. The incorporation of copper into SOD in mammals occurs via a direct protein−protein interaction[34,35] between SOD and the copper chaperone for SOD (CCS).[36]

Because ^{64}Cu-SOD was the major observed metabolite of ^{64}Cu-TETA-octreotide in rat liver,[23] we anticipated similar metabolism of non-peptide-conjugated ^{64}Cu-azamacrocyclic complexes in the present study. In liver metabolism experiments, we observed an exchange of radioactive copper from Cu(II)-tetraazamacrocycle complexes to SOD. We have further examined this phenomenon using a combination of techniques including size-exclusion chromatography, SDS−PAGE, and immunoblot analysis. In this paper, we present the metabolism data for four ^{64}Cu-labeled macrocyclic complexes, including two cross-bridged macrocycles, CB-TE2A and CB-DO2A, and corresponding nonbridged analogues, TETA and DOTA.

Table 1. Selected Bond Distances (Å) and Bond Angles (deg) for Cu-CB-DO2A[a]

Cu(1)−O(1)	1.971(6)	O(1)−Cu(1)−O(3)	90.1(3)
Cu(1)−O(3)	2.009(6)	O(1)−Cu(1)−N(4)	164.6(3)
Cu(1)−N(1)	2.279(8)	N(4)−Cu(1)−N(2)	86.4(3)
Cu(1)−N(2)	2.079(7)	N(4)−Cu(1)−N(3)	82.2(3)
Cu(1)−N(3)	2.192(7)	N(2)−Cu(1)−N(3)	84.0(3)
Cu(1)−N(4)	2.019(8)	N(4)−Cu(1)−N(1)	83.1(3)
		N(3)−Cu(1)−N(1)	159.0(3)
		N(2)−Cu(1)−N(1)	80.2(3)

[a] Standard deviations in parentheses.

Results

Copper(II) Complex of CB-DO2A. The complexation of cold Cu(II) to CB-DO2A can be readily accomplished by reacting either $Cu(ClO_4)_2$ or $Cu(NO_3)_2$ with the neutral ligand H_2CB-DO2A in methanol. Two equivalents of aqueous NaOH was added followed by refluxing to give a dark-blue solution. Diethyl ether diffusion into this yielded crystals of the Cu-CB-DO2A complex with cocrystallized $NaClO_4$ or $NaNO_3$. These complexes both have a single broad d−d absorption maximum in their aqueous electronic spectra (λ_{max} 642 nm, $\epsilon = 75\ M^{-1}\ cm^{-1}$; and λ_{max} 647 nm, $\epsilon = 75\ M^{-1}\ cm^{-1}$, respectively; each also has a CT band at 280 nm) and two strong carboxylate infrared absorption bands at 1595/1624 cm^{-1} and 1595/1638 cm^{-1}, respectively. A single-crystal X-ray diffraction study of Cu-CB-DO2A·$NaNO_3$ confirmed hexadentate coordination of CB-DO2A using all four of its tertiary nitrogens and both carboxylate donor sites (Table 1, Figure 2).

Radiochemistry. The general procedures for radiolabeling the conventional macrocyclic ligands TETA and DOTA in aqueous buffer were followed as previously described.[17] Formation of ^{64}Cu complexes was verified by radio-TLC using a mobile phase consisting of 50:50 MeOH/10% ammonium acetate on silica plates. A single peak was observed for ^{64}Cu-TETA ($R_f \sim 0.6$), as well as for ^{64}Cu-DOTA ($R_f \sim 0.5$). The absence of uncomplexed ^{64}Cu-acetate at the origin indicated that the formation of the complexes was complete.

The formation of ^{64}Cu-CB-TE2A and ^{64}Cu-CB-DO2A required more vigorous radiolabeling conditions. In aqueous solution, the slower kinetics of ^{64}Cu complexation with the CB-TE2A or CB-DO2A macrocycle cavity resulted in the formation of a significant amount of kinetically favored complexes with trace ligand impurities. To overcome this problem, complexation was

Table 2. Rat Biodistribution Data of ^{64}Cu-CB-DO2A in Selected Organs, Presented as %ID/Organ ± SD (n = 4)

	5 min	30 min	2 h	24 h
blood	13.45 ± 0.96	3.79 ± 0.54	0.32 ± 0.029	0.14 ± 0.049
liver	2.47 ± 0.17	1.47 ± 0.11	0.93 ± 0.047	0.39 ± 0.030
kidney	2.64 ± 0.14	1.14 ± 0.16	0.44 ± 0.040	0.064 ± 0.007
lung	0.72 ± 0.07	0.21 ± 0.033	0.044 ± 0.0024	0.019 ± 0.007
muscle	18.27 ± 1.74	6.42 ± 0.80	1.39 ± 0.71	0.28 ± 0.059
fat	3.99 ± 1.60	2.14 ± 0.86	0.22 ± 0.039	0.12 ± 0.11
heart	0.22 ± 0.028	0.067 ± 0.005	0.0095 ± 0.0007	0.0065 ± 0.001
brain	0.097 ± 0.009	0.036 ± 0.005	0.008 ± 0.001	0.002 ± 0.001
bone	4.52 ± 0.86	1.69 ± 0.55	0.56 ± 0.20	0.24 ± 0.13

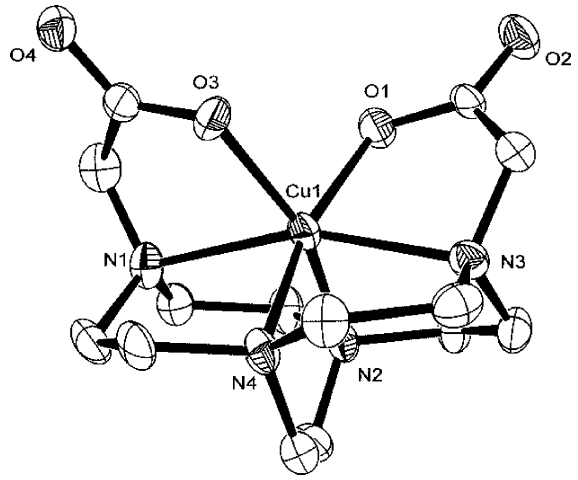

Figure 2. ORTEP drawing of the Cu-CB-DO2A complex with hydrogens omitted for clarity.

Table 3. Comparison of Rat Biodistribution Data for ^{64}Cu-DOTA, ^{64}Cu-CB-DO2A, ^{64}Cu-TETA, and ^{64}Cu-CB-TE2A at 24 h Postinjection (Data presented as %ID/organ ± SD)

ligand	blood	liver	kidney
DOTA[a]	0.58 ± 0.19	1.05 ± 0.16	0.54 ± 0.08
CB-DO2A	0.14 ± 0.05	0.39 ± 0.03	0.051 ± 0.009
TETA[a]	0.21 ± 0.05	0.49 ± 0.11	0.21 ± 0.03
CB-TE2A[b]	0.032 ± 0.014	0.14 ± 0.03	0.064 ± 0.012

[a] Reference 17. [b] Reference 39.

achieved under basic conditions in ethanol using Cs_2CO_3 to deprotonate the amines of CB-TE2A or CB-DO2A, making the complexation of ^{64}Cu more kinetically favorable prior to addition of $^{64}CuCl_2$. A single peak (R_f = 0.60−0.70) for ^{64}Cu-CB-TE2A and for ^{64}Cu-CB-DO2A was observed on radioTLC using a 9:1 MeOH/0.1 M ammonium citrate (pH 6.5) mobile phase on C-18 plates.

Biodistribution of ^{64}Cu-CB-DO2A. The biodistribution of ^{64}Cu-CB-DO2A was determined in normal rats (Table 2) and demonstrated rapid clearance of the agent through the blood, liver, and kidneys. A comparison of the rat biodistribution of ^{64}Cu-CB-DO2A to the three other ^{64}Cu-macrocycles evaluated at 24 h postinjection is presented in Table 3. These data demonstrate that the ^{64}Cu-labeled cross-bridged chelates are cleared more completely from the blood, liver, and kidneys than the non-cross-bridged analogues, with ^{64}Cu-CB-TE2A having the lowest amount of ^{64}Cu remaining in the blood and tissues.

Metabolism. The injectates of the four ^{64}Cu complexes were evaluated by size-exclusion HPLC, and all eluted with retention times in the <5-kDa ranges as indicated in Figure 3 (data not shown). Interestingly, the cross-bridged complexes have a slightly higher affinity for the stationary phase of the size-exclusion column than the non-cross-bridged complexes (t_R = ~39 min vs ~37 min), presumably due to their overall neutral charge or possibly due to their markedly less branched structures. After injection of ^{64}Cu-CB-TE2A, ^{64}Cu-TETA, ^{64}Cu-CB-DO2A, and ^{64}Cu-DOTA in rats, liver and blood sample homogenates from 1, 4, and 20 h postinjection were analyzed by size-exclusion chromatography to determine the extent to which the ^{64}Cu transchelated to proteins. Representative size-exclusion chromatograms of liver extracts obtained from rats sacrificed at 4 h postinjection revealed that ^{64}Cu-TETA undergoes transchelation of ^{64}Cu, mostly into a ~34 kDa protein, to a much greater extent than ^{64}Cu-CB-TE2A under the same conditions (Figures 3A, 3B). A similar trend was observed between the corresponding non-bridged and cross-bridged cyclen compounds ^{64}Cu-DOTA and ^{64}Cu-CB-DO2A at the 4 h time point (Figures 3C, 3D).

The majority of protein-bound ^{64}Cu eluted in a peak corresponding to a molecular weight of 34 kDa, consistent with previous results showing SOD as the primary radiolabeled protein in rat liver.[11,23] This protein had the same retention time as ^{64}Cu-labeled human SOD (Sigma, St. Louis, MO) under the same conditions (data not shown). Another radiolabeled protein with an apparent molecular weight of ~14 kDa was also observed in the size-exclusion chromatograms for all four complexes. This is consistent with the apparent molecular weight of metallothioneins, a class of ubiquitous, low molecular weight, cysteine-rich, metal binding proteins that function in the metabolism of zinc and copper. The ~14-kDa protein had a retention time identical to that of ^{64}Cu-labeled rabbit liver metallothionein (Sigma, St. Louis, MO) under the same conditions (data not shown). The relative amounts of the major (34-kDa) and minor (14-kDa) ^{64}Cu-labeled proteins varied; however, metabolism of ^{64}Cu-CB-DO2A seemed to result in a greater percentage of the radiolabeled ~14-kDa protein than the other ^{64}Cu-labeled complexes, resulting in approximately a 1:1 ratio of the two radiolabeled proteins at 4 h, in contrast to a 5−10-fold excess of the ~34-kDa protein relative to the ~14-kDa protein for ^{64}Cu-TETA and ^{64}Cu-DOTA (Figure 3).

The percentage of protein-bound ^{64}Cu in the liver (Figure 4) was calculated for each of the ^{64}Cu-macrocyclic complexes as previously reported (see Experimental Section).[37] This calculation accounts for ^{64}Cu incorporation into protein as determined by integration of peaks from size-exclusion HPLC chromatograms and also corrects for protein extraction efficiency. ^{64}Cu-CB-TE2A resulted in significantly lower values of liver protein-associated ^{64}Cu than ^{64}Cu-TETA [13 ± 6% vs 75 ± 9% at 4 h] (Figure 4A). A similar trend was

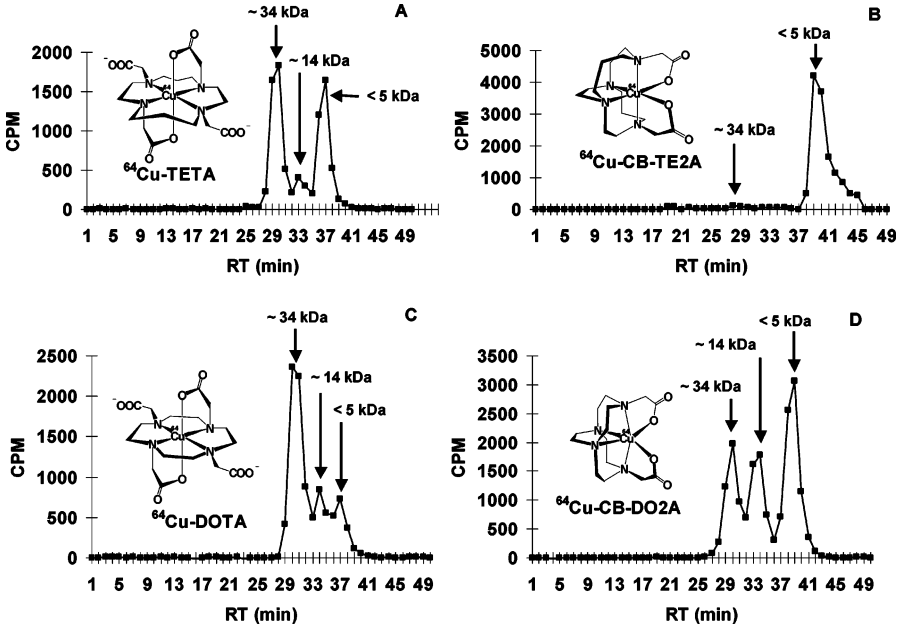

Figure 3. Size-exclusion chromatograms of liver extracts from male Lewis rats following tail vein injection of no-carrier-added ^{64}Cu-tetraazamacrocycle complexes: (A) ^{64}Cu-TETA, (B) ^{64}Cu-CB-TE2A, (C) ^{64}Cu-DOTA, and (D) ^{64}Cu-CB-DO2A at 4 h postinjection.

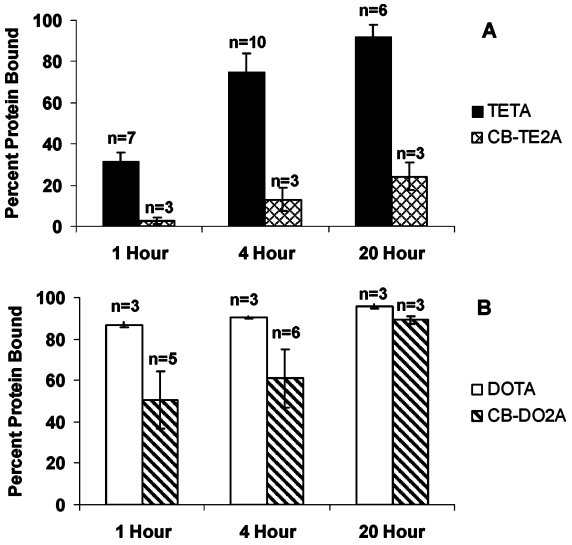

Figure 4. Percent protein-bound ^{64}Cu calculated from integration of size-exclusion chromatograms of male Lewis rat liver extracts following tail vein injection of ^{64}Cu-azamacrocycle complexes at 1, 4, and 20 h postinjection: (A) ^{64}Cu-TETA vs ^{64}Cu CB-TE2A, (B) ^{64}Cu-DOTA vs.^{64}Cu-CB-DO2A.

observed for the corresponding cyclen derivatives, with ^{64}Cu-CB-DO2A undergoing less transchelation than ^{64}Cu-DOTA [61 ± 14% vs 90.3 ± 0.5% at 4 h] (Figure 4B). While ^{64}Cu-CB-TE2A was most resistant to transchelation in rat liver at all time points, its greatest stability compared to the other complexes studied was most evident at 20 h postinjection. At 20 h postinjection, ^{64}Cu-TETA had almost completely dissociated in the liver (92 ± 5%), in contrast to ^{64}Cu-CB-TE2A, which was only 24 ± 7% dissociated (Figure 4A). Even at 1 h postinjection, almost 90% of the radiometal from ^{64}Cu-DOTA in rat liver was protein-associated, indicating that this complex was highly susceptible to transchelation (Figure 4B).

The blood data reveal similar trends of stability for the ^{64}Cu-labeled complexes as in the liver (Figure 5); however, different ^{64}Cu-labeled proteins were observed. In the blood, there did not appear to be appreciable levels of the 34-kDa protein that was present in the liver. Two overlapping peaks with retention times of ~24 min and ~26 min were observed for ^{64}Cu-CB-TE2A at 4 h postinjection (Figure 5B), corresponding to calculated molecular weights of 84 kDa and 64 kDa, respectively. For ^{64}Cu-CB-TE2A, there were very low levels of radioactivity in the blood at 4 h compared to the other three complexes, demonstrating its superior blood clearance. Because of the limited resolution and low intensity of the protein-associated radioactivity for ^{64}Cu-CB-TE2A, the presence of two ^{64}Cu-labeled plasma proteins was confirmed using a nonreducing, nondenaturing SDS−PAGE followed by phosphorimaging (data not shown). For the other three ^{64}Cu-complexes, the 64-kDa protein was the major observed radiolabeled protein (Figures 5A, 5C, 5D); however, small amounts of the 84-kDa peak were possibly masked due to poor resolution of the gel filtration chromatography. The retention time of the 64-kDa protein is identical to that of ^{64}Cu-labeled rat albumin (Sigma, St. Louis, MO), while the 84-kDa radiolabeled protein has a retention time corresponding to the UV peak of human ceruloplasmin (Sigma, St. Louis, MO). Ceruloplasmin is a 132-kDa blue α-2-glycoprotein that contains 95% of the copper found in human plasma. It has been reported that, although human apo-ceruloplasmin invariably runs as a 130-kDa band in nondenaturing SDS−PAGE, *holo*-ceruloplasmin results in a doublet of bands with apparent molecular weights of 78 kDa and 84 kDa.[38] The appearance of radiolabeled ceruloplasmin has been reported for ^{67}Cu-labeled monoclonal antibodies in humans.[12] Ceruloplasmin is synthesized in hepatocytes, where conversion from the apo to holo form is followed by secretion from the liver into the plasma.[38]

The percentage of protein-bound ^{64}Cu in the blood for each of the ^{64}Cu-macrocycles was calculated by the same method used for the liver data (Figure 6). At 4 h postinjection, ^{64}Cu-TETA resulted in significantly more

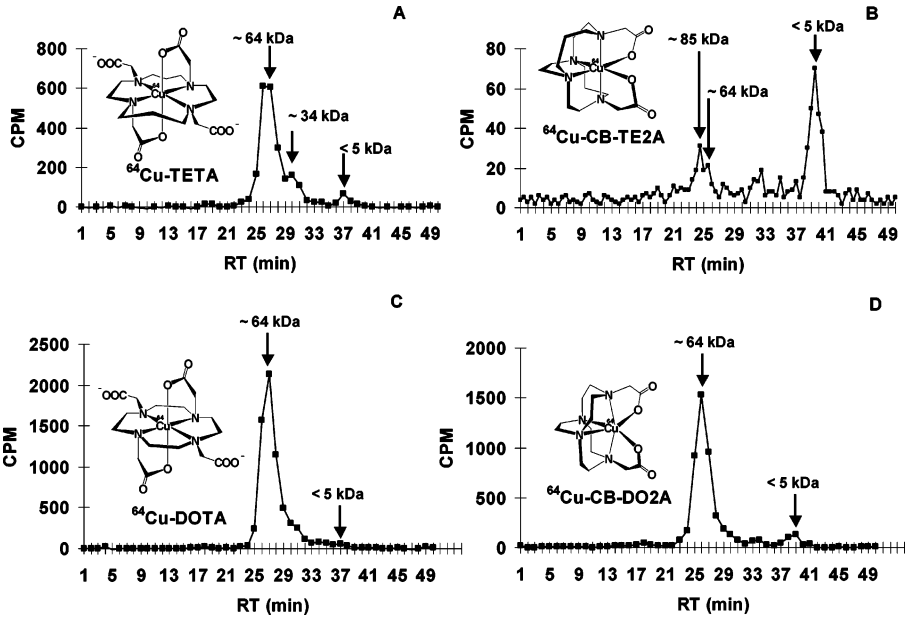

Figure 5. Size-exclusion chromatograms of blood extracts from male Lewis rats following tail vein injection of no-carrier-added ^{64}Cu-tetraazamacrocycle complexes at 4 h postinjection: (A) ^{64}Cu-TETA, (B) ^{64}Cu-CB-TE2A, (C) ^{64}Cu-DOTA, and (D) ^{64}Cu-CB-DO2A.

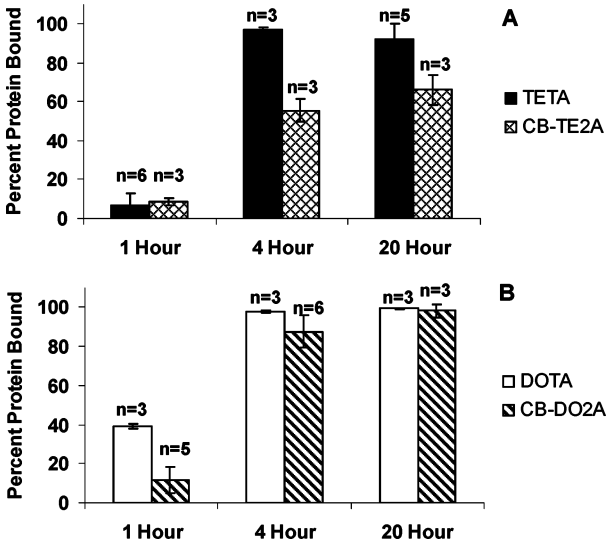

Figure 6. Percent protein bound ^{64}Cu calculated from integration of size-exclusion chromatograms of male Lewis rat blood extracts following tail vein injection of ^{64}Cu-azamacrocycle complexes at 1, 4, and 20 h postinjection: (A) ^{64}Cu-TETA vs ^{64}Cu CB-TE2A, (B) ^{64}Cu-DOTA vs.^{64}Cu-CB-DO2A.

protein-bound ^{64}Cu than ^{64}Cu-CB-TE2A [97 ± 1% vs 55 ± 6 % at 4 h] (Figure 6A). Nearly complete incorporation into plasma proteins was observed at 4 h postinjection for both ^{64}Cu-DOTA [98.0 ± 0.5% at 4 h] and ^{64}Cu-CB-DO2A [88 ± 8% at 4 h], illustrating the decreased stability associated with the cyclen backbone (Figure 6B). The increased stability provided by the cross-bridge was more evident at 1 h postinjection, with ^{64}Cu-DOTA resulting in a greater percentage of protein-bound ^{64}Cu than ^{64}Cu-CB-DO2A [39 ± 1% vs 12 ± 7% at 1 h] (Figure 6B). By 20 h postinjection, almost all radioactivity in the blood was protein-associated; however, this accounts for a very small percentage of the injected dose due to rapid renal clearance from the blood.

Organ blank experiments were carried out to ensure that the species observed after iv injection of the radiolabeled complexes were indeed the result of in vivo processes versus extracellular incorporation of ^{64}Cu into SOD1 released upon lysing the cells during homogenization and/or sonication. The ^{64}Cu-CB-TE2A, ^{64}Cu-TETA, ^{64}Cu-CB-DO2A, or ^{64}Cu-DOTA injectate was added ex vivo to liver and blood samples from non-injected rats, and the metabolism procedures were performed. These control experiments indicated that the observed incorporation of radiometal into SOD1 was indeed due to metabolic processes in vivo and was not merely the result of the techniques used to extract the metabolites from the organs.

Rat liver and blood extracts obtained following metabolism of the ^{64}Cu-complexes were subjected to analysis by radio-TLC in order to further characterize the radiolabeled low-molecular weight species present. A portion of the ethanol-containing extract used for size-exclusion HPLC analysis was subjected to radio-TLC. These experiments indicated the presence of ^{64}Cu-labeled low-molecular weight species with R_f values identical to the injected complexes. This finding suggests that the ^{64}Cu-labeled low-molecular weight species present after in vivo metabolism (i.e. the intact radiometal−ligand complexes that have not undergone metal loss to proteins) are chemically identical to the injected radiometal complexes.

^{64}Cu Incorporation into SOD. Binding of ^{64}Cu to SOD in rat liver was demonstrated by detecting ^{64}Cu-SOD using SDS−PAGE and phosphorimaging (Figure 7). Copper-64-labeled holoSOD1 was detected in the total liver extract from the ^{64}Cu-TETA-injected rat (lane 1) as well as the corresponding SOD-containing size-exclusion HPLC fraction (lane 3). In contrast, ^{64}Cu-SOD could not be detected by phosphorimaging in the total liver extract from the^{64}Cu-CB-TE2A-injected rat (lane 2), and very little ^{64}Cu-SOD was observed for the corresponding size-exclusion HPLC fraction (lane 4). Negative controls were performed using the 34-min eluting fraction (~14 kDa) demonstrating no ^{64}Cu incorporation (lanes 5 and 6). The presence of SOD for

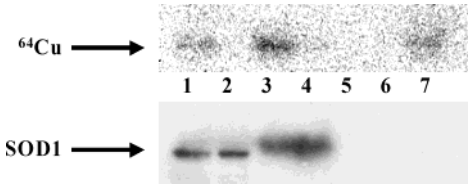

Figure 7. Phosphorimaging of an SDS−PAGE showing ^{64}Cu incorporation into SOD1 (top) and the corresponding immunoblot using a rat SOD1-specific antibody (bottom). **1**, ^{64}Cu-TETA total liver extract, **2**, ^{64}Cu-CB-TE2A total liver extract, **3**, ^{64}Cu-TETA fraction 30, **4**, ^{64}Cu-CB-TE2A fraction 30, **5**, ^{64}Cu-TETA fraction 34, **6**, ^{64}Cu-CB-TE2A fraction 34, **7**, ^{64}Cu-hSOD1.

both the TETA and CB-TE2A-derived samples was confirmed by Western blot using an anti-SOD antibody. The Western blot confirmed that equal amounts of protein were loaded on the SDS−PAGE gel for the ^{64}Cu-TETA and ^{64}Cu-CB-TE2A rat liver metabolism samples. A radiolabeled human SOD1 standard was also detected by phosphorimaging (**lane 7**) but is not detected in the Western blot because the antibody was raised to a rat Cu/Zn SOD immunogen.

Discussion

The goal of this work was to determine whether the cross-bridged macrocycles offer advantages with respect to thermodynamic and/or kinetic stability of the Cu(II) complexes compared to the corresponding nonbridged analogues for radiopharmaceutical applications. We chose to examine macrocyclic polycarboxylate ligands based on the cyclam and cyclen backbones, both of which bind Cu(II) with high thermodynamic stability and were found to be relatively stable in vivo compared to acyclic polyaminopolycarboxylate chelators.[16,17] Preliminary studies showed that ^{64}Cu-CB-TE2A was more stable in rat liver in vivo than ^{64}Cu-TETA.[39] In this study, further metabolism studies were performed with these cyclam-based macrocycles, and comparisons were made with cyclen analogues.

The synthesis and characterization of the Cu(II) complex of the cyclen-based cross-bridged chelator, CB-DO2A, have not previously been reported. The spectral data of this Cu-CB-DO2A complex are consistent with the previously published characterization of Cu-CB-TE2A (λ_{max} 629 nm, ϵ = 20 M^{-1} cm^{-1} and infrared carboxylate bands at 1590/1616 cm^{-1}) whose X-ray structure also confirmed the complete envelopment of the Cu(II) within each ligand's six-coordinate N_4O_2 donor set including *cis*-carboxylate arms.[26] Interestingly, the Jahn−Teller distortion in Cu-CB-DO2A is along the axial N(1)−Cu-N(3) axis with elongated Cu−N(1) of 2.279(8)Å and Cu−N(3) of 2.192(7)Å instead of along a N−Cu−O axis as in Cu-CB-TE2A. Other Cu−N distances range between 2.02 and 2.08 Å while Cu-O distances are approximately 2.0 Å in both structures. Another notable difference from the Cu-CB-TE2A structure is the more pronounced protrusion of the Cu(II) from the ligand cleft due to its poorer fit as revealed by the axial N(1)−Cu−N(3) angle of 159.0(3)° compared to the nearly linear 177.5(1)° in Cu-CB-TE2A. A very similar structure to Cu-CB-DO2A in the literature is that of Cu-Me_2DO2A (Me_2DO2A is 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate).[40] In this structure, the cyclen also coordinates in a *cis*-folded manner with two cis-equatorial carboxylates. The Jahn−Teller elongations are also along the axial N−Cu−N axis with Cu−N distances of 2.30−2.32 Å. This Cu(II) is even more distended from the ligand cavity as the axial N−Cu−N angle is further compressed to 151.2°.

The greater in vivo stability of the Cu(II) cross-bridged macrocycles compared to their non-cross-bridged counterparts may be due in part to differences in their coordination chemistry. For example, the structures of the Cu(II) complexes of TETA and CB-TE2A are quite dissimilar despite their related frameworks (Figure 1). The Cu(II)-CB-TE2A complex is characterized as an octahedral complex with two axial nitrogens, two equatorial nitrogens, and carboxylate groups at the remaining two cis-equatorial positions.[26] In contrast, the Cu(II)-TETA complex is a distorted octahedron with two weakly coordinated axial carboxylates, while the four nitrogens of the tetraazamacrocycle are equatorial.[41] The two complexes also differ in overall charge; CB-TE2A forms a neutral complex, completely enveloping the Cu(II) cation, while Cu(II)-TETA has an overall −2 charge due to two free carboxylates. The structure of the Cu(II)-DOTA complex is analogous to that of Cu(II)-TETA,[41,42] while the structure of Cu(II)-CB-DO2A resembles that of Cu-CB-TE2A in having cis-coordinated pendant arms (vide supra).[26]

The reaction conditions (basic ethanol) for preparing ^{64}Cu-CB-TE2A and ^{64}Cu-CB-DO2A are relatively harsh compared to those for the conventional complexes. At the no-carrier added level, these conditions were required to favor the formation of ^{64}Cu-CB-TE2A or ^{64}Cu-CB-DO2A over possible kinetically favored trace impurities. Under 1:1 carrier-added conditions (ligand:Cu(II) = 1:1), ^{64}Cu-CB-TE2A formed easily in aqueous buffer, while a mixture of both ^{64}Cu-CB-TE2A and ^{64}Cu-labeled trace impurities was observed at a 1:100 ligand:Cu(II) ratio.

It is important to note that data from both in vivo clearance (biodistribution) and metabolism studies indicate the enhanced stability of ^{64}Cu-CB-TE2A compared to the other complexes. As shown in Table 3, at 24 h postinjection for all ^{64}Cu complexes there are varying amounts of ^{64}Cu remaining in the blood and liver, but in general these amounts are relatively small. By performing the metabolism studies, we verified the presence of both protein-bound ^{64}Cu and intact complex. The protein-bound form may be transported back into the blood,[12] potentially increasing bone marrow toxicity in radiotherapeutic applications. In addition, increasing amounts of ^{64}Cu in the blood over time will decrease target tissue:blood ratios, thus degrading PET image quality. Therefore, even though the actual amount of ^{64}Cu accumulating in the liver is low, it is important for both PET imaging and therapy with ^{64}Cu-BFC-biomolecule conjugates.

The data presented here demonstrate that the size of the parent macrocyclic structure has a significant impact on the in vivo stability. Based on in vivo clearance experiments in rats, it was previously suggested that ^{64}Cu-TETA was more stable in vivo than ^{64}Cu-DOTA, although metabolism experiments were not performed as verification.[17] Here, metabolism experiments indeed confirm that significantly more transchelation of ^{64}Cu(II) to liver proteins occurs with ^{64}Cu-

DOTA than with ^{64}Cu-TETA. The addition of the ethylene cross-bridge to both DOTA and TETA (CB-DO2A and CB-TE2A, respectively) imparts a greater in vivo stability to their ^{64}Cu-complexes, with the combination of the cyclam backbone and the cross-bridge providing the greatest enhancement of in vivo stability.

The significant differences in metabolism observed between ^{64}Cu-TETA and ^{64}Cu-DOTA demonstrate that kinetic stability plays a more central role in biological stability of metal-chelate complexes than thermodynamic stability. The effect of increasing macrocycle size from DOTA to TETA results in a small decrease in log K_1 for the relatively small (ionic radius 0.57) Cu^{2+} ion (log K (H_2O) DOTA = 22.25; log K (H_2O) TETA = 21.13).[43] However, this decrease in thermodynamic stability is inconsistent with the greater in vivo stability of ^{64}Cu-TETA. This is consistent with a report by McMurry et al. on the determination of the optimal Y(III) chelator for attaching yttrium radionuclides to monoclonal antibodies.[44] This group reported that thermodynamic stability and acid stability measurements of ^{88}Y complexes did not accurately predict in vivo stability.

Along with the coordination chemistry of copper, the fundamental role of copper as an essential trace metal in human biochemistry is an important consideration in copper radiopharmaceuticals. Virtually no "free" or aqueous copper(II) exists in cells due to its toxicity in its free form.[45] Specific cuproproteins exploit copper's unique redox behavior to achieve electron-transfer reactions in several key metabolic pathways in all aerobic organisms.[46] A membrane-bound copper transporter, CTR1, imports copper into cells,[47,48] whereas subsequent trafficking of copper to specific intracellular compartments is mediated by a unique class of metalloproteins termed copper chaperones.[49,50] Copper chaperones have been identified that transport copper to cytochrome *c* oxidase,[51] copper-transporting ATPase,[52] and Cu/Zn superoxide dismutase.[53] In this study, size-exclusion chromatography, in combination with Western blot analysis, clearly demonstrate that ^{64}Cu transchelated to SOD in rat liver in vivo. Questions are raised regarding the precise location and mechanism of transchelation of Cu(II) from the macrocyclic complexes, and whether the copper chaperones play a role in incorporation of ^{64}Cu into SOD1 following the dissociation of ^{64}Cu from chelators in vivo.

Human PET imaging studies with ^{64}Cu-TETA-octreotide showed slow blood clearance and accumulation of ^{64}Cu in the liver over time.[22] In human radioimmunotherapy trials with a ^{67}Cu-labeled monoclonal antibody, ^{67}Cu-BAT-2IT-Lym-1, where the BFC was a TETA derivative, it was found that ^{67}Cu dissociated from the chelator, and ^{67}Cu-labeled ceruloplasmin was found in the blood.[12] The data presented here confirm these findings and demonstrate a great need for a chelator that forms even more stable complexes with Cu(II) in vivo. Although there is still a slight degree of transchelation of ^{64}Cu-CB-TE2A to proteins, it is a major improvement compared to the more widely used TETA chelator. Further research in developing Cu(II) chelators with even greater stability is clearly warranted.

Conclusions

The synthesis and characterization of the new Cu-CB-DO2A complex confirmed full hexadentate Cu(II) coordination by the dianionic pendant-armed ligand as was reported for Cu-CB-TE2A.[26] The biodistribution of ^{64}Cu-CB-DO2A showed similar trends as the other ^{64}Cu macrocyclic complexes, but further demonstrated the more favorable in vivo clearance of the ^{64}Cu-labeled cross-bridged complexes. The biodistribution data were consistent with the metabolism data, demonstrating that greater in vivo clearance correlated with decreased transchelation of ^{64}Cu to proteins such as SOD. The metabolism data for four ^{64}Cu-labeled tetraazamacrocyclic ligands confirmed that the ^{64}Cu complex of CB-TE2A showed a significantly higher resistance to transchelation than ^{64}Cu-TETA by rat liver and blood metabolism studies, providing further evidence that a BFC of CB-TE2A has significant potential for labeling copper radionuclides to biological molecules for diagnostic imaging and targeted radiotherapy.

Experimental Section

General Methods and Materials. CAUTION! Although we encountered no problems, perchlorate metal salts with organic ligands are explosive hazards and should be handled with great care and only in small quantities.

Cold copper salts and solvents were reagent grade. Cross-bridged ligands H_2CB-TE2A[26] and H_2CB-DO2A[54,55] were prepared as trifluoroacetate salts by modifications of published methods.[25,26] Infrared spectra were obtained as KBr pellets on a Nicolet MX-1 FTIR spectrophotometer while electronic spectra were recorded on a Cary 5E spectrophotometer. Elemental analyses were performed at Atlantic Microlab. Inc. Norcross, GA. Copper-64 was prepared on the Washington University Medical School CS-15 cyclotron by the ^{64}Ni(p,n)^{64}Cu nuclear reaction at a specific activity range of 50−200 mCi/μg as previously described.[7] Waters C18 silica gel TLC plates (KC18F, 60 Å, 200 μm) were purchased from Fisher Scientific (Pittsburgh, PA). Radio-TLC was accomplished using a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA).

Synthesis of Cu-CB-DO2A. (a) An amount of 57.0 mg (0.154 mmol) of $Cu(ClO_4)_2 \cdot 6H_2O$ and 64.3 mg (0.150 mmol) of H_2CB-DO2A$\cdot CF_3COOH \cdot H_2O$ was dissolved in 12 mL of methanol. A 0.46-mL portion of a 0.970 N aq NaOH solution was added and the clear blue reaction mixture refluxed for 2 h. A small amount of precipitate was centrifuged off and the supernatant was decanted off and subjected to diethyl ether diffusion to give 79.6 mg (98% yield) of blue crystalline product. IR (KBr pellet): 1624(s), 1595 (s) (CO_2^- stretches). Electronic spectrum: λ_{max} (H_2O) 642 nm (ϵ = 75 M^{-1} cm^{-1}). Anal. Calcd For $CuC_{14}H_{24}N_4O_4 \cdot (NaClO_4)_2(H_2O)_{2.5}$: C, 30.95; H 5.38; N, 10.31%. Found: C, 30.89; H, 5.03; N, 10.12%.

(b) An amount of 53.6 mg (0.222 mmol) of $Cu(NO_3)_2 \cdot 3H_2O$ and 89.0 mg (0.208 mmol) of H_2CB-DO2A$\cdot CF_3COOH \cdot H_2O$ was dissolved in 25 mL of methanol. A 0.64 mL portion of a 0.970 N aq NaOH solution was added and the clear blue reaction mixture refluxed for 8 h. A small amount of precipitate was centrifuged off; the supernatant was decanted and subjected to diethyl ether diffusion to give a 38% yield of blue block-shaped crystals. IR (KBr pellet): 1638(s), 1595 (s) (CO_2^- stretches). Electronic spectrum: λ_{max} (H_2O) 647 nm (ϵ = 57 M^{-1} cm^{-1}). Anal. Calcd for $CuC_{14}H_{24}N_4O_4 \cdot (NaNO_3)_2(H_2O)_{0.5}$: C, 30.30; H 4.54; N, 15.14; F, 0.00%. Found: C, 30.60; H, 4.15; N, 14.85; F, <0.25%.

X-ray Crystallography. Selected bond-lengths and angles are listed in Table 1. Additional X-ray crystallographic data can be found in the Supporting Information in the CIF file.

All details of the complex composition of the Cu clusters and hydrated $NaNO_3$ in the crystal lattice could not be fully resolved. The composition presented is the result of charge balance considerations, but varying degrees of disorder in the regions external to the Cu clusters made it impossible to provide a definitive number of water molecules present. Results obtained using the void analysis program SQUEEZE (A. Spek, Platon Program Library) agree with the composition we report.

Radiochemistry. The general procedures for radiolabeling non-cross-bridged ligands were followed as previously described.[17] Complexation of ^{64}Cu with TETA and DOTA was achieved by the addition of no-carrier-added $^{64}CuCl_2$ in 0.1 N HCl to a 5 mM solution of ligand in 0.1 M ammonium acetate, pH 6.5, followed by a 30-min incubation at 25°C, although incubation at 75°C facilitated more rapid complexation. Formation of ^{64}Cu-TETA and ^{64}Cu-DOTA complexes was verified by radio-TLC using a mobile phase consisting of 50:50 MeOH/10% ammonium acetate on silica plates.

Complexation of ^{64}Cu with CB-TE2A and CB-DO2A was achieved by a 30-min preincubation of the ligand in EtOH with an excess of Cs_2CO_3 at 75 °C with constant mixing. Following centrifugation, addition of no-carrier-added $^{64}CuCl_2$ to the isolated supernatant was accompanied by precipitation of CsCl. The mixture was vortexed and incubated at 75°C for another 30 min. The supernatant was removed, and the pellet was further extracted with an additional volume of ethanol. The combined supernatants were transferred to 0.1 M ammonium citrate buffer (pH 6.5), and the ethanol was evaporated. Radio-TLC of ^{64}Cu-CB-TE2A and ^{64}Cu-CB-DO2A was performed using a 9:1 MeOH/0.1 M ammonium citrate (pH 6.5) mobile phase on C-18 plates.

Biodistribution. The biodistribution study was performed as previously described.[39] Briefly, mature, female, Sprague−Dawley rats (180−200 g) were injected with ^{64}Cu-CB-DO2A diluted with saline (ca.15 μCi in 150 μL per rat). Animals were sacrificed at selected time points postinjection. Organs of interest were removed, weighed, and counted. The %ID per gram (%ID/g) and %ID per organ (%ID/organ) were calculated by comparison to a weighted, counted standard.

Metabolism. The metabolic fates of ^{64}Cu-CB-TE2A, ^{64}Cu-TETA, ^{64}Cu-CB-DO2A, and ^{64}Cu-DOTA in the liver and blood of male Lewis rats were analyzed using reported methods.[23] Organ blank control experiments were also carried out by adding the injectate ex vivo to liver or blood samples from noninjected rats prior to workup. Radiolabeled complexes were injected into rats via the tail vein. The rats were sacrificed at 1, 4, and 20 h postinjection, blood was aspirated, and the livers were immediately excised and placed on ice. Tissue samples were homogenized in 65:35 ethanol/ammonium acetate buffer (0.1 M, pH 5.5) using a Tissumizer tissue homogenizer (Tekmar, Cincinnati, OH) followed by a 1-min tip sonication using a Sonifier 185 cell disruptor [setting 7, microtip limit] (Branson, Danbury, Connecticut). The insoluble protein and cellular debris were removed by centrifugation at 23 500*g* for 30 min at 4 °C. The cleared lysate was counted for radioactivity and analyzed by size-exclusion HPLC on a Superose 12 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.3. A radioactivity balance was performed for each sample run on the column. For all samples analyzed, greater than 95% of the radioactivity was recovered from the column.

The percent protein bound ^{64}Cu in the liver and blood was calculated using the following equation: % protein bound = 100 − % ^{64}Cu-ligand complex. The % ^{64}Cu-ligand complex values were calculated in a manner similar to the methods previously reported by Bass et al.[37] as follows: % ^{64}Cu-ligand complex = % P × % C × [1 + (% Pellet/% Super)] × % E, where % P = purity of injectate determined by TLC; % C = ^{64}Cu-ligand complex determined by integration of the size-exclusion HPLC chromatogram; % Pellet/% Super = the ratio of the radioactivity in the pellet and supernatant of the organ blank; and % E = the extraction efficiency of the harvested organ after injection of ^{64}Cu-TETA or ^{64}Cu-CB-TE2A.

A standard curve was constructed for the Superose column using proteins of an appropriate molecular mass range; this curve was used to estimate the molecular masses of ^{64}Cu-containing species formed in vivo. Human SOD1 (hSOD1) isolated from human erythrocytes, rabbit liver metallothionein (MT), and rat serum albumin (RSA) are all commercially available (Sigma, St. Louis, MO) and were radiolabeled in 1 h with $^{64}CuCl_2$ at 25°C in PBS. Human ceruloplasmin (Sigma, St. Louis, MO), ^{64}Cu-hSOD1, ^{64}Cu-rabbit liver MT, and ^{64}Cu-RSA were analyzed by the same size-exclusion HPLC method used for blood and liver samples.

Radio-TLC studies were performed using the same conditions described for the radiolabeled complexes. Crude extracts were subjected to radio-TLC analysis. Co-spot control experiments were also performed to ensure that the protein present in liver samples did not significantly alter the R_f values of the pure radiolabeled complexes.

SOD1 Incorporation of ^{64}Cu. Binding of ^{64}Cu to SOD1 in rat liver was demonstrated using SDS−PAGE under nonreducing conditions, followed by autoradiography and immunoblot analysis using a rat SOD1-specific antibody. ^{64}Cu-TETA or ^{64}Cu-CB-TE2A (20 mCi) was injected into the tail vein of normal 175−200-g male Lewis rats. At 4 h postinjection, two distinct extractions were performed on 1-g portions of the harvested liver: one extraction using a 2-mL portion of 65:35 ethanol/ammonium acetate buffer (0.1 M, pH 5.5), and a parallel aqueous extraction in a 2-mL portion of NP-40 Lysis Buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 0.1% Nonidet P-40, and 5 mM EDTA). Both extractions were supplemented with a 30-μL aliquot of protease inhibitor mixture (Sigma, St. Louis, MO) consisting of pepstatin, leupeptin, and phenylmethylsulfonyl fluoride, kept on ice, and subjected to tissue homogenization, a 1-min tip sonication, and centrifugation at 25 000*g*, 30 min, 4 °C. The ethanol-containing extract was subjected to size-exclusion chromatography as described above, and fractions were collected.

Human SOD1 (hSOD1) (Sigma, St. Louis, MO), isolated from human erythrocytes, was radiolabeled with ^{64}Cu by incubation at 25 °C for 1 h in PBS. A portion of this reaction mixture was subjected to size-exclusion HPLC as described above to obtain a pure sample of ^{64}Cu-hSOD1 for SDS−PAGE analysis.

The size-exclusion HPLC fractions containing radiolabeled protein, the total aqueous liver extracts (100 μg), and the radiolabeled human SOD1 standard were resolved by nonreducing, nondenaturing 4−15% SDS−PAGE followed by exposure to PhosphorImager (Amersham, Sunnyvale, CA) to detect holoSOD1.[36,53] For immunoblot analysis, proteins from this gel were transferred to nitrocellulose membranes. The blots were then UV-cross-linked, boiled in 2% SDS/50 mM Tris·HCl pH 7.6 for 10 min, and probed using a polyclonal anti-SOD1 antiserum[56] at 1:20 000 dilution, followed by horseradish peroxidase-conjugated ImmunoPure anti-rabbit IgG from goat (Pierce Biotechnology, Inc., Rockford, Illinois) at 1:20 000 dilution. The secondary antibody was detected using the ECL Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL).

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Supporting Information Available: X-ray crystallographic information for Cu-CB-DO2A including experimental crystal data, refinement details, final atomic position parameters, atomic thermal parameters, complete bond distances and angles. This material is available free of charge via the Internet at http://pubs.acs.org.

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