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Regulation of aortic CuZn-superoxide dismutase with copper

Caeruloplasmin and albumin re-activate and transfer copper to the enzyme in culture

Charles T. DAMERON and Edward D. HARRIS*
Department of Biochemistry and Biophysics and the Texas Agricultural Experiment Station, Texas A & M University, College Station, TX 77843-2128, U.S.A.

Caeruloplasmin and albumin were compared as potential donors of copper to Cu_2Zn_2-superoxide dismutase (CuZn-SOD) in culture. Aortas from 15-day copper-deficient chicks were suspended in oxygenated, serum-free, Waymouth medium (752/1) for 24 h. SOD activity was restored when the medium was supplemented with $CuCl_2$, a copper–albumin complex or caeruloplasmin, all present at a level equivalent to 5 μM-copper. Activation did not occur at 4 °C or with Cu-EDTA as the supplement. Mn^{2+} and Zn^{2+}, alone or in combination, did not activate nor enhance the activation achieved by $CuCl_2$. The activation with $CuCl_2$ was not inhibited by cycloheximide or cordycepin. [^{67}Cu]Caeruloplasmin and albumin when added to the medium transferred radioactive copper to at least three cytosolic protein fractions, one of which was determined by immunoprecipitation to be CuZn-SOD. The transfer of ^{67}Cu from caeruloplasmin was inhibited by increasing amounts of unlabelled caeruloplasmin; disodium EDTA (1.0 mM) had no effect on the transfer of copper from caeruloplasmin. These data show that aortic SOD activity, suppressed in copper deficiency, can be restored by incubating the aortas in culture medium supplemented with copper salts. In this system, caeruloplasmin and Cu–albumin appear equally capable of activating aortic CuZn-SOD. Moreover, the transfer of copper into the enzyme structure appears to be the primary event restoring catalytic activity to the enzyme.

INTRODUCTION

Aortic tissue contains both copper/zinc (EC 1.15.1.1) and mangano forms of superoxide dismutase (SOD). Both are suppressed when young chicks are raised on diets lacking copper. About 80% of the CuZnSOD activity was restored when deficient animals were refed diets containing copper or given the copper salts intraperitoneally; no activity was restored by adding copper salts directly to homogenates [the preceding paper (Dameron & Harris, 1987)]. The copper-dependent regulation of SOD activity in animal tissues has been observed previously (Jenkinson *et al.*, 1984; Paynter *et al.*, 1979; Bettger *et al.*, 1979) and is reminiscent of the control copper exerts over the enzyme lysyl oxidase (protein lysine 6-oxidase, EC 1.4.3.13) (Harris *et al.*, 1974; Harris, 1976).

Considerable interest has focused on identifying the mechanisms and intermediates that intercede transfer of copper to intracellular copper enzymes. Anticipating this to be a complex mechanism, attention has been directed primarily to the extracellular transfer stage, specifically the plasma proteins that transfer copper complexes to the cell. Caeruloplasmin, albumin and a newly discovered protein called 'transcuprein' (Weiss & Linder, 1985) have been hypothesized to perform this function *in vivo*. Support for caeruloplasmin in this role has been bolstered by the discovery of a membrane receptor for chick caeruloplasmin in heart and aorta (Stevens *et al.*, 1984). Membrane receptors for caeruloplasmin have also been reported in mature erythrocytes (Barnes & Frieden, 1984), leucocytes (Kataoka & Tavassoli, 1985), pig heart (Harris, 1987) and rat liver endothelium (Kataoka & Tavassoli, 1984). These observations strongly support the contention that caeruloplasmin is a transport protein for copper and give suggestive insight into how the protein may function.

In aorta the CuZn-SOD activity responds reversibly to copper. Copper salts given intraperitoneally rapidly restore CuZn-SOD activity in aortic tissue *in vivo* [the preceding paper (Dameron & Harris, 1987)]. These data suggest that the activation of aortic CuZn-SOD will allow us to compare the relative efficacy of potential copper donors. In the present study free copper, albumin-bound copper and caeruloplasmin were allowed to react with copper-deficient aortas in a culture medium. The use of radioactive copper has provided additional insights into the mechanism.

EXPERIMENTAL

Animals and diets

The source of animals (hybrid Leghorn cockerels), care, feeding and preparation of copper-deficient diets were described previously (Rayton & Harris, 1979). Deficient diets contained less than 1 μg of copper per kg as determined by atomic-absorption analysis. Suffice it to say that the animals were judged to be copper-deficient by analysis of serum caeruloplasmin oxidase activity and serum copper; both parameters are lowered by copper deficiency in chicks (Harris & DiSilvestro, 1981).

Abbreviation used: CuZn-SOD, Cu_2Zn_2-superoxide dismutase.

* To whom correspondence and reprint requests should be sent.

Preparation of aortic cultures

Aortas were removed aseptically from 15-day copper-deficient and control chicks and immersed in sterile medium by using a modification of the procedure described previously (Rayton & Harris, 1979). Incubations were performed in freshly prepared, serum-free, Hepes-buffered Waymouth medium (752/1) supplemented with 0.035 mg of penicillin G and 0.05 mg of streptomycin sulphate/ml of medium. Aortas were cleaned of fascia while immersed in fully oxygenated incubation medium maintained at 37 °C. Intact aortas were used in all culture procedures. Deficient aortas required careful handling because of their tendency to tear. It is important that aortas remain fully immersed in medium before incubation. The aortas were transferred to 1.5 cm × 15 cm culture tubes containing 1.0 ml of medium per aorta and the appropriate test substances. The tubes were flooded with 100% O_2 for 15 s, sealed tightly with screw caps and incubated in a shaking water bath at 37 °C for the specified time. The reaction was stopped by decanting the medium and freezing the aortas.

Assay of SOD activity.

Frozen aortic tissue was thawed and blotted dry. The tissue was homogenized in 10 vol. of 0.12 M-NaCl/0.015 M-potassium phosphate, pH 7.6, with a motor-driven Teflon pestle and 2.0 ml-capacity Potter–Elvehjem homogenizer. The buffers were treated with Chelex-100 to rid them of copper contaminants. After centrifugation (100000 *g*, 30 min, 4 °C), portions of the supernatant were tested for SOD activity by using the procedure of Marklund & Marklund, 1974), employing pyrogallol (1,2,3-trihydroxybenzene; BDH Chemicals, Poole, Dorset, U.K.). CuZn-SOD was determined by measuring SOD activity in the presence and absence of NaCN (1.0 mM) and subtracting the CN-sensitive activity from total activity. Corrections were made for direct effects of cyanide on the pyrogallol autoxidation. One unit of activity is defined as that amount of enzyme that half inhibits the autoxidation of pyrogallol.

Preparation of chicken caeruloplasmin

Caeruloplasmin was isolated from the serum of mature chickens. Each chicken received $CuSO_4$ (0.33 mg of Cu/kg body wt.) intraperitoneally, followed 4 h later with 1.0 ml of turpentine intramuscularly to induce caeruloplasmin synthesis (Giclas *et al.*, 1985). At 24 h after the copper injection, blood was collected from the severed jugular vein in 250 ml centrifuge bottles containing 5.0 ml of 0.2 M-6-aminohexanoic acid in 0.15 M-NaCl for each 50 ml of blood. The whole blood was set aside at room temperature until clotting was complete. The serum was isolated from two centrifugations (8000 *g*, 30 min, 4 °C).

Purification of chicken caeruloplasmin used a modification of the procedure described by DiSilvestro & Harris (1985), designed for rapid purification with small samples. Briefly, solid $(NH_4)_2SO_4$ was slowly dissolved in serum to 25% saturation of 4 °C, followed by centrifugation. The precipitate was discarded and the supernatant was adjusted to 50% saturation with solid $(NH_4)_2SO_4$. After centrifugation the precipitate was suspended in 0.05 M-potassium phosphate/0.1 M-NaCl/0.02 M-6-aminohexanoic acid, pH 7.6, and dialysed against this buffer overnight with three changes of 50 vol. each. The dialysed protein was applied to a 1.5 cm × 8 cm DEAE-Sephadex column equilibrated against the same buffer. The adsorbed enzyme was eluted from the column with a linear 800 ml NaCl gradient (0.0–0.3 M-NaCl) and the fractions containing caeruloplasmin, indicated by the blue colour and an absorbance ratio (A_{610}/A_{280}) of greater than 0.02, were pooled for further purification. After dialysis against 0.01 M-NaCl, the solution was applied to a Bio-Rad Affi-Gel Blue column (0.8 cm × 1.5 cm), the adsorbed caeruloplasmin being eluted with 1.5 M-NaCl. The caeruloplasmin was judged to have a minimum of 80% purity on the basis of absorbance ratio, and showed no evidence of albumin or lower-M_r proteins when analysed on reducing SDS/polyacrylamide gels.

Preparation of ^{67}Cu-labelled caeruloplasmin

Intrinsically bound copper was exchanged with ^{67}Cu by incubating caeruloplasmin with 220 μCi of carrier-free $^{67}CuCl_2$ (Los Alamos National Laboratory; sp. radioactivity 2000 Ci/mg) in the presence of 0.05% (w/v) L-ascorbate for 30 min at room temperature. The labelled protein was treated with 1.0 ml of Chelex-100 (0.5 g/ml) for 30 min with intermittent stirring to remove loosely bound and free ^{67}Cu. The Chelex-100 was removed by centrifugation and the supernatant containing the ^{67}Cu-labelled caeruloplasmin was filter-sterilized. ^{67}Cu-labelled caeruloplasmin showed a single peak of radioactivity on Sephadex G-25; free copper or lower-M_r complexes were not evident.

Preparation of ^{67}Cu-labelled albumin

Chicken serum albumin was obtained from U.S. Biochemical Corp., Cleveland, OH, U.S.A. The protein showed one major band when subjected to SDS/polyacrylamide-gel electrophoresis and was not purified further. A 1.0 mg/ml solution of the protein in distilled water showed no detectable copper. ^{67}Cu-labelled albumin was prepared by adding 220 μCi of carrier-free $^{67}CuCl_2$ to a 1.0 mg/ml solution of chick serum albumin for 30 min at room temperature. This was followed by rapid mixing with Chelex-100 (0.5 g/ml) to sequester free copper. Short exposures to Chelex did not decrease the protein-bound copper. Longer exposures (beyond 30 min) did result in less copper in the protein. The Chelex-treated albumin contained no evidence of free copper when chromatographed on Sephadex G-25.

Reaction of ^{67}Cu-labelled complexes with aortas

Aortas (three per tube) were incubated in fully oxygenated, serum-free, Waymouth medium (752/1), 1 ml per aorta, for 24 h at 37 °C. The medium was supplemented with 1% (w/v) chicken serum albumin. Background copper levels were tested for each medium prepared and were found to be below 0.1 μg of Cu/ml. The results reported reflect the amount of copper that was added as supplements to the medium. The aortas were transferred to 1.5 cm × 15 cm culture tubes containing the appropriate test substances, flooded with O_2 for 15 s, sealed, and incubated in a shaking water bath at 37 °C for 24 h. All incubations contained the same final concentration of radioactivity (5.6×10^5 c.p.m./ml), present either as $^{67}CuCl_2$, ^{67}Cu-albumin or [^{67}Cu]-caeruloplasmin. Also, the copper concentration of the medium supplied by non-radioactive complexes was

the same for each incubation (0.1 μg/ml). Atomic-absorption analyses were performed before the experiment. At termination, the medium was pipetted off and discarded. The aortas were stored frozen (−20 °C).

Fractionation of labelled aortic proteins

The aortic proteins were characterized on Sephadex G-75. Six aortas (two groups of three) were rinsed in cold 0.15 M-NaCl, at 4 °C, and homogenized in a 2 ml-capacity Potter–Elvehejm homogenizer with a motor-drive Teflon pestle. A 5% (w/v) homogenate was prepared in Chelex-100 treated 0.12 M-NaCl/0.015 M-potassium phosphate, pH 7.6. A supernatant was prepared by centrifuging at 100000 *g* for 30 min at 4 °C. A 5 ml portion of extract was applied to the column (2.4 cm × 90 cm). Absorption measurements were taken to be sure that the same amount of protein was applied to each column. The column was developed at a flow rate of 1 ml/min, and 4 ml fractions were collected. Exactly 0.5 ml of each fraction was tested for radioactivity. Corrections were applied for decay ($t_{\frac{1}{2}} = 62$ h) from the start of the experiment. Peak tubes were analysed for immunoprecipitable CuZn-SOD protein, again correcting for radioactive decay.

Immunological procedures

Rabbit anti-(chicken CuZn-SOD) and control IgG fractions were prepared as described in the preceding paper (Dameron & Harris, 1987). Neither chicken caeruloplasmin nor albumin reacted with the antibodies. Immunoprecipitation of CuZn-SOD in aortic tissue employed the procedure of Kessler (1975), with certain modifications. Protein A from *Staphylococcus aureus* (Cowan strain), was obtained from Sigma Chemical Co. as a 10% (w/v) crude cell suspension. Extracts of aortic tissue were incubated with purified SOD antibody; controls received the IgG fraction from non-immunized rabbits. Incubations were conducted in the presence of 6% (w/v) bovine serum albumin for 1 h and were terminated by adding 5 μl of the protein A suspension. After 15 min, the precipitate was collected by centrifugation in a Beckman Microfuge (12000 *g*, 5 min) and the pellet fraction was washed three times with 0.4 ml of buffer (0.12 M-NaCl/0.015 M-potassium phosphate, pH 7.6) and solubilized by heating in 0.1 ml of 1 M-HCl for 10 min at 60 °C. Exactly 0.08 ml of the suspension was transferred to liquid-scintillation tubes for quantification of radioactivity.

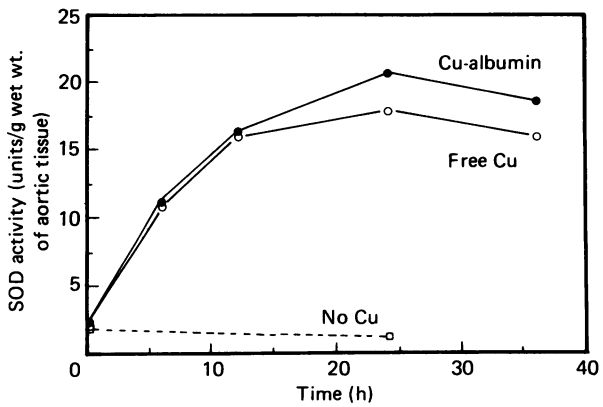

Fig. 1. Time-dependent activation of aortic CuZn-SOD *in vitro* by Cu–albumin and free Cu

Aortas were from copper-deficient chicks. Each copper source contributed 0.3 μg Cu/ml. Data points represent the average results for two groups (five aortas each).

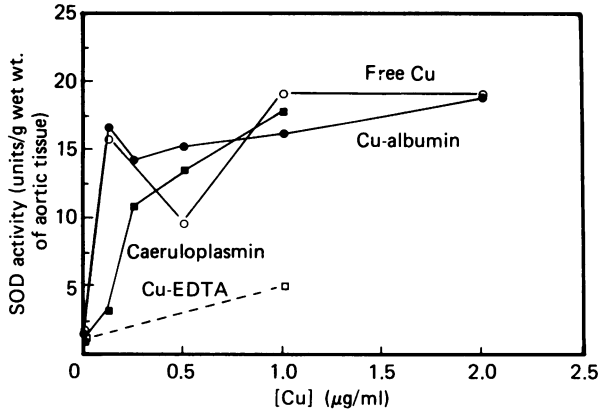

Fig. 2. Dependence of CuZn-SOD activation on the medium copper concentration

Incubations were at 37 °C for 24 h with each source present at zero time. Data represent the average results for three groups (five aortas each). Copper–EDTA was added as the holocomplex.

Measurements of radioactivity

These were performed in a Beckman model LS-250 liquid-scintillation counter with a wide-spectrum setting. Exactly 0.5 ml portions from even-numbered fractions were transferred to 3 ml Nalgene filmware bags and dissolved in 3 ml of Opti-fluor (Packard Instruments).

Other procedures

Copper–albumin complexes were prepared by incubating chicken serum albumin with two equivalents of $CuCl_2$ as described by Dixon & Sarkar (1974). Unbound copper was removed by Centricon-30 (Amicon, Lexington, MA, U.S.A.) ultrafiltration with four washes, 2 ml each, of 0.15 M-NaCl. Cu–EDTA was prepared by mixing equimolar disodium EDTA,$5H_2O$ and 1.6 mM-$CuCl_2$ in water and leaving this solution overnight at 4 °C. The Cu–albumin and Cu–EDTA solutions were adjusted to pH 7.0 and filter-sterilized before use.

RESULTS

Activation of CuZn-SOD *in vitro*

In the preceding paper (Dameron & Harris, 1987), we showed that copper-deficient diets fed to young chicks lowered total aortic SOD activity to non-detectable levels in 8–10 days. Dietary copper or $CuSO_4$ injected intraperitoneally restored the CuZn-SOD activity in 48 and 8 h respectively. To characterize copper-induced activation further requires a controlled environment in which the possible copper donors can be tested apart from other influences. In this study we suspended aortas from 15-day copper-deficient chicks in Waymouth medium supplemented with either free copper, copper–albumin or caeruloplasmin. Fig. 1 shows that aortas suspended in the medium regained SOD activity within hours. In fact, maximal enzyme activation was achieved at or before 24 h. No activation was seen when copper or

Table 1. Activation of aortic CuZn-SOD: response to metal cations

All data are means ± S.D. All metal salts were present at 3.0 μg/ml. Incubations were for 24 h at 37 °C.

Cation tested	SOD activity (units/g wet wt. of aortic tissue)
No addition	1.1 ± 2.4
$CuCl_2$	15.8 ± 2.0
$ZnCl_2$	2.8 ± 2.3
$MnSO_4$	2.2 ± 1.5
$CuCl_2 + ZnCl_2$	19.6 ± 2.6
$CuCl_2 + ZnCl_2 + MnSO_4$	16.2 ± 0.7

Table 2. Activation of aortic CuZn-SOD *in vitro*: effect of antimetabolites and copper proteins

All data are means ± S.D. SOD activity was measured after 24 h.

Addition	SOD activity (units/g wet wt. of aortic tissue)
None	1.1 ± 1.8
$CuCl_2$ (0.3 μg of Cu/ml) (4 °C)	3.3 ± 2.2
$CuCl_2$ (0.3 μg of Cu/ml) (37 °C)	16.3 ± 1.1
+ Cordycepin (12.5 μg/ml)	13.8 ± 2.2
+ Cycloheximide (15 μg/ml)	13.8 ± 0.7
+ NaN_3 (0.3 μM)	17.1 ± 0.1
+ 2,4-Dinitrophenol (0.3 μM)	15.5 ± 0.8
Cu–EDTA (0.3 μg of Cu/ml)	5.0 ± 7.0
Cu–albumin (0.3 μg of Cu/ml)	16.2 ± 1.4
Caeruloplasmin (0.3 μg of Cu/ml)	17.8 ± 2.3

a copper source was omitted. Fig. 1 also shows that free copper ($CuCl_2$) and Cu–albumin, each tested separately at 0.3 μg of copper/ml, induced activation at almost identical rates. Cu–albumin appeared to offer, perhaps, a slight advantage in the later time points. However, this difference was not observed consistently.

The extent of activation was a function of the amount of copper added to the medium (Fig. 2). Comparing $CuCl_2$, Cu–albumin and caeruloplasmin, all seemed to achieve maximum activation at copper concentrations below 1.0 μg/ml. Note that, as above, $CuCl_2$ and Cu–albumin showed similar activation curves. Caeruloplasmin also activated the CuZn-SOD in the tissue. At the lowest copper concentration tested (0.1 μg/ml), caeruloplasmin was not as effective as the other copper sources. With increasing concentrations of the protein, caeruloplasmin appeared to achieve the same plateau activity. Cu–EDTA added as a complex did not restore appreciable activity. One could surmise that each copper source, with the exception of Cu–EDTA, was an effective activator of the enzyme at what may be considered physiological concentrations of copper. For Cu–albumin and free copper, this value was less than 0.5 μg of Cu/ml,

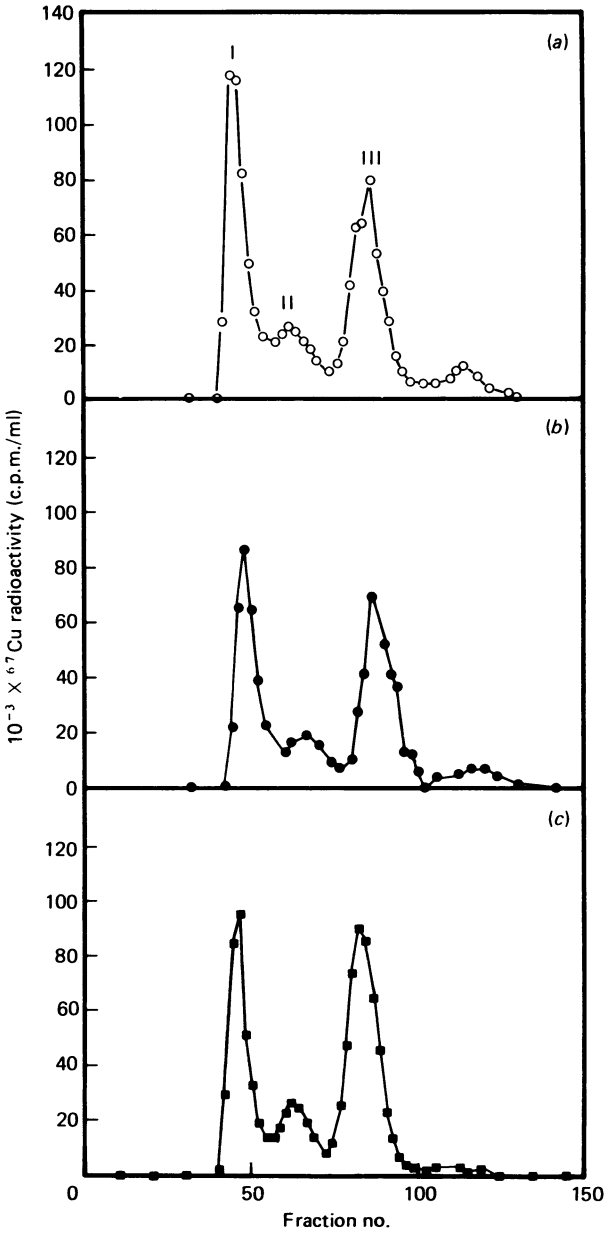

Fig. 3. Resolution of ^{67}Cu-labelled components in aortic cytosol

The three ^{67}Cu sources [$CuCl_2$ (*a*), Cu–Albumin (*b*) or caeruloplasmin (*c*)] were incubated with Cu-deficient-chick aortas for 24 h. Sephadex G-75 was used to separate the labelled components in the 100000 *g* supernatant. The void volume of the column determined by separate analysis was 46 ml. The ratio of ^{67}Cu to the stable isotope was the same for each tube. All incubations were performed in the presence of $CuCl_2$ (0.1 μg/ml) and 1% (w/v) chicken serum albumin.

which approximates the level of copper in the serum of these growing animals (Harris & DiSilvestro, 1981). Additions of $MnSO_4$ or $ZnCl_2$, alone or in combination, neither restored aortic SOD activity nor enhanced the activity achieved by $CuCl_2$ (Table 1). This indicates that the activation response was showing a high degree of specificity for copper.

Additional factors that may have an impact on the

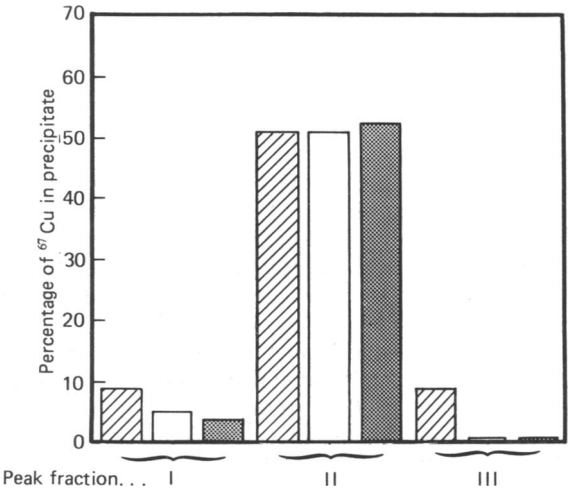

Fig. 4. Percentage of ^{67}Cu in Fig. 3 precipitated by anti-CuZn-SOD serum

Duplicate 0.2 ml samples from each peak fraction seen in Figs. 3(*a*)–3(*c*) were used. Protein A was used to precipitate the CuZn-SOD–anti-SOD complex. ▩, $CuCl_2$; □, albumin; ▨, caeruloplasmin.

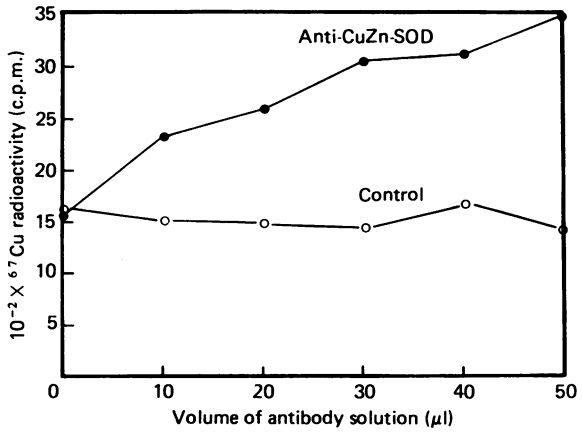

Fig. 5. Necessity of antibodies to CuZn-SOD for precipitation of ^{67}Cu-labelled components in aortic extracts

Control antibodies were obtained from serum of non-immunized rabbits.

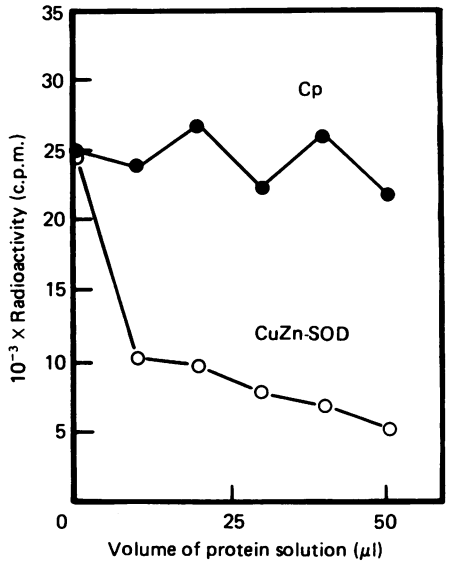

Fig. 6. Comparison of the ability of caeruloplasmin (Cp) and CuZn-SOD to inhibit precipitation of ^{67}Cu-labelled components

Caeruloplasmin solution contained 1.0 mg/ml, whereas CuZn-SOD contained 1.5 mg/ml. Both proteins were in 0.15 M-NaCl.

activation are shown in Table 2. As above, $CuCl_2$, Cu–albumin and caeruloplasmin all seemed to achieve the same degree of activation. Although activation occurred only when copper was present, the response was considerably decreased when incubations were conducted at 4 °C. Activation by $CuCl_2$ addition was not inhibited by cordycepin or cycloheximide. Cycloheximide at the concentration tested (15 μg/ml) is known to inhibit lysine incorporation into aortic proteins in culture (Rayton & Harris, 1979). NaN_3 and 2,4-dinitrophenol, inhibitors of electron transport and ATP formation, did not influence the activation.

Role of copper in *in vitro* activation

The precise role of copper proteins in the activation was unknown. We hypothesized that the proteins themselves provided the copper that was transferred to the enzyme. To investigate this possibility, ^{67}Cu-labelled albumin and caeruloplasmin were prepared. Assurances were made that the copper ions were tightly bound to the protein structure as described in the Experimental section. Aortas were incubated with the $^{67}CuCl_2$, ^{67}Cu-labelled albumin or [^{67}Cu]caeruloplasmin for 24 h. The supernatant fractions from homogenates were chromatographed on identical Sephadex G-75 columns to resolve and characterize the labelled components. The same amount of protein was applied to each column. The results, shown in Figs. 3(*a*)–3(*c*), are profiles of soluble radioactive components from the labelled aortas. Each gave rise to three major peaks. It is evident that no major differences were observed in their position or overall distribution.

Of the various fractions shown in Fig. 3, only the radioactivity in peak II was precipitable by antiserum to CuZn-SOD. When the immunoprecipitated radioactivity was expressed as a percentage of the total radioactivity in the fraction, no differences were apparent for each of the copper donors (Fig. 4). In a control experiment, the anti-(chicken SOD) as opposed to non-immune serum specifically precipitated ^{67}Cu-labelled aortic proteins (Fig. 5), giving assurances that radioactivity in the pellets depended on the presence of the antibody to CuZn-SOD. These data support the hypothesis that the copper bound to caeruloplasmin and albumin can be potential sources of copper for CuZn-SOD. They give no insight into the transfer mechanism.

Transfer of copper from caeruloplasmin

Attention was then focused on copper transfer from caeruloplasmin specifically. Again, immunoprecipitable radioactivity was used to gauge the binding of ^{67}Cu to SOD. Fig 6 shows that anti-(CuZn-SOD) serum did not recognize caeruloplasmin in a competition assay. This is

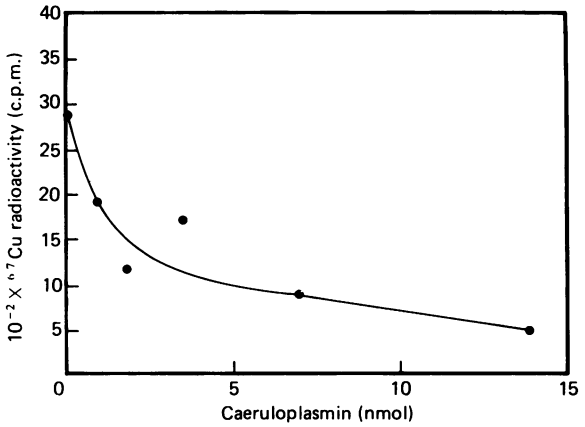

Fig. 7. Unlabelled caeruloplasmin inhibits the incorporation of ^{67}Cu from caeruloplasmin into CuZn-SOD

Immunoprecipitable CuZn-SOD was used to determine the amount of ^{67}Cu incorporated.

further assurance that radioactivity precipitated from the extracts by the antibody could not be [^{67}Cu]-caeruloplasmin. It had been established previously by Ouchterlony double-diffusion analysis that the antibody to chicken SOD will not cross-react with chick caeruloplasmin [the preceding paper (Dameron & Harris, 1987)]. The ^{67}Cu from caeruloplasmin was incorporated optimally when no unlabelled caeruloplasmin was present in the medium (Fig. 7). Increasing concentrations of unlabelled caeruloplasmin decreased exponentially the radioactive copper incorporated. Clearly unlabelled caeruloplasmin was able to compete with the radioactive caeruloplasmin in some phase of the delivery mechanism. Disodium EDTA (0.1 mM) was added to chelate loosely bound or free radioactive copper that could have dissociated from the protein during the incubation. The EDTA did not inhibit the incorporation of ^{67}Cu into CuZn-SOD (results not shown). These results favour the hypothesis that caeruloplasmin copper stays firmly bound to the protein through early phases of the transfer process.

DISCUSSION

The suppression of CuZn-SOD in aortic tissue of animals fed low-copper diets has confirmed the importance of cofactor regulation of the enzyme. Here we addressed the question of copper transport, more specifically, the mechanism for transferring copper ions to CuZn-SOD. Two phases were evaluated: the reactivation of CuZn-SOD with $CuCl_2$ or copper complexes added exogenously, and the transfer of radiolabelled copper from donor complex to the enzyme. Since the studies used aortic tissue suspended in culture medium, the interplay with other factors that could influence or regulate enzyme activation was minimal.

Caeruloplasmin isolated from chick serum contains 4 copper atoms per molecule (DiSilvestro & Harris, 1985). On the other hand, albumin (from bovine sources) has been shown by Scatchard analysis to have two independent binding sites for copper (Mohanakrishnan & Chignell, 1982). The copper–albumin complex in the present study was prepared by mixing two equivalents of copper with one equivalent of albumin. Thus, assuming average distribution, one can deduce that each albumin contained half the number of copper atoms as caeruloplasmin and that only half as much caeruloplasmin as albumin was added for each increment in copper. If the holocomplex is the active component transferring copper, this may explain why caeruloplasmin was not as effective as albumin or free copper at the lower concentrations (Fig. 2). Recently, it was shown that free Cu and Cu(II)-nitrilotriacetate both inhibited the binding and uptake of ^{67}Cu from caeruloplasmin into Chinese-hamster ovary cells and showed similar effects with rat heart, brain, liver and kidney (Orena *et al.*, 1986). Those data would suggest the metal dissociates from caeruloplasmin before uptake. Here we saw no inhibition of copper transfer from caeruloplasmin when 0.1 mM-EDTA was added to the medium and a strong inhibition by nanomolar amounts of holo-caeruloplasmin. Our observations suggest that copper is transferred to the tissue as a holocomplex with caeruloplasmin. Moreover, the apparent antagonism of unlabelled caeruloplasmin would suggest competition for an entrance portal in the cell membrane which is consistent with receptors for caeruloplasmin in chick aortic membranes (Stevens *et al.*, 1984). With a K_d of about 45 nM, receptor–caeruloplasmin interactions would occur at the concentration of caeruloplasmin in the medium and are well within the range of caeruloplasmin concentrations in chick serum (Stevens *et al.*, 1984). This gives physiological significance to the results.

In the present study, free copper, Cu–albumin, and caeruloplasmin independently restored the CuZn-SOD activity in copper-deficient aortas. Each donor appeared to use a mechanism in which activation was accompanied by an exchange of copper from the complex to the enzyme. Apparently a rather complicated, but undefined, ligand-exchange mechanism must be involved. Copper from albumin or caeruloplasmin became bound to three major copper-binding fractions in aorta. In this respect, aortic copper-binding proteins show consistency with intracellular copper-binding proteins in rat organs. Terao & Owen (1974, 1976) identified three cytosolic fractions labelled by copper and resolved by procedures similar to those employed in the present work. One protein of intermediate M_r was identified as SOD (Terao & Owen, 1976). Moreover, ^{67}Cu derived from caeruloplasmin binds to liver SOD (cytocuprein) when the labelled protein is injected into the tail vein of rats (Marceau & Aspin, 1973). In the present study, the identity of SOD protein in peak II was confirmed by immunochemical procedures. The distribution of radioactivity intracellularly reportedly can vary with the type of organ and the nature of the copper-bearing transport ligand (Terao & Owen, 1974). That result was not seen in the present study. Our results tended to point more to similarities than differences between caeruloplasmin and albumin as copper transporters, although the aortas were exposed to higher concentrations of Cu–albumin copper and for much longer durations than would exist *in vivo*. Nonetheless, albumin would appear to be an effective transporter of copper to CuZn-SOD.

In conclusion, in the simple '*in vitro*' system described here, Cu–albumin complexes and caeruloplasmin appear equally effective in restoring catalytic activity and delivering copper to CuZn-SOD. These observations have several bearings on copper metabolism. First, in

culture medium, copper can be provided to cells by a mechanism that does not depend on caeruloplasmin. That observation is consistent with the findings of Barnes & Sato (1980), who concluded that caeruloplasmin is not a growth factor for cells in tissue culture. Also, cells appear to have specific, high-affinity, membrane receptors for free copper as was reported by Schmitt *et al.* (1983). Since free copper, Cu–albumin and caeruloplasmin transferred copper to the enzyme, the copper ions must converge to a common pool before binding to the CuZn-SOD. Secondly, these data suggest that re-activating CuZn-SOD in aortas from copper-deficient animals need only require the transfer of copper to a preformed apoenzyme and, for that reason, may be limited only by cofactor availability. Recently, the existence of a precursor CuZn-SOD has been reported in prokaryotes (Steinman, 1986). Evidence for a precursor form of the CuZn-SOD in aorta has also been obtained [see the preceding paper (Dameron & Harris, 1987)] and is consistent with the apparent failure of cycloheximide and cordycepin to inhibit activation. Finally, the data suggest that metal-ion-induced activation requires metabolic organization in an intact tissue environment. Intact aortas and not homogenates of the tissue responded to copper addition. The need for the intact animal or tissue is further proof that copper incorporation occurs by a defined pathway with specific intermediates.

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