

# The iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity

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Excess copper is poisonous to all forms of life, and copper overloading is responsible for several human pathologic processes. The primary mechanisms of toxicity are unknown. In this study, mutants of *Escherichia coli* that lack copper homeostatic systems (*copA cueO cus*) were used to identify intracellular targets and to test the hypothesis that toxicity involves the action of reactive oxygen species. Low micromolar levels of copper were sufficient to inhibit the growth of both WT and mutant strains. The addition of branched-chain amino acids restored growth, indicating that copper blocks their biosynthesis. Indeed, copper treatment rapidly inactivated isopropylmalate dehydratase, an iron-sulfur cluster enzyme in this pathway. Other enzymes in this iron-sulfur dehydratase family were similarly affected. Inactivation did not require oxygen, in vivo or with purified enzyme. Damage occurred concomitant with the displacement of iron atoms from the solvent-exposed cluster, suggesting that Cu(I) damages these proteins by liganding to the coordinating sulfur atoms. Copper efflux by dedicated export systems, chelation by glutathione, and cluster repair by assembly systems all enhance the resistance of cells to this metal.

*copA* | *Escherichia coli* | glutathione | hydrogen peroxide | Suf

Copper is universally toxic. Indian childhood cirrhosis and Tyrolean infantile liver cirrhosis are human pathologic processes that result from high environmental exposure (1, 2), whereas Wilson disease is a copper-overloading genetic disorder that stems from the dysfunction of a copper pump that normally keeps levels within tolerable limits (3–6). Microbes are particularly vulnerable to metal poisoning because they cannot control their extracellular environment, and so studies into the mechanisms of metal toxicity have often exploited them as model systems.

*Escherichia coli* elaborates 3 homeostatic systems that try to maintain low levels of intracellular copper. CopA is a P-type ATPase that pumps copper from the cytoplasm into the periplasm; it is a homologue of the ATP7B protein that is defective in Wilson disease (7). CueO is an oxidase that is believed to oxidize periplasmic Cu(I) to Cu(II), possibly to slow its adventitious entry into the cytoplasm (8, 9). Both CopA and CueO are strongly induced when cells are exposed to low levels of copper (7, 10–12). Higher levels additionally activate the Cus system, which pumps copper from the periplasm back to the extracellular environment (13). Mutants that lack these systems stop growing when they are exposed to copper levels that WT cells can tolerate (14).

The mechanism by which copper poisons cells has been elusive. A long-standing hypothesis is that copper reacts with endogenous H<sub>2</sub>O<sub>2</sub> to generate hydroxyl radicals in a process analogous to the Fenton reaction:



When the analogous reaction is driven by iron in vivo, the hydroxyl radicals are potent oxidants of DNA and cause both mutagenesis and lethality (15, 16). However, when *copA cueO cus* mutants of *E. coli* were overloaded with copper, growth was

suppressed, but no DNA damage was detected (17). Further inspection suggested that intracellular metabolites, including glutathione, might chelate copper so that it fails to associate with DNA and/or undergo cycles of oxidation and reduction (17).

How, then, does copper “toxify” cells? The present study used *copA cueO cus* mutants to identify primary routes of intracellular damage. It also exploited the ability of *E. coli* to grow anaerobically, so that the role of oxygen in copper toxicity could be directly evaluated.

## Results

### Copper Is Highly Toxic Under Environmentally Relevant Conditions.

Our goal was to determine the mechanism of copper toxicity under physiological conditions. In a simple salts medium that contained glucose as the sole carbon source, WT *E. coli* exhibited a growth defect when Cu(II) concentrations exceeded 8 μM (Fig. 1A). Further, this sensitivity increased when systems that control copper levels were removed. A *copA cueO cusCFBA* mutant showed an aerobic growth defect with as little as 0.25 μM Cu(II) (Fig. 1B). This extreme sensitivity contrasts with the millimolar doses that are needed to inhibit growth in complex media (12, 14, 17).

**Copper Caused a Branched-Chain Amino Acid Auxotrophy.** It has been hypothesized that copper produces toxicity in cells in part by generating reactive oxygen species. Indeed, we found that copper-stressed cells produced endogenous H<sub>2</sub>O<sub>2</sub> at a rate several fold higher than did untreated cells (data not shown). Excessive levels of superoxide (O<sub>2</sub><sup>•-</sup>) and H<sub>2</sub>O<sub>2</sub> can disrupt several amino acid biosynthetic pathways (18–20); therefore, we tested whether copper creates similar blocks. (This experiment was complicated by the fact that α-amino acids chelate copper and thereby increase the dose necessary for toxicity; to correct for this effect, alanine was added in equivalent doses to cultures from which amino acids of interest were withheld.) The *copA cueO cusCFBA* mutant could not grow aerobically in glucose/alanine medium to which 10 μM copper was added (Fig. 2A). The addition of branched-chain amino acids partially restored growth, indicating that this pathway is a primary target of copper toxicity. The failure to fully restore growth indicates that additional growth-limiting damage also occurred outside of this pathway.

### Copper Inactivated Iron-Sulfur Cluster Dehydratases Inside Aerobic Cells.

Reactive oxygen species block branched-chain biosynthesis because they directly damage the iron-sulfur clusters of 2 dehydratases: dihydroxy-acid dehydratase in the common branched-chain pathway and isopropylmalate isomerase (IPMI) in the leucine-specific branch (18, 21–23). In conformity with the

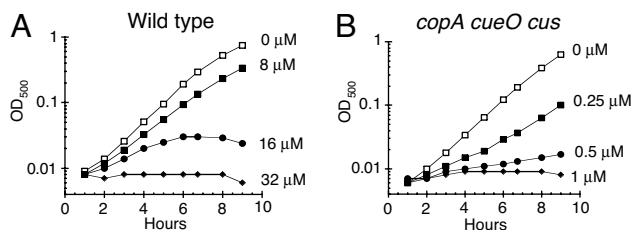
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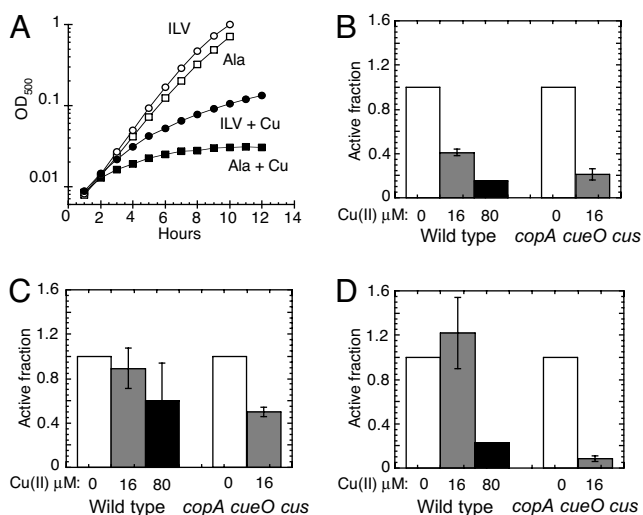


**Fig. 1.** Copper is toxic to aerobic cells at micromolar doses. *E. coli* cultures were grown at 37 °C in aerobic glucose medium, and CuSO<sub>4</sub> was added. (A) W3110 (wild type) cultures were challenged with 0 μM (open squares), 8 μM (closed squares), 16 μM (closed circles), or 32 μM CuSO<sub>4</sub> (closed diamonds). (B) LEM33 (*copA cueO cusCFBA*) cultures were challenged with 0 μM (open squares), 0.25 μM (closed squares), 0.5 μM (closed circles), or 1 μM CuSO<sub>4</sub> (closed diamonds). The data are representative of 3 independent experiments.

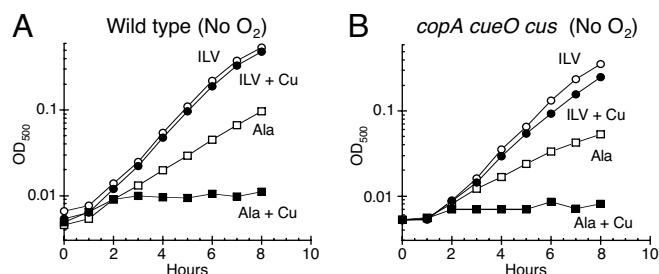
growth data, we found that the IPMI activity of WT cells did not decrease when they were exposed to 10 μM Cu(II) for 8 h, whereas a similar treatment to *copA cueO cusCFBA* mutant cells reduced the activity to less than 15% (the detection limit) of untreated WT controls.

In principle, protracted exposure to copper might diminish enzyme activity either by damaging extant enzymes or by blocking new enzyme synthesis or activation. A higher dose of 16 μM copper diminished the total IPMI activity of a culture of WT cells by 60% within 30 min, suggesting that the former explanation pertains. Similarly, activity decreased in *copA cueO cusCFBA* mutant cells by 80% (Fig. 2B).

Furthermore, the activities of fumarase A and 6-phosphogluconate dehydratase also declined when cells were exposed to copper (Fig. 2C and D). Like IPMI and dihydroxy-acid dehydratase, these enzymes are dehydratases that employ iron-sulfur



**Fig. 2.** Copper damages iron-sulfur-cluster dehydratases. (A) LEM33 (*copA cueO cusCFBA*) was grown at 37 °C in aerobic glucose medium with 1.5 mM alanine (Ala) (squares) or 0.5 mM each of isoleucine (I), leucine (L), and valine (V) (circles), and CuSO<sub>4</sub> was added to 0 μM (open symbols) or 10 μM (closed symbols). The data are a representative of 3 independent experiments. (B-D) W3110 (WT) and LEM33 (*copA cueO cusCFBA*) were grown aerobically to an OD<sub>500</sub> of ≈0.1, then challenged with 0 μM (open bars), 16 μM (gray bars), or 80 μM CuSO<sub>4</sub> (black bars) for 30 min. (B and C) Cells were grown in glucose/alanine, and IPMI (B) and fumarase (C) activities were measured. (D) Cells were grown aerobically in gluconate medium supplemented with 1.5 mM alanine, and 6-phosphogluconate activity was measured. (B-D) Data are the average of 3 independent experiments, and the error bars represent SD. (B) WT cells exposed to 80 μM Cu had IPMI activities below the detection limit (<15%).



**Fig. 3.** Copper inhibits branched-chain biosynthesis even in the absence of oxygen. (A and B) *E. coli* cultures were grown anaerobically in glucose medium supplemented with either 1.5 mM alanine (Ala) (squares) or 0.5 mM each of isoleucine (I), leucine (L), and valine (V) (circles), and they were then challenged with 0 μM (open symbols) or 2 μM CuSO<sub>4</sub> (closed symbols). (A) W3110 (WT). (B) LEM33 (*copA cueO cusCFBA*). The data are representative of 3 independent experiments.

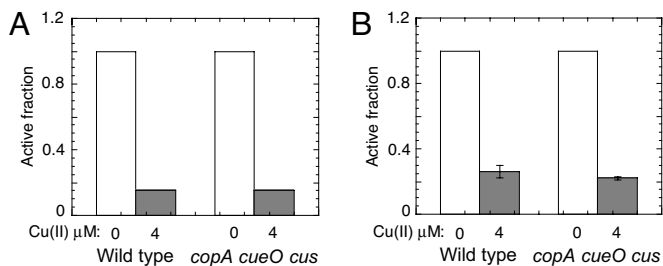
clusters, and so it appears that all members of this enzyme class are vulnerable to copper stress. Whereas growth on glucose does not require large substrate fluxes through these enzymes, other growth conditions do: the *copA cueO cusCFBA* mutant failed to grow aerobically on succinate medium even when it was supplemented with branched-chain amino acids [supporting information (SI) Fig. S1A], a phenotype consistent with the inactivation of fumarase A. Indeed, overexpression of fumarase A restored growth.

**The Mechanism of Copper Toxicity Is Independent of Oxygen.** Other investigators have noted that copper can poison bacterial cells in anaerobic medium (12, 17, 24), and we observed, in fact, that both WT cells and the *copA cueO cusCFBA* mutant were much more sensitive to growth inhibition by copper when oxygen was absent (Fig. S2). The anaerobic growth of the WT and mutant strains was effectively blocked by 1 μM and 125 nM copper, respectively. At the very least, this means that copper has an acute mechanism of toxicity that does not involve reactive oxygen species. Surprisingly, experiments suggested that the primary target of anaerobic toxicity was no different from that in aerobic cells. Growth resumed when branched-chain amino acids were added to anaerobic medium (Fig. 3). This restoration of growth was not caused by chelation of copper, because the D-enantiomers of the amino acids failed to restore growth (data not shown). Further, an overexpression plasmid carrying *leuCD* fully suppressed the growth defect, indicating that it arose from the lack of IPMI activity (Fig. S1B).

Indeed, during anaerobic exposure to 4 μM copper, the IPMI activities of both WT cells and *copA cueO cusCFBA* mutant cells decreased to less than 15% of the activity of untreated cells (Fig. 4A). Copper also damaged fumarase B, the anaerobic isozyme of fumarase A (Fig. 4B). These results indicated that the poisoning of these enzymes by copper occurred through a non-oxidative mechanism. It is likely that the same was true in aerobic media.

CueR is the regulatory protein that senses cytoplasmic copper and activates synthesis of the efflux pump CopA and the copper oxidase CueO. When WT cells were exposed to 2 μM copper—half the level that inactivated IPMI and fumarase A in the preceding experiment—transcription of a *copA-lacZ* fusion was activated 5-fold (Fig. S3A). Strikingly, transcription of the fusion was fully activated in a *copA cueO cus* mutant even without copper supplements, indicating that the efflux system was needed to avoid stress from the trace copper that contaminates standard media.

**Copper-Damaged Fumarase A Was Repaired in Vivo.** *E. coli* can repair damaged clusters (25), and so we tested whether the activity of

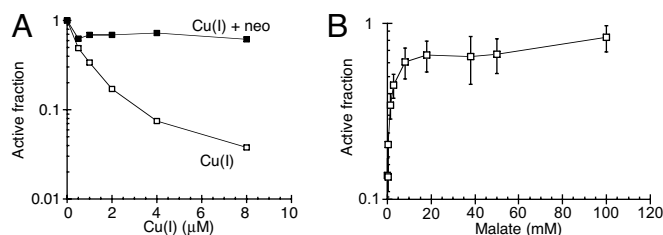


**Fig. 4.** Copper damages iron-sulfur cluster dehydratases in the absence of oxygen. (A and B) W3110 (WT) and LEM33 (*copA cueO cusCFBA*) cultures were grown anaerobically in glucose/alanine medium to an  $\text{OD}_{500}$  of  $\approx 0.1$ , and then challenged with 0  $\mu\text{M}$  (open bars) or 4  $\mu\text{M}$   $\text{CuSO}_4$  (gray bars) for 30 min. (A) IPMI activity: copper-treated cells had IPMI activities below the detection limit ( $<15\%$ ). (B) Fumarase activity: the data are the average of 3 independent experiments, and the error bars represent SD.

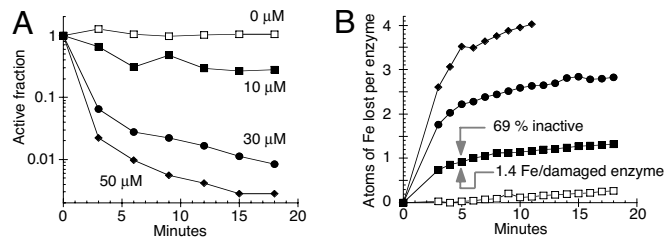
these enzymes would be restored *in vivo* after toxic doses of copper were removed (see *SI Methods*). These experiments were conducted in aerobic medium, to facilitate the export of residual copper by the pmf-driven Cus system. Chloramphenicol was included to ensure that any regained activity was not caused by new protein synthesis. We observed that the activity that had been lost during copper treatment gradually reappeared after copper was removed, with half of the activity regained within 30 min (Fig. S4). The fact that the enzyme damage was reversible is consistent with the notion that inactivation was caused by damage to the cluster itself, rather than to polypeptide.

**Cuprous Copper Directly Damages Fumarase A *In Vitro*.** Our previous EPR analyses showed that intracellular copper in overloaded *E. coli* is in the reduced, Cu(I), valence (17). Therefore, we tested the ability of Cu(I) to directly damage a purified iron-sulfur cluster dehydratase in an anaerobic reaction system. Because of the poor solubility of Cu(I) salts, we generated Cu(I) by co-incubation of Cu(II) with ascorbic acid before its addition to the target enzyme.

Low micromolar concentrations of Cu(I), but not ascorbate alone, rapidly inactivated purified fumarase A (Fig. 5A). The Cu(I)-specific chelator neocuproine completely protected fumarase activity. To emphatically exclude the possibility that reactive oxygen species might be generated through leaked oxygen, superoxide dismutase (SOD) and catalase were added to the reaction. As expected, these enzymes, together or separately, did not protect fumarase at all (data not shown). We conclude that the enzyme is directly damaged by Cu(I). Separate experiments



**Fig. 5.** Fumarase A is damaged by Cu(I) and protected by substrate. (A) Purified fumarase A (0.1  $\mu\text{M}$ ) was anaerobically challenged in 50 mM Tris-HCl, pH 7.6, with Cu(I) for 3 min at 27  $^{\circ}\text{C}$  either without a chelator (open squares) or in the presence of 100  $\mu\text{M}$  neocuproine (*neo*) (closed squares). The data are a representative of 3 independent experiments. (B) Fumarase A was exposed to 3  $\mu\text{M}$  Cu(I) for 3 min at 27  $^{\circ}\text{C}$  in the presence of the indicated concentrations of malate, and final activity was determined. The data are normalized to the starting activity. The data are the average of 3 independent experiments, and the error bars represent the SD.



**Fig. 6.** Cu(I) damages the iron-sulfur cluster of fumarase A. (A and B) Fumarase A (4  $\mu\text{M}$ ) was challenged anaerobically at 27  $^{\circ}\text{C}$  with 0  $\mu\text{M}$  (open squares), 10  $\mu\text{M}$  (closed squares), 30  $\mu\text{M}$  (closed circles), or 50  $\mu\text{M}$  (closed diamonds) Cu(I) for 3 min. (A) Fumarase activity after challenge with Cu(I). (B) Time course of iron release from fumarase during copper challenge in A. The data are representative of 3 independent experiments.

showed that Cu(II) is also capable of damaging fumarase, but Cu(II) does so at a rate significantly slower than Cu(I) (data not shown).

**Copper Damaged the Iron-Sulfur Cluster of Fumarase A.** Saturating concentrations of malate, a substrate of fumarase A, fully protected the enzyme activity from copper (Fig. 5B). This effect was probably mediated by its shielding the enzyme active site, as the protective effect tracked closely to the dose needed to form an ES complex.

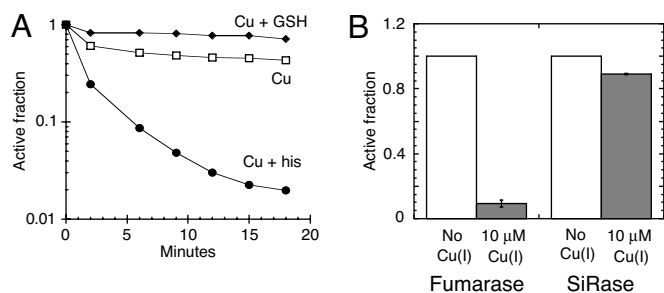
The ferrous-iron chelator dipyriddy was used to monitor any loss of iron from the enzyme. The addition of 10  $\mu\text{M}$  Cu(I) caused a rapid release of more than one atom of Fe(II) per inactivated enzyme (Fig. 6B), with the loss of a second iron atom following quickly after the first (e.g., arrows in Fig. 6B). In contrast, oxidants displace a single iron atom, and the residual [ $^3\text{Fe-}^4\text{S}$ ] cluster is stable to further exposure (18, 26). Higher concentrations of copper lead to total disintegration of the cluster.

Two aspects of the kinetics were unexpected. First, the concentration of copper that was needed to inactivate fumarase A was higher in experiments that used elevated concentrations of fumarase A, even though copper was substantially superstoichiometric to enzyme. Second, the rate of enzyme inactivation quickly declined during time courses. We speculated that both phenomena reflect the adventitious binding of copper to the protein surface, causing the copper to become progressively immobilized and unable to generate further damage.

We tested this notion by adding 100  $\mu\text{M}$  histidine as a moderate-affinity solubilizing agent that might lessen the tendency of copper to adhere to the surface of the protein. The histidine significantly enhanced the rate of enzyme inactivation, and it extended the period during which copper was active against the enzyme (Fig. 7A). Presumably histidine and analogous metabolites mobilize copper *in vivo*, too, and enhance its ability to selectively access higher-affinity binding sites. Conversely, glutathione is a small molecule that binds copper very tightly, and its addition (100  $\mu\text{M}$ ) to the reaction system completely blocked damage to the enzyme (Fig. 7A).

When a copper-binding protein, mammalian CuZnSOD, was heterologously expressed in WT anaerobic *E. coli*, 40 U/mg SOD activity was recovered in cell extracts, representing the activation of 100  $\mu\text{M}$  active CuZnSOD monomers in the cytoplasm. At the same time, the presence of the CuZnSOD protein shielded fumarase from inactivation when these cells were exposed to copper (Fig. S3B), presumably by immobilizing the copper (the experiment was performed anaerobically to ensure that the catalytic function of CuZnSOD could not play a role in the protection of fumarase). These results support the implications of the histidine and glutathione experiments: inside the cell, me-





**Fig. 7.** The ability of Cu(I) to damage iron-sulfur clusters is modulated by copper-binding metabolites and requires that clusters be solvent-exposed. (A) Fumarase A ( $4 \mu\text{M}$ ) was anaerobically challenged with  $10 \mu\text{M}$  Cu(I) at  $27^\circ\text{C}$  without supplementation (open squares) or in the presence of  $100 \mu\text{M}$  histidine (*his*) (closed circles) or  $100 \mu\text{M}$  reduced glutathione (*GSH*) (closed diamonds). The data are a representative of 3 independent experiments. (B) Fumarase A ( $0.1 \mu\text{M}$ ) was mixed with sulfite reductase (*SiRase*), and the enzyme mixture was then challenged anaerobically with  $0 \mu\text{M}$  (open bars) or  $10 \mu\text{M}$  Cu(I) (gray bars) for 3 min at  $27^\circ\text{C}$ . The data are the average of 3 independent experiments, and the error bars represent SD.

tabolites allow copper to find tight binding sites, and sequestration can restrict the tendency of copper to damage dehydratases.

**What Is the Structure of the Damaged Cluster?** Copper-damaged fumarase could be substantially reactivated *in vitro*. The enzyme was incubated with Cu(I) until 5% of the initial activity remained; neocuproine was then added to chelate the copper and block further damage. The subsequent addition of ferrous iron and DTT reactivated fumarase to 50% of its initial activity, suggesting that a significant fraction of the damaged enzyme molecules contained  $[\text{}^3\text{Fe-}^4\text{S}]$  clusters that retained their inorganic sulfur atoms. The remaining enzymes that could not be reactivated likely had clusters that had degraded further, which would have required the provision of a sulfur species for repair. Unfortunately, the addition of a moderate level of sulfide was inhibitory, causing less recovery of activity than did iron/DTT alone, and higher concentrations led to precipitation. As these treatments have been successful for us in other circumstances (26), we suspect that residual copper interfered with the process.

The stoichiometry of iron release suggested that a population of copper-treated fumarase molecules would likely include clusters at different stages of disintegration. EPR spectroscopy has been used to identify  $[\text{}^3\text{Fe-}^4\text{S}]^+$  (18, 27),  $[\text{}^2\text{Fe-}^2\text{S}]^+$  (28), and  $[\text{Cu-}^3\text{Fe-}^4\text{S}]^+$  clusters (29, 30), and we attempted to find evidence for any of these species (see *SI Methods*). Enzyme was exposed to sufficient copper to inactivate 85% of the population. This treatment did not generate any significant  $[\text{}^3\text{Fe-}^4\text{S}]^+$  signal, in contrast to a large signal obtained with  $\text{H}_2\text{O}_2$  (Fig. S5); this result was expected, as a non-oxidative event that released ferrous iron would be expected to create the EPR-silent  $[\text{}^3\text{Fe-}^4\text{S}]^0$  species. No  $[\text{}^2\text{Fe-}^2\text{S}]^+$  signal was detected, even after the addition of dithionite to reduce any  $[\text{}^2\text{Fe-}^2\text{S}]^{2+}$  clusters to the EPR-visible  $[\text{}^2\text{Fe-}^2\text{S}]^+$  form, consistent with the limited extent of cluster destruction. We had thought that we might detect a  $[\text{Cu-}^3\text{Fe-}^4\text{S}]$  cluster species similar to those that have been artificially generated in ferredoxins (29, 30). However, no such signal was observed, suggesting that the presumptive displacement of iron atoms by copper does not produce a stable intermediate form that contains copper. Thus, the current data support the idea that copper treatment first converts the  $[\text{}^4\text{Fe-}^4\text{S}]^{2+}$  native enzyme to a  $[\text{}^3\text{Fe-}^4\text{S}]^0$  form, and that subsequent events further degrade the enzyme through undefined intermediates ultimately to an apoenzyme.

### Enzymes with Buried Iron-Sulfur Clusters Were Resistant to Copper.

The vulnerability of dehydratases to chemical agents reflects the accessibility of their clusters to solutes. This principle was reaffirmed by the protective effect that bound substrate had upon the fumarase cluster (Fig. 5B). It follows that enzymes whose clusters are largely occluded by polypeptide are unlikely to be targets of dissolved copper. Indeed, metabolic pathways that include non-dehydratase cluster enzymes—such as sulfate reduction (sulfite reductase), nicotinamide biosynthesis (quinolinate synthase), and ammonia assimilation (glutamate synthetase)—remained functional when cells were exposed to copper, as evidenced by growth in glucose medium supplemented with branched-chain amino acids. Further, when purified fumarase A was co-incubated with sulfite reductase or with everted membrane vesicles containing succinate dehydrogenase, copper doses that eliminated fumarase activity had virtually no effect on these other enzymes, which rely upon buried iron-sulfur clusters (Fig. 7B and Fig. S6). Thus, the toxicity of copper is focused upon cell processes that rely upon proteins with solvent-exposed clusters.

### Glutathione and the Suf Iron-Sulfur Cluster Assembly System Contributed to Copper Resistance.

As demonstrated earlier, glutathione protected purified fumarase from damage by copper *in vitro*. To test whether glutathione might also play this role *in vivo*, we deleted the gene (*gshA*) that catalyzes the first step in glutathione biosynthesis. The resultant  $\Delta\text{gshA}$  mutant was indeed more sensitive to growth inhibition by copper than were WT cells (Fig. S7A).

The iron-sulfur cluster assembly system (Isc) is responsible for *de novo* synthesis and transfer of iron-sulfur clusters. The exact mechanism of Isc function is not yet clear; however, the assembly and/or transfer of an iron-sulfur cluster could plausibly involve its exposure to solvent and, as a consequence, vulnerability to small solutes like copper. In fact, the Isc system is inhibited by  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  (S. Jang, unpublished observations). In *E. coli*, when Isc function is inhibited, the cell induces a second iron-sulfur cluster assembly system, the Suf system. The Suf system, unlike the Isc system, is resistant to  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ , perhaps because it builds and transfers clusters in a more cloistered environment (S. Jang, unpublished observations). Therefore, we wondered whether copper might similarly inhibit the Isc system and create a requirement for the Suf system during copper stress. To test this idea, cells were grown anaerobically on medium containing glycerol and nitrate to maximize the need for iron-sulfur cluster enzymes. The *suf* mutant was more sensitive to copper than was the WT strain (Fig. S7B), suggesting both the failure of the Isc system and the resistance of the Suf system.

### Discussion

Life evolved in an anaerobic world that was rich in iron. Evolution exploited the versatile surface chemistry of this metal by integrating it into iron-sulfur clusters that serve to bind substrates into enzyme active sites. This arrangement succeeded for 2 billion years, until the emergence of photosystem 2 caused the gradual oxygenation of the environment. As molecular oxygen accumulated, it oxidized dissolved sulfur compounds, which until that time had sequestered soft metals into mineral precipitates. The subsequent solubilization of these metals evidently posed a new problem for extant organisms. The present study reveals that one of these metals, copper, rapidly inactivates the catalytic clusters of dehydratases. This enzyme family has representatives in central catabolic and biosynthetic pathways, which therefore become dysfunctional in copper-exposed cells.

These enzymes are uniquely vulnerable to chemical damage because their clusters are substantially exposed to solvent (31). Previous studies show that  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  are small enough to invade the active site, where they coordinate and oxidize the

iron-sulfur cluster to an unstable valence (18, 21, 32). In this work, we found that these clusters are also the primary targets of copper, which evidently displaces their iron atoms when it coordinates their thiolate or inorganic sulfur ligands. The extreme avidity with which it does so means that cellular environments that contain such enzymes cannot tolerate even modest levels of active copper.

**Mechanisms of Protection: Compartmentalization or Chelation?** After the biosphere became aerobic, rather than discard and replace their many iron-sulfur-based enzymes in piecemeal fashion, ancient organisms developed comprehensive strategies that shield them from the toxic agents that might damage them. Peroxidases and SODs reduce reactive oxygen species to tolerable levels by converting them to innocuous products. Copper (and other soft metals) cannot be chemically degraded, and so *E. coli* employs efflux systems that drive copper out of the cytosol (7, 13). Mutants that lacked these enzymes were damaged by nanomolar levels of copper, indicating that such defenses must be requisite for organisms to live in many natural habitats.

The redox activity of copper also induced microbes to integrate it into novel enzymes that facilitate aerobic metabolism, including the copper-zinc SOD, cytochrome bo oxidase, and amine oxidase. However, this advance posed the problem of ensuring that copper was available as a cofactor for these proteins but somehow still blocked from damaging hypersensitive iron-sulfur enzymes. *E. coli* appears to have solved this conundrum by compartmentalizing its copper-dependent enzymes in the periplasm or the outer aspect of the cytoplasmic membrane, whereas its unstable dehydratases are exclusively inside the cytoplasm. The periplasm accumulates significant copper (17); it contains the Cus copper export system, but the synthesis of the exporter is not triggered until copper levels substantially exceed what is needed to charge these enzymes. In contrast, CopA, which pumps copper out of the cytoplasm, is activated by very low environment copper levels (12) and may be designed to drive the amount of cytoplasmic copper as low as possible. Interestingly, the periplasmic Cus system probably assists the CopA system: by limiting the copper concentration in the periplasm to a reasonable level, it can slow the incidental leakage of copper into the cytoplasm.

Eukaryotes appear to shield dehydratases from copper by chelation rather than compartmentalization. In their cytosol, enzymes that require copper, such as SOD, commingle with enzymes of the dehydratase class, including IPMI. To circumvent possible problems, chaperone systems deliver copper directly to SOD and cytochrome oxidase (33–37). Calculations demonstrate that the level of fully aquated copper inside these cells must be vanishingly low (38), although an unknown amount of un-incorporated copper might still be bound to metabolites and to the surfaces of bio-molecules. It is possible that glutathione plays an accessory role in sequestering loose copper, as it does in *E. coli*.

**Secondary Effects of Cluster Damage.** Workers in eukaryotic systems have reported that copper stress elicits markers of oxidative damage, including DNA damage (39–42). Speculation as to the basis of this phenomenon has centered on the possibility that copper generates hydroxyl radicals by participating in Fenton-like chemistry, after the fashion of iron. However, the discovery that copper destroys iron-sulfur clusters points to another possibility: that the released iron atoms overload the cell and proportionately accelerate iron-based Fenton chemistry. Superoxide stress facilitates DNA damage through a similar mechanism (43, 44). Further, because the eukaryotic iron-responsive protein belongs to the dehydratase class of cluster enzymes (45), it follows that copper may adventitiously convert it into the apo-protein form and thereby stimulate excessive iron import.

Consistent with these ideas, workers have shown that iron chelators can suppress some aspects of copper toxicity (40).

It is notable that copper may also inhibit the Isc iron-sulfur-cluster assembly system. Many bacteria express the Suf system as a more-robust back-up, as in the case of the *E. coli* studied here, but eukaryotes rely exclusively on Isc and therefore may suffer a more expansive set of disabled pathways. Interestingly, Ranquet et al. recently reported that cobalt disrupts Isc function (46). We found that 100  $\mu\text{M}$  cobalt did not directly inactivate fumarase A in vitro (data not shown), which suggests that the mechanism by which metals impede Isc may not directly mimic the mechanism by which they displace iron from dehydratases.

## Materials and Methods

**Reagents.** Djenkolic acid and D-leucine were obtained from Aldrich; glycerol, D-glucose, potassium cyanide, and potassium nitrate from Fisher Scientific; sodium dithionite from Fluka; Amplex red from Invitrogen; and neocuproine hydrochloride from MP Biomedicals. L-amino acids, D-isoleucine, D-valine, L-ascorbic acid, bovine liver catalase, bovine erythrocyte SOD, bovine milk xanthine oxidase, horse heart cytochrome C, rabbit muscle lactate dehydrogenase, chloramphenicol, copper sulfate, ferrous ammonium sulfate, 2,2'-dipyridyl, disodium L(-) malic acid, D,L-DTT, flavin mononucleotide, hydrogen peroxide, isopropyl  $\beta$ -D-thiogalactopyranoside,  $\beta$ -lactose, NADH, NADPH, potassium ferricyanide, potassium ferrocyanide, potassium D-gluconate, glutathione (reduced), sodium sulfide, succinic acid, tri(cyclohexylammonium) 6-phosphogluconic acid, plumbagin, thiamine, and xanthine were obtained from Sigma.

Fumarase A was purified and quantified (18, 27) from anaerobic cultures of an overexpressing strain (SJ50) that lacks catalase and peroxidase, to ensure that hydrogen peroxide could be used to inactivate the enzyme in subsequent experiments. All purification steps (27) were conducted anaerobically at chamber temperature (27  $^{\circ}\text{C}$ ), and all buffers were anaerobic. Sulfite reductase was purified aerobically from an overproducing strain (PD401) grown with djenkolic acid as the sole sulfur source (47).

**Bacterial Growth.** Strains are listed in Table S1 in the *SI Methods*. Luria broth and base M9 minimal salts were of standard composition (48). Where indicated, M9 medium was supplemented with 0.2% glucose, 0.4% gluconate, 0.2% lactose, 40 mM succinate, 40 mM glycerol/40 mM nitrate, 1.5 mM alanine, 0.5 mM L-isoleucine/L-leucine/L-valine, or 0.5 mM of the standard amino acids. Media were supplemented with 100  $\mu\text{g}/\text{mL}$  ampicillin, 20  $\mu\text{g}/\text{mL}$  chloramphenicol, 30  $\mu\text{g}/\text{mL}$  kanamycin, or 12  $\mu\text{g}/\text{mL}$  tetracycline when antibiotic selection was needed. Anaerobic cultures were grown in a Coy chamber (Coy Laboratory Products) using pre-equilibrated growth media under 85%  $\text{N}_2$ , 10%  $\text{H}_2$ , and 5%  $\text{CO}_2$ .

To ensure that all studies were conducted upon exponentially growing cells, overnight cultures were diluted to  $\text{OD}_{500}$  0.005, and cells were grown at 37  $^{\circ}\text{C}$  to an  $\text{OD}_{500}$  of  $\approx 0.1$ . They were then subcultured again to  $\text{OD}_{500}$  0.005 and grown at 37  $^{\circ}\text{C}$  to an  $\text{OD}_{500}$  of  $\approx 0.1$  before analyses. Measurements were made at 500 nm to minimize interference by dissolved copper.

Enzyme activities from copper-treated cells were typically measured after 30 min of incubation with copper at 37  $^{\circ}\text{C}$ .

**Enzyme Assays.** To prepare extracts, cells were washed twice and resuspended in anaerobic buffer. Lysis and the removal of cell debris by centrifugation were also performed in the anaerobic chamber. All enzyme reactions were monitored at room temperature (25  $^{\circ}\text{C}$ ). IPMI (49), fumarase (50),  $\beta$ -galactosidase (48), SOD (51), 6-phosphogluconate dehydratase (52), and sulfite reductase (47) activities were measured according to the cited methods. Protein concentrations were determined using the Coomassie protein assay reagent (Pierce) with BSA as the standard.

**In Vitro Studies.** Stock solutions of Cu(II) were prepared by mixing Cu(II) with 350  $\mu\text{M}$  ascorbic acid in anaerobic 50 mM Tris-HCl, pH 7.6. The reduction of Cu(II) to Cu(I) was confirmed by the complete loss of the Cu(II) absorption peak at 630 nm.

Purified fumarase was inactivated by addition of copper or  $\text{H}_2\text{O}_2$  at 27  $^{\circ}\text{C}$  (i.e., ambient chamber temperature) to enzyme dissolved in anaerobic buffer (50 mM Tris-HCl/10 mM  $\text{MgCl}_2$ , pH 8.0). Where indicated, 100  $\mu\text{M}$  neocuproine, 100  $\mu\text{M}$  reduced glutathione, 100  $\mu\text{M}$  histidine, varied L-malic acid, 160 U of SOD, or 28,000 U of catalase was present.

In some experiments, damaged fumarase was reactivated after copper

treatment. Neocuproine (100  $\mu\text{M}$ ) was added after the damage period to sequester copper, and 10  $\mu\text{M}$   $\text{Na}_2\text{S}/500 \mu\text{M}$  D,-DTT/100  $\mu\text{M}$   $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  were then added.

The loss of iron from the cluster of fumarase was monitored by trapping with dipyriddy ( $\epsilon_{\text{mM}}$ , 9.2  $\text{mM}^{-1} \text{cm}^{-1}$  at 522 nm for the ferrous complex). The reaction contained 4  $\mu\text{M}$  fumarase A/1 mM dipyriddy/50 mM Tris-HCl/10 mM  $\text{MgSO}_4$ , pH 8.  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  was used to create a standard curve.

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