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FORUM REVIEW ARTICLE

Redox Regulation of SCO Protein Function: Controlling Copper at a Mitochondrial Crossroad

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Abstract

Reversible changes in the redox state of cysteine residues represent an important mechanism with which to regulate protein function. In mitochondria, such redox reactions modulate the localization or activity of a group of proteins, most of which function in poorly defined pathways with essential roles in copper delivery to cytochrome *c* oxidase (COX) during holoenzyme biogenesis. To date, a total of 8 soluble (COX17, COX19, COX23, PET191, CMC1–4) and 3 integral membrane (COX11, SCO1, SCO2) accessory proteins with cysteine-containing domains that reside within the mitochondrial intermembrane space (IMS) have been identified in yeast, all of which have human orthologues. Compelling evidence from studies of COX17, SCO1, and SCO2 argues that regulation of the redox state of their cysteines is integral to their metallochaperone function. Redox also appears to be crucial to the regulation of a SCO-dependent, mitochondrial signaling pathway that modulates the rate of copper efflux from the cell. Here, I review our understanding of redox-dependent modulation of copper delivery to COX and IMS-localized copper-zinc superoxide dismutase (SOD1) during the maturation of each enzyme, and discuss how this in turn may serve to functionally couple mitochondrial copper handling pathways with those localized elsewhere in the cell to regulate cellular copper homeostasis. *Antioxid. Redox Signal.* 13, 1403–1416.

Introduction

Mitochondria are cellular organelles that are essential to aerobic ATP production and, as such, energy homeostasis. ATP is generated by the coordinate activity of five multimeric enzyme complexes, most of which rely on iron–sulfur, heme, or copper centers for their catalytic competence. Mitochondria therefore have a vested interest in the cellular metabolism of metal ions. It is perhaps not surprising then that bioactive pools of several metals, including iron and copper, are localized to the mitochondrial matrix (73), and are crucial to the sustained synthesis and delivery of prosthetic groups that are required by these enzymes for their assembly and proper function (21, 52).

As mitochondria contain bioactive pools of copper and iron, mechanisms must exist to acquire, store, and mobilize these elements. Many of the proteins that mediate the uptake of iron into mitochondria and its subsequent assimilation into heme and iron–sulfur clusters have been identified (64, 83); however, those critical to mitochondrial copper metabolism remain unknown. What is clear is that copper export from the matrix to the mitochondrial intermembrane space (IMS) is required for its use in metallating cytochrome *c* oxidase (COX) subunits I and II during the biogenesis of COX, and in maturing the small fraction of the total cellular copper–zinc superoxide dismutase (SOD1) that resides in this compartment (21). Delivery and insertion of copper into each of these enzymes is surprisingly complex. To date, 12 accessory proteins with essential roles in the maturation of COX, IMS-localized SOD1 or both enzymes have been identified in yeast (34, 55). Each factor has a human orthologue, and contains highly conserved cysteines that are organized as Cx_3C or Cx_9C motifs. While it is known that these motifs are used to oxidatively trap soluble accessory proteins within the IMS (60, 76), thereby ensuring their appropriate localization, most of these proteins have yet to be functionally characterized. However, studies of COX17, SCO1, and SCO2 in a number of *in vivo* and *in vitro* systems have provided robust evidence that redox regulation of their cysteine residues is crucial to their metallochaperone functions (9, 12, 13, 22, 35). Recent data also support an important role for redox regulation of the copper-binding cysteines of SCO1 in the transduction of a SCO-dependent, mitochondrial signal that modulates cellular copper homeostasis (47). Here, I review the proteins and pathways responsible for delivering copper to COX and IMS-localized SOD1 during their maturation, with a particular focus on the importance of redox in regulating the ligand-exchange reactions that ultimately mediate copper transfer. I also discuss

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the role for redox in modulating the SCO-dependent, mitochondrial regulation of copper efflux from the cell, and consider how redox may provide a general mechanism to functionally couple copper handling pathways within the organelle to those that reside in other cellular compartments.

COX Assembly

COX, the terminal enzyme of the respiratory chain, is embedded in the inner mitochondrial membrane where it catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen. In mammals, it is composed of 13 structural subunits, with the three mitochondrially-encoded subunits (COX I–III) forming the catalytic core of the enzyme. Highly conserved domains within subunits I and II contain two heme (a, a_3) and two copper (Cu_A, Cu_B) moieties essential to catalytic competence (17, 101). The catalytic core of the enzyme is surrounded by the ten remaining nuclear-encoded subunits, which are thought to play a role in stabilizing the enzyme and modulating its activity (42). Additional, nuclear-encoded accessory factors are required for the assembly of individual structural subunits into a functional holoenzyme complex (29). Their identification has been greatly facilitated by large-scale, genetic screens of respiratory mutants from budding yeast (57, 93), with at least 20 of the more than 30 complementation groups that have been identified as COX-deficient encoding factors dedicated to various aspects of COX assembly. More than half of these proteins in turn have known human homologues.

The assembly of a fully functional holoenzyme is a rather complicated, and as yet poorly understood, process. Studies of a human leukemia cell line have revealed three distinct assembly intermediates that precede the formation of the mature holoenzyme, suggesting that COX assembly likely proceeds in a modular fashion (63). The first (S1) consists of COX I alone, the second (S2) contains COX I-IV-Va, and the third (S3) contains the bulk of the remaining structural subunits. Two of these assembly intermediates, S1 and S2, have been observed in cell lines and tissues from COX-deficient patients by blue native gel electrophoresis, and infer that progression from S1–S3 requires the full metallation of both COX I and COX II. Several factors critical to the metallation of COX I have been identified; COX10, COX15, and SURF1 participate in the synthesis, delivery, or insertion of the heme a and a_3 moieties (15, 85), and COX11 is required for formation of the mononuclear Cu_B site (32). Patients with mutations in *COX10* and *COX15* fail to accumulate the S2 assembly intermediate (3, 4), while its accumulation is observed in *SURF1* patients (98). This argues that COX I needs a portion of its total heme complement to associate with COX IV and COX Va in order to form S2, and its full heme complement in order for assembly to progress to the S3 stage. Progression to the S3 stage of COX assembly is likewise dependent on the metallation of COX II, as accumulation of the S2 assembly intermediate is also observed in patients with mutations in *SCO1* and *SCO2* (48, 89, 98), paralogous genes whose protein products play a critical role in the maturation of its mixed valence, binuclear Cu_A site. In humans, the specific stages of COX assembly during which the prosthetic groups are added to the nascent holoenzyme complex have yet to be directly demonstrated. Recent studies in yeast have identified several high molecular weight complexes consisting of various COX assembly factors whose temporally discrete interactions with newly synthesized COX I are crucial to heme incorporation (43, 72); however, most of these accessory proteins (Mss51, Coa1, Coa2, Cox14) do not appear to have human homologues.

Copper Delivery to COX

Perhaps the most thoroughly characterized aspect of COX assembly is that of mitochondrial copper delivery to the nascent holoenzyme complex, and in particular delivery of copper to the Cu_A site contained within COX II at the S2 stage of holoenzyme assembly. The first accessory factor in this pathway to be identified was yeast COX17, a small molecular weight protein containing highly conserved, twin Cx_9C motifs that is localized both within the cytoplasm and IMS (26). It was subsequently shown that copper-binding is essential to its function (30), and that, in spite of its dual localization, its role in mitochondrial copper delivery to COX is restricted to the IMS (56). Germline deletion of the gene in mice is embryonic lethal, further arguing that the function of COX17 is critical during early mammalian development (91). Several additional, small molecular weight COX assembly factors with twin Cx_9C motifs have since been identified in yeast, all of which have human homologues (Fig. 1). These include COX19, COX23, PET191, and CMC1-4 (14, 33, 55, 66). Very little is currently known about the function of these proteins in either organism.

A high copy suppressor screen of a yeast *COX17* null strain led to the identification of *SCO1* and *SCO2* (27), two closely related genes whose protein products share a high degree of sequence similarity, particularly in their C-terminal region that protrudes into the IMS and contains a conserved CxxxC motif involved in copper-binding (40, 65, 69) (Fig. 2). Further biochemical studies of the yeast proteins demonstrated specific transfer of copper from COX17 to SCO1 (35) and physical interactions between both the SCO proteins and COX II (53, 54). Although this suggested that both SCO proteins act downstream of COX17 in the delivery of copper to COX (27), only the deletion of the yeast *SCO1* gene resulted in a respiratory phenotype. In contrast, both *SCO1* and *SCO2* are essential in humans, with mutations in either gene resulting in a severe, isolated COX deficiency and an early onset, fatal clinical outcome. *SCO2* mutations are associated primarily with neonatal encephalocardiomyopathy; however, clinical symptoms identical to those observed in spinal muscular atrophy (SMA) type I patients have also been reported in some *SCO2* patients (82, 92). *SCO1* patients ultimately die from either neonatal hepatic failure and ketoacidotic coma (94) or a fatal hypertrophic cardiomyopathy (88). These distinct clinical phenotypes are not the result of tissue-specific expression of the two genes, as *SCO1* and *SCO2* are ubiquitously expressed and exhibit a similar expression pattern in different human tissues (69).

All reported *SCO2* patients, save one (62), carry an E140K missense mutation on one allele. In general, patients are either homozygous for this mutation or are compound heterozygotes; however, hemizogosity at the *SCO2* genomic locus has also been reported (49). Patients homozygous for the E140K mutation have a delayed onset of the disease pathology and a more prolonged course of disease as compared to compound heterozygotes (39). To date, *SCO1* mutations have only been

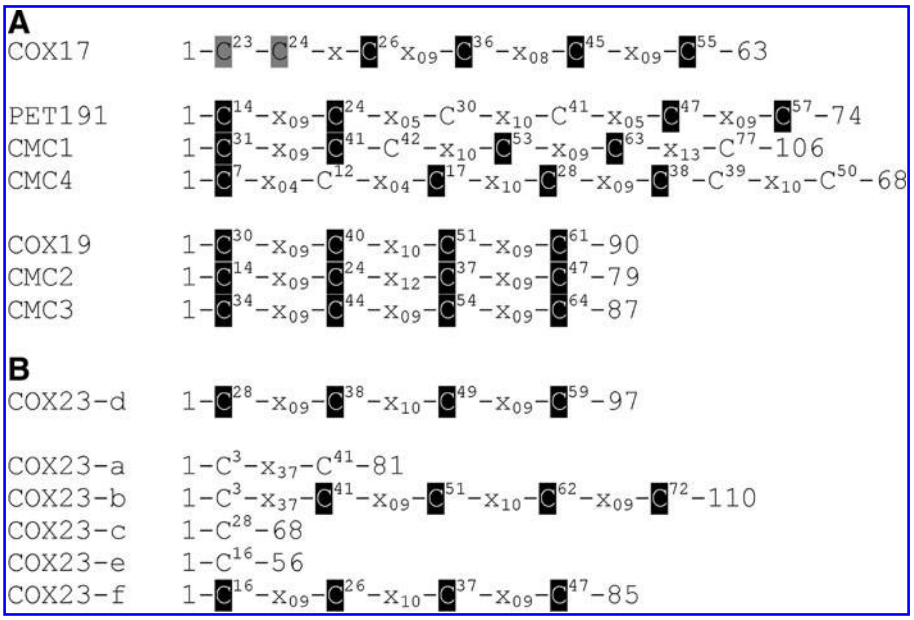

FIG. 1. Schematic representation of the human orthologues of yeast IMS COX assembly factors with twin Cx_9C motifs. With the exception of COX17, where the copper-binding cysteines are known (27) and have been shaded in *gray*, conserved cysteines are shaded in *black*. *Superscripts* indicate the relative position of each cysteine within the primary sequence, while *subscripts* denote the number of residues between two cysteines. (**A**) COX19, CMC2, and CMC3 are physically separated from the remaining IMS proteins to emphasize the fact that their only cysteines are contained within the twin Cx_9C motifs. (**B**) the 6 variants (a–f) of COX23 (NCBI, Entrez Gene) are depicted. Variant d is physically separated from the others because it is the most widely recognized primary sequence of the protein reported in the literature (55).

reported in two pedigrees. Patients were either homozygous for the G132S mutation (88) or compound heterozygotes, with a nonsense mutation on one allele and a P174L missense mutation on the second allele (94). Both the E140K and P174L substitutions are adjacent to the conserved CxxxC motif (Figs. 2 and 3), and are thought to compromise either the copper-binding properties or redox functions of the SCO proteins (50). Interestingly, mapping of the known pathogenic mutations for *SCO2* onto its three dimensional structure reveals that a significant number of them (6/8, 75%) cluster closely around the CxxxC motif and a histidyl ligand (Figure 2), all of which are crucial to Cu(I) binding (36).

The specific function of the cysteines of the CxxxC motif of SCO proteins, and of human SCO1 in particular, has been the subject of some debate in the literature. Several groups have clearly demonstrated that the ability to bind both Cu(I) and Cu(II) is crucial to the function of each human SCO protein (9, 36), as well as that of *Bacillus subtilis* SCO (*Bs*SCO) (2, 38). Accordingly, supplementation of the growth media with copper salts results in either a partial or complete rescue of the observed COX deficiency in *SCO1* and *SCO2* patient cell lines (41, 48, 81). Others, however, have suggested that the CxxxC motif of human SCO1 and *Bs*SCO confers a critical redox function (8, 19, 84, 97), a contention that in some studies is based largely on its *in vitro* sensitivity to hydrogen peroxide (84, 97). Although this may simply be an oxidative effect that is unrelated to the function of the cysteine residues, daily subcutaneous injections of copper histidine into a *SCO2* patient failed to completely reverse disease progression (25), suggesting that copper supplementation alone cannot overcome all of the defects associated with loss of function mutations in these genes. Indeed, functional studies (7) and phylogenetic analyses (5, 10) are consistent with the idea that SCO proteins fulfill multiple functions, and additional roles for SCO1 and SCO2 in the regulation of cellular copper homeostasis in humans have been identified (47, 88) (see below).

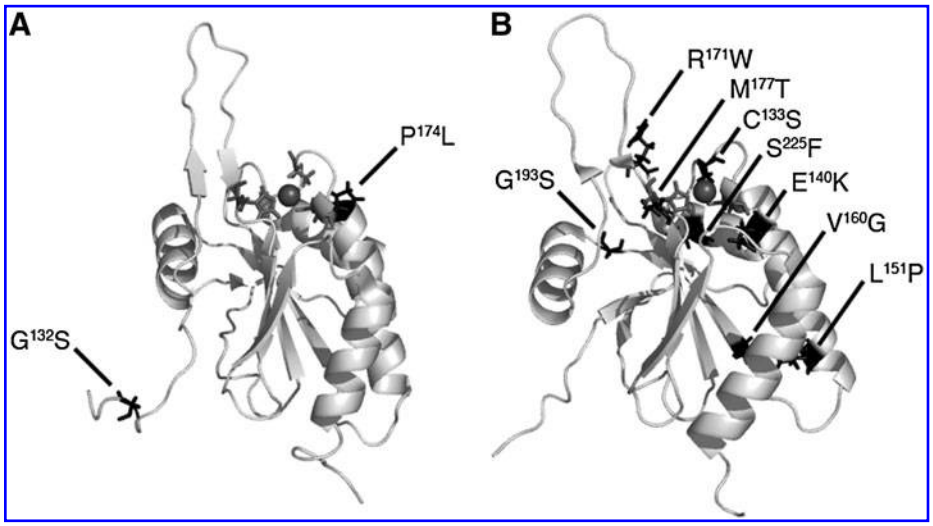

FIG. 2. Three dimensional structures of human SCO1 and SCO2. PyMOL-derived ribbon diagrams of the Cu(I)-bound conformers of human SCO1 [(**A**) (9)] and SCO2 [(**B**) (11)] are shown, with the cysteines and the highly conserved histidine that coordinate Cu(I) all shaded in a *darker gray* relative to the remainder of the protein backbone. Residues for which pathogenic mutations have been identified are highlighted in *black* (SCO1; G132S (88) and P174L (94): SCO2; C133S (92), E140K (69), L151P (80), V160G (46), G193S (62), R171W (40), M177T (74), and S225F (69)).

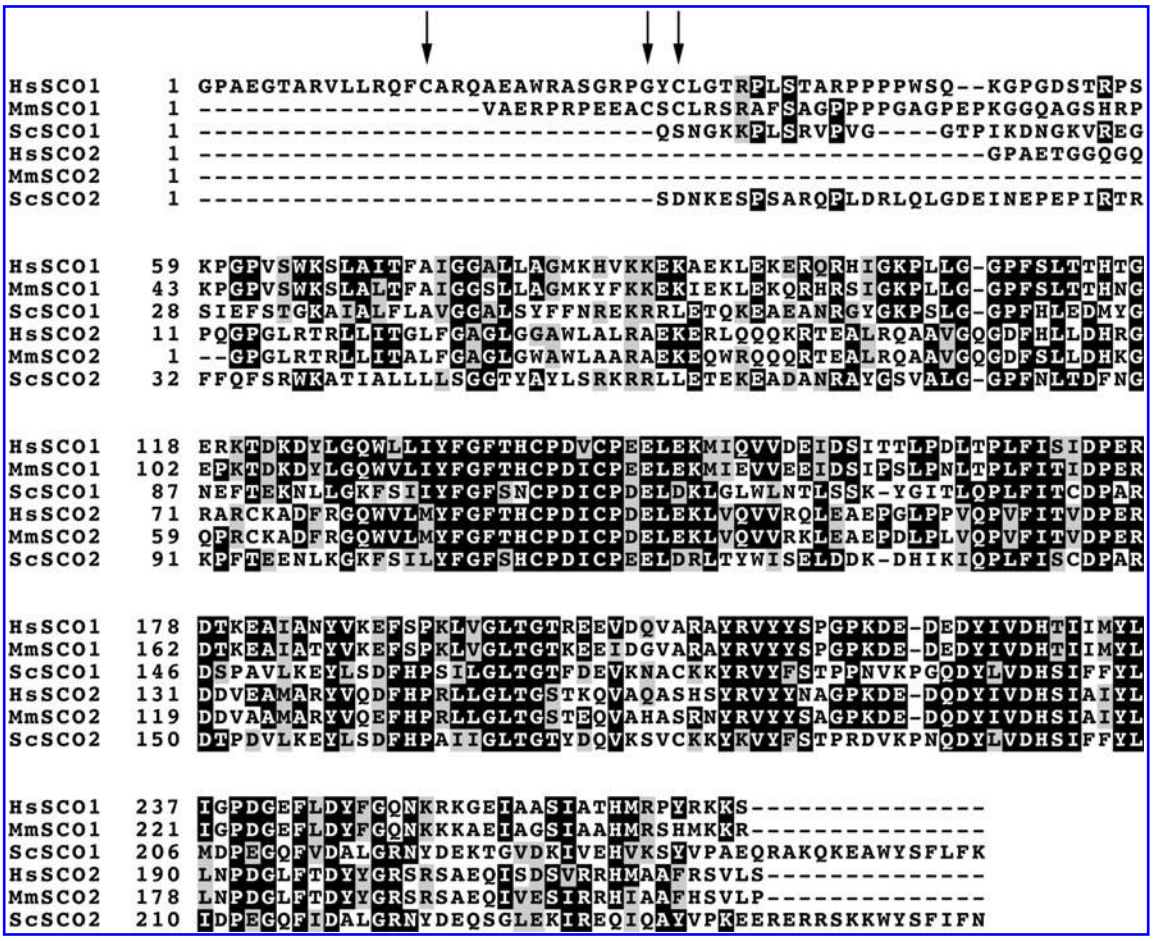

FIG. 3. Sequence alignments of SCO proteins. Alignments of mature SCO1 and SCO2 lacking their N-terminal mitochondrial targeting sequences from human (Hs), mouse (Mm), and budding yeast (Sc) are shown. *Solid lines* that flank the sequences indicate the position of the transmembrane region. *Arrows* highlight the N-terminal, matrix-localized cysteines of human and murine SCO1.

In recent years, considerable progress has been made with respect to identifying the protein–protein interactions crucial to the biogenesis of the Cu_A site of COX II, and characterizing their redox dependency (Figs. 4 and 5). Human SCO1 and SCO2 have been shown to fulfill distinct, stage-specific functions during COX II synthesis and Cu_A site maturation that are absolutely dependent on their CxxxC motifs (48, 50). SCO2 acts upstream of SCO1 in this pathway, and is indispensable for COX II synthesis (48, 50). *In vitro* studies from Bertini and colleagues have established that ligand-exchange reactions between COX17 and SCO2 require that the cysteines of the latter protein be fully reduced for its copper-loading (12). *In vivo* data support this model, and suggest that this metallation step is crucial for either the synthesis of COX II or stabilization of the newly synthesized polypeptide (50). Subsequent maturation of COX II requires both SCO proteins, with SCO2 transferring its copper to COX II and then acting as a thiol–disulfide oxidoreductase to oxidize the cysteines of the CxxxC motif of SCO1, which in turn promotes a second round of copper transfer to COX II and completes the biogenesis of the Cu_A site. Although it has been shown that SCO1 can be metallated by COX17 irrespective of the redox state of its cysteines (9, 12), the disproportionate reduction of the cysteines of SCO1 in *SCO2* patient fibroblasts, and the associated defects at the level of COX II expression (50), suggest that COX17 simultaneously transfers Cu(I) and two electrons to the oxidized cysteines of SCO1 *in vivo*. Thus, another possibility is that the thiol–disulfide oxidoreductase activity of SCO2 may be more important to priming the cysteines of SCO1 for their metallation in subsequent ligand-exchange reactions with COX17. Interestingly, the cysteines in P174L SCO1 are almost entirely oxidized and are relatively resistant to chemical reduction (50), arguing that the accessibility of the CxxxC motif *in vivo* is sterically hindered by the missense mutation. This observation may explain why ligand-exchange reactions between mutant SCO1 and COX17 are severely compromised (13, 22).

It is important to stress that the redox-dependent interactions proposed above represent a working model of Cu_A site biogenesis. In fact, much remains to be learned about the molecular events that govern copper transfer to COX II during its maturation, and about the relative contributions of SCO1 and SCO2 during this process. Dominant-negative phenotypes obtained upon overexpression of SCO variants incapable of binding copper in each *SCO* patient background suggest that their physical interactions with COX II do not require that SCO proteins are copper-loaded (36, 50). This contention is supported by the observation that a SCO1 var-

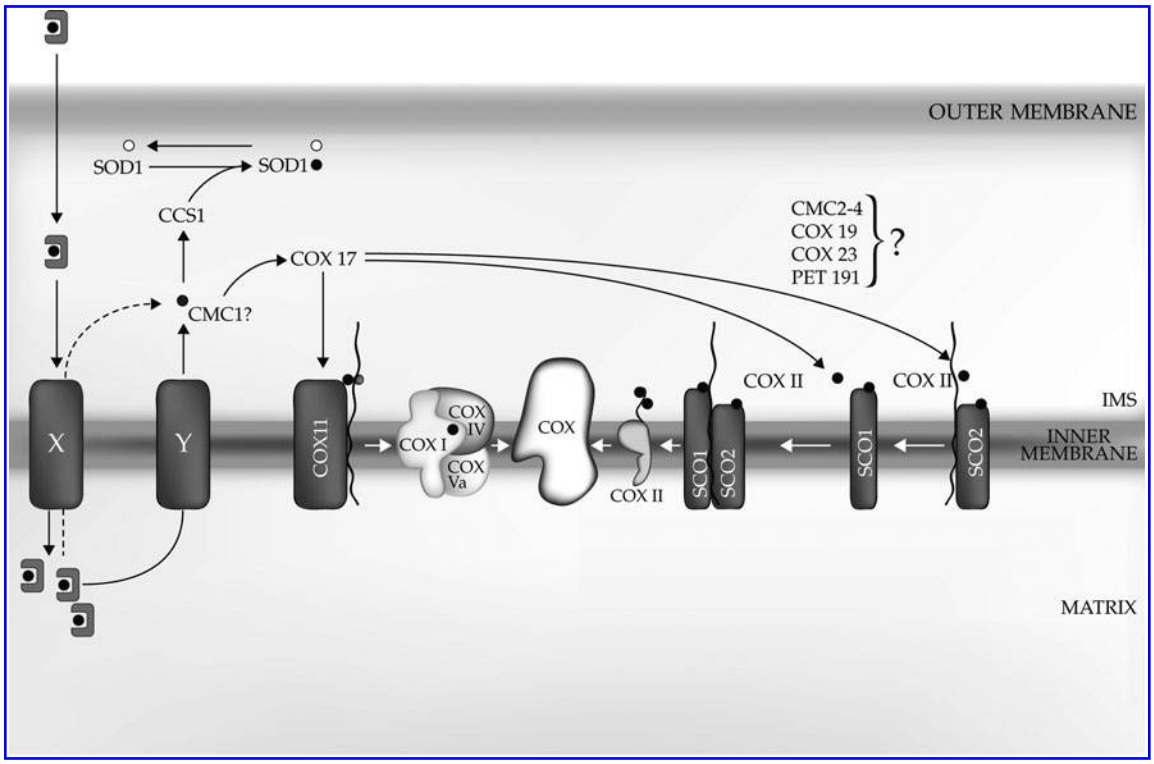

FIG. 4. Schematic illustration of mitochondrial copper trafficking pathways. Upon binding copper (*black ball*), a small, nonproteinaceous ligand trafficks to mitochondria, and passively diffuses across the outer membrane into the IMS. Its subsequent interaction with an inner membrane transporter (X) results in translocation to the matrix for its storage in a labile copper pool. Subsequent mobilization of the copper-bound ligand and its export to the IMS is mediated by a bi-directional (X) or unidirectional (Y) inner membrane transporter. Copper is then chaperoned by CMC1 or other, as yet unidentified metallochaperones to COX17, which uses distinct interfaces to transfer copper to COX11 for maturation of the Cu_B site of COX I, or to SCO1 and SCO2 for maturation of the Cu_A site of COX II. Metallation of COX I and COX II allows for progression through the early stages of COX assembly and maturation of the fully assembled holoenzyme (COX). Alternatively, upon its matrix export, copper is delivered to CCS1 for the full maturation of SOD1, which exists as a mixed population in the IMS, with a significant fraction of the total pool being zinc (*white ball*) but not copper-loaded. *N.B.* While SCO1 and SCO2 are present as homodimers in human cells (48), they are presented here in their monomeric state for ease of depiction.

iant incapable of binding copper complements the peroxide sensitivity of a yeast *SCO1* null strain, by physically capping a COX assembly intermediate that is otherwise capable of potentiating free radical production (44). The dissociation between the abilities to bind copper and physically interact with COX II is also supported by additional studies of yeast SCO1, in which variants with mutations of highly conserved residues within loop 8 had wild-type copper-binding properties but nonetheless failed to restore growth of a *SCO1* null strain on a nonfermentable carbon source (78). Although these data argue strongly that SCO1 uses distinct interfaces to physically interact with COX17 and COX II, more refined domain mapping is required to understand the specific nature of these physical interactions and appreciate how they affect ligand-exchange reactions between interacting partners. Sequential delivery of copper to COX II during Cu_A site biogenesis was recently described for a bacterial COX II *in vitro* (1), and has been proposed in humans (48, 50); however, whether two copper atoms are simultaneously donated to COX II *in vivo* to form the Cu_A site remains an open question. Our understanding of these, and other, events awaits the development of *in vitro* systems in which recoded COX II can be expressed and studied, and the functional characterization of additional COX assembly factors with proposed roles in this pathway like COX20 (31).

Copper Delivery and COX Assembly Factors: Functional Divergence Between Orthologues?

The analysis of the >30 different genetic complementation groups in budding yeast with an isolated COX deficiency has been invaluable to the identification of COX assembly factors (57, 93). More than half of these factors have human orthologues, and many have equivalent functions in both species (28). There are, however, clear examples of differences between the two species with respect to the role of a given COX assembly factor (95), many of which are relevant to the pathways that deliver copper to COX.

The most obvious example is that only a single SCO protein, SCO1, is necessary for Cu_A site formation in yeast (23, 27), while both SCO1 and SCO2 are required in humans (40, 69, 94). In humans, this requirement reflects the fact that each SCO protein fulfills distinct, stage-specific functions during COX II synthesis and maturation of the Cu_A site (48, 50). Whether yeast SCO1 fulfills all of these functions or, like its homologue in *Thermus thermophilus* (1), collaborates with

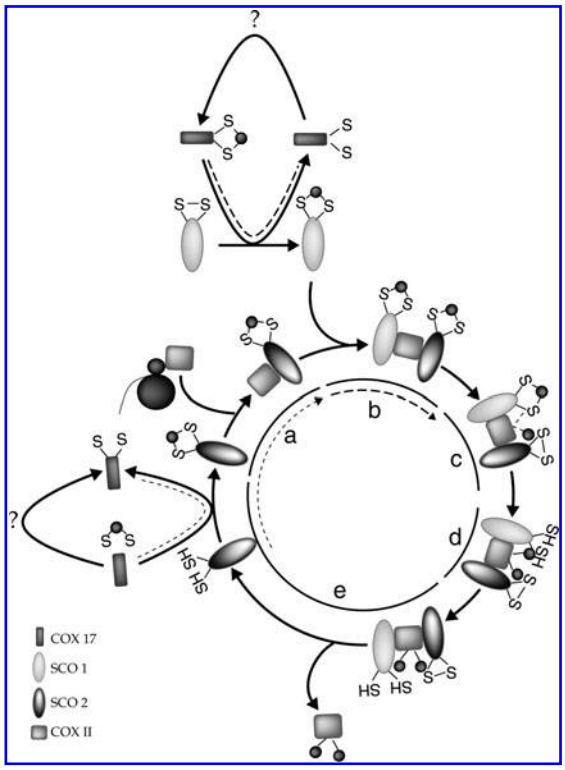

FIG. 5. Schematic illustration of SCO-dependent regulation of COX II synthesis and subsequent maturation of its Cu_A site during holoenzyme assembly. A working model is presented of the relative roles of human SCO1 and SCO2 in regulating the synthesis, maturation, and insertion of COX II into the nascent holoenzyme complex (50). First, SCO2 interacts with newly synthesized COX II, either as it is inserted into the inner membrane or immediately thereafter, an association that depends on its prior metallation by COX17 (*step a*, *thin dashed lines*). Second, the physical interaction between SCO2 and COX II triggers the metallation of SCO1 by COX17 and its recruitment to the SCO2-COX II complex (*step b*, *medium dashed lines*). Third, each SCO protein sequentially delivers copper to COX II to form the Cu_A site (*steps c & d*), an event that results in the dissociation of the ternary complex and incorporation of the mature polypeptide into the nascent holoenzyme complex (*step e*). Although further mechanistic investigations are required, we favor a scenario in which SCO2 donates its copper initially, with its cysteine thiols becoming oxidized in the process. After transfer of copper from SCO1 to COX II, SCO2 acts as a thiol–disulfide oxidoreductase to reoxidize the cysteines in SCO1. Alternatively, SCO2 may induce disulfide bond formation in copper-loaded SCO1 to facilitate copper transfer from SCO1 to COX II. Either mechanism, however, would effectively prime the cysteines of both proteins for their subsequent metallation by COX17 and another round of copper delivery to COX II. *N.B.* While SCO1 and SCO2 are present as homodimers in human cells (48), they are presented here in their monomeric state for ease of depiction.

another, as yet unidentified factor has not been established. It is important to note, however, that a subtle role for yeast SCO2 in optimizing SCO1 function during COX assembly cannot be ruled out, because the observed, wild-type growth of a *SCO2* null strain on a nonfermentable carbon source (27) does not preclude the existence of modest reductions in COX activity. Accordingly, the reduced steady-state levels of Cox1p and Cox2p observed in a *SCO1* null background are further attenuated upon the subsequent deletion of *SCO2* (54).

It is equally plausible, however, that yeast SCO2 evolved a function that is unrelated to COX assembly *per se*. Indeed, evolutionary divergence in SCO protein function is emphasized by the observation that many organisms lacking an aa_3-type oxidase with a Cu_A site nonetheless express one or more SCO proteins (5, 10). Sequence similarity to peroxiredoxins and thiol–disulfide oxidoreductases that contain a conserved thioredoxin fold led to the proposal that an oxidoreductase activity may be an important aspect of SCO protein function (19). This hypothesis has since been substantiated for several SCO proteins, including PrrC in *Rhodobacter sphaeroides* (7), SCO1 in *T. thermophilus* (1), and SCO2 in humans (50). While *T. thermophilus* SCO1 and human SCO2 both function as thiol–disulfide oxidoreductases during Cu_A site formation in COX II, recent data also support an additional and important role for this activity in SCO2 in the regulation of a SCO1-dependent, mitochondrial signaling pathway that affects the rate of copper efflux from human cells (47). The thiol–disulfide oxidoreductase activity of PrrC is similarly crucial to modulating the activity of PrrAB, a two-component signal transduction system that induces the expression of photosynthetic genes in response to a decrease in oxygen tension (24). Thus, in some species, SCO proteins may have evolved to function strictly as thiol–disulfide oxidoreductases that regulate the activity of signaling pathways containing proteins with redox-active cysteines.

Early evidence also suggests that the soluble IMS COX assembly factors represent a second example of functional divergence between yeast proteins and their human orthologues. Yeast SCO1 and human SCO proteins are all copper-loaded by COX17 (9, 12, 22, 35), yet 6 other soluble IMS proteins with highly conserved, twin Cx_9C motifs that are also crucial to COX assembly have been identified in yeast (34, 55). All have human orthologues, and CMC1 has been proposed to be functionally equivalent to its yeast counterpart based on sequence similarity and mitochondrial localization (33). With the exception of COX17, however, very little is known about the function of these factors in either species, and it remains unclear why such a large number of like proteins are presumably required for IMS copper delivery to COX during holoenzyme assembly. We therefore decided to functionally characterize a subset of the human orthologues (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). While cell fractionation experiments established that all of these proteins localize to mitochondria, only knockdown of PET191 produced an isolated COX deficiency in human fibroblasts. Significant reductions in the steady-state levels of both COX19 and COX23 had very modest effects on COX activity, arguing that each factor fulfills either a subtle or redundant role in holoenzyme assembly. Thus, we propose that among the twin Cx_9C motif-containing IMS factors, at least human COX19 and COX23 have additional functions within the cell that are unrelated to COX assembly *per se*. An attractive possibility currently under investigation is that some of these proteins have evolved redox functions that are crucial to the mitochondrial regulation of cellular copper homeostasis (see below).

The "ABCs" of Mitochondrial Copper Dynamics: Regulated Uptake, Storage, and Efflux

Genetic and biochemical studies identified the existence of a bioactive copper pool within the mitochondrial matrix that is used at least in part to metallate both COX and SOD1 (20, 21). Copper is delivered to the organelle by a small, non-proteinaceous ligand that likely translocates from the cytosol to the matrix upon metal ion binding (51) (Fig. 4). It is thought that the copper-loaded, anionic ligand diffuses across the outer membrane into the IMS through either porin or the TOM complex; however, the highly impermeable nature of the inner membrane necessitates its protein-mediated transport to and from the matrix. At present, it is not clear how copper is moved across the inner membrane. Its translocation could be achieved by either a single, bidirectional transporter or two, uni-directional transporters (51) (Fig. 4). Several forward genetic approaches used by our group and that of others have all failed to identify candidate transporters, suggesting that functional redundancy may be a key feature of this transport system. Future screening efforts will benefit from a more directed approach afforded by the successful isolation and identification of the ligand.

Although the identity of the ligand remains unknown, preferential accumulation of the apo- conformer in the cytosol and the anionic, copper-bound conformer in mitochondria in both yeast (21) and human cells (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data) suggests that it functions to maintain a labile store of copper within the organelle. The dual localization of Cox17 to the cytosol and the IMS originally made it an attractive candidate to act as a copper shuttle to mitochondria (27). However, it has since been established that all of the twin Cx_9C motif-containing IMS COX assembly factors, with the exception of Pet191, are imported into the IMS as either apo-proteins or zinc-bound conformers by the Mia40-Erv1 pathway (59, 60). The ability of a Cox17 variant that is tethered to the inner membrane to complement the COX deficiency in a *COX17* null strain demonstrates that its function is restricted to the IMS (56), and further supports the idea that copper-loading occurs after the cysteines of its twin Cx_9C motifs have been oxidatively trapped by the import machinery. Deletion of *COX17, CCS1*, *COX19,* or *COX23* does not significantly perturb mitochondrial copper levels in yeast (20), and provides perhaps the strongest evidence against a widespread role for soluble IMS COX assembly factors in delivering copper to the organelle.

Unlike copper uptake, a role for COX assembly factors in regulating copper export from the mitochondrial matrix has been demonstrated in yeast. Deletion of *COA1* or *SHY1*, two integral inner membrane accessory proteins with roles in COX I maturation, reduces the size of the mitochondrial copper pool, a phenotype that can be partially complemented by co-culturing either deletion strain in media supplemented with exogenous copper salts (72). These data argue that complexes containing each protein physically interact with an inner membrane copper transporter(s) to regulate its activity, a mechanism that ultimately may serve to coordinate the functions of multiple pathways that are all crucial at this stage of holoenzyme biogenesis (51). While similar mechanisms must exist in mammals, an orthologue for Coa1 has yet to be identified, and patients with mutations in *SURF1*, the human orthologue of Shy1, have wild-type levels of anionic, copper-bound ligand (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). However, equivalent roles to those of yeast Coa1 and Shy1 may be fulfilled in mammals by SCO proteins. Reduced mitochondrial copper levels were reported recently in *SCO2* transgenic mice harboring either a knockout allele paired with an *E129K* missense allele, which is equivalent to the common *E140K* substitution in humans, or two *E129K* missense alleles (99). Although the mitochondrial copper pool is unaffected by mutations in human *SCO1* or *SCO2*, copper phenotypes generated in *SCO1* patient fibroblasts upon co-expression of a matrix-targeted version of SOD1 and various forms of SCO1 suggest that its N-terminus functions as a crucial interface between mitochondrial and cellular copper handling pathways (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). Interestingly, the matrix localized, N-terminal tail of human SCO1 is considerably longer than that of human SCO2 or SCO proteins of yeast and mice, and contains two cysteine residues whose spatial separation is much greater than those found in murine SCO1 (Fig. 3). One potential model for copper translocation from the matrix to the IMS in mammals therefore is that SCO2 modulates conformational changes in the N-terminus of SCO1, thereby providing a gating mechanism that regulates the ability of copper to access the channel of an inner membrane transporter.

Given the potential for free copper to generate reactive oxygen species (ROS) via Fenton chemistry, copper translocation from the matrix to the IMS is likely coordinated with its direct transfer to at least one specific metallochaperone (Fig. 4). Although it is well established that COX17 donates copper to SCO proteins and COX11 for the biogenesis of the Cu_A and Cu_B sites (9, 12, 22, 35), respectively, and CCS1 transfers copper to SOD1 during its maturation (90), how these chaperones are themselves metallated within the IMS remains an open question. Deletion or overexpression of Cmc1 in yeast shifts the relative proportion of catalytically active COX and IMS-localized SOD1 (33), suggesting that its abundance is an important, upstream determinant of copper prioritization to each of these pathways. A direct relationship between the pathways that route copper to COX and IMS-localized SOD1 is further supported by the isolated COX deficiency observed in spinal cords of mice that overexpress CCS1 and G93A SOD1 (86), an established model of amyotrophic lateral sclerosis (ALS) (87). It has been proposed that copper routing through discrete IMS copper trafficking pathways may be explained by a "daisy chain" model, in which the directionality of copper transfer reactions is driven by step-wise differences in the redox potential of individual metallochaperones (34). However, the large number of soluble, twin Cx_9C-motif containing proteins localized to the IMS with roles in COX assembly and SOD1 maturation emphasizes the multiple, potential fates for copper upon its export from the matrix, and suggests that this model is too simplistic to account for the regulatory inputs that are likely required to control copper dynamics within the IMS.

The selective advantage of maintaining a labile pool of mitochondrial copper for the IMS metallation of COX and SOD1 is obvious, given the respective importance of each enzyme in aerobic ATP production and free radical detoxification. Its size, however, is in vast molar excess of that required for these reactions, and can be readily expanded and contracted upon manipulation of cellular copper levels in

both yeast (21) and human cells (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). In fact, synchotron x-ray fluorescence microscopy (SXRF) has demonstrated that even under basal conditions, the two most significant *labile* stores of copper in the cell are contained within mitochondria and the Golgi apparatus (100). These observations suggest that the absolute size of the mitochondrial copper pool may function as a rheostat to monitor, and perhaps even regulate, cellular copper status (51). How this would be achieved mechanistically remains an open question. Although the apo- and copper-bound conformers of the ligand are predominantly cytosolic and mitochondrial, respectively, minor amounts of each species are clearly present in both compartments (21). One possibility therefore is that regulated trafficking of different conformers of the ligand may report on the activity of discrete copper trafficking pathways.

Mitochondrial Regulation of Cellular Copper Homeostasis

Because copper is stored in the matrix and the inner membrane is impermeable, integral membrane proteins with soluble domains that localize to both the matrix and the IMS are required for dynamic communication between organellar and cellular copper handling pathways. While not an obligate requirement, the ability of such proteins to bind copper further strengthens their candidacy to function in such pathways. At present, only three human proteins have been identified that fit these criteria; COX11, SCO1, and SCO2. Stable knockdown of COX11 in human fibroblasts does not affect cellular copper homeostasis (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data); however, a severe cellular copper deficiency is observed in affected tissues and cell types of *SCO1* and *SCO2* patients (47, 88). In *SCO2* patients, a genotype–phenotype relationship is also evident, with the cellular copper deficiency being more severe in patients expressing a single missense allele as compared to those expressing two missense alleles (47). Overexpression of SCO2 functionally complements the copper deficiency phenotype in *COX15* and *SCO2* patient fibroblasts, and partially restores cellular copper levels in *SCO1* patient fibroblasts. Further molecular genetic analyses established that the copper deficiency in *SCO* patient fibroblasts was caused by the inappropriate stimulation of copper efflux from the cell rather than a defect in its high affinity uptake (47). Intriguingly, the COX and copper deficiency phenotypes were dissociable in all patient backgrounds investigated, arguing that SCO1 and SCO2 are bifunctional molecules that fulfill important regulatory roles in each of these distinct processes. These results collectively led us to propose a model in which SCO2 modifies an aspect of SCO1 function that is crucial to the generation and transduction of a mitochondrial signal that regulates copper efflux from the cell (47) (Fig. 6).

At the time, we postulated that the molecular basis for this mitochondrial signal might be generated by SCO2-dependent modulation of the redox state of the cysteines within the CxxxC motif of SCO1. We subsequently demonstrated that SCO2 acts as a thiol–disulfide oxidoreductase to oxidize the copper-binding cysteines of SCO1 during maturation of the Cu_A site of COX II (50). Importantly, significant perturbations were also detected in the redox state of the cysteines of SCO1 in both *SCO1* and *SCO2* patient backgrounds (50), and these correlate well with the severity of the observed cellular copper deficiency (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). Although the cysteines of SCO1 P174L are almost entirely oxidized, we favor a scenario in which the missense mutation alters the local conformation of the protein such that it mimics that of wild-type SCO1 in the *SCO2* patient background, and similarly signals a state of cellular copper overload (47, 50) (Fig. 6). Two additional pieces of evidence are consistent with active signaling through the cysteine thiols of SCO1 (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). First, disproportionate reduction of the cysteines of SCO1 is observed in fibroblasts derived from patients with mutations in *ATP7A*, a genetic lesion known to produce several fold increases in cellular copper levels in this cell type (18). Second, pharmacological depletion of cellular copper levels in control cells, which mimics the copper deficiency observed in *SCO* patient backgrounds, results in a significant enrichment in the relative proportion of oxidized to reduced cysteines of SCO1, a response that is significantly attenuated in *SCO2* patient cells. It is important to note, however, that differences in the redox state of the cysteines of SCO1 do not fully explain the observed variation in cellular copper content (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). Therefore, it will also be important to consider the potential for regulating signaling through SCO1 via sulfenylation, glutathionylation, or S-nitrosylation of its cysteines, as there is a growing appreciation of the involvement of these cysteine modifications in the physiological regulation of protein function (45, 68, 77).

The disproportionate reduction of the copper-binding cysteines of SCO1 may affect its ability to bind copper, and thereby alter the activity of a SCO-dependent, mitochondrial signaling pathway (Fig. 6). Although it has been demonstrated *in vitro* that COX17 can metallate the cysteines of the CxxxC motif of SCO1 independent of their redox state (9, 12), it is not known whether one of these ligand-exchange reactions is more kinetically favorable *in vivo*. The relative proportion of the total SCO1 pool within the inner membrane that is metallated under basal conditions also has yet to be established experimentally. Thus, metabolic ^{64}Cu-labeling studies that quantify the percentage of total cysteines of SCO1 that are copper-loaded in control and *SCO* patient cells are crucial to determining if the overrepresentation of thiols potentiates the metallation of SCO1, and contributes to the generation of a SCO-dependent, mitochondrial signal that regulates copper efflux from the cell.

Even in the absence of a change in their metallation state, the relative enrichment of thiols may affect SCO-dependent, mitochondrial signaling by perturbing the ability of SCO1 to function as a thiol–disulfide oxidoreductase. Although such an activity has yet to be detected, this may reflect the fact that *in vitro* assays have used soluble truncates incapable of forming oligomers (97), even though it is well known that disulfide isomerase activity is enhanced by protein dimerization (102), and that SCO proteins exist as homodimers within the inner mitochondrial membrane (48). Interestingly, evolutionary studies of bacterial thioredoxin have established that the amino acids internal to the highly conserved CxxC motif are critical determinants of the redox properties and thiol–disulfide oxidoreductase activity of the protein (75). The sequence of the CxxxC motif of human SCO1 is unique

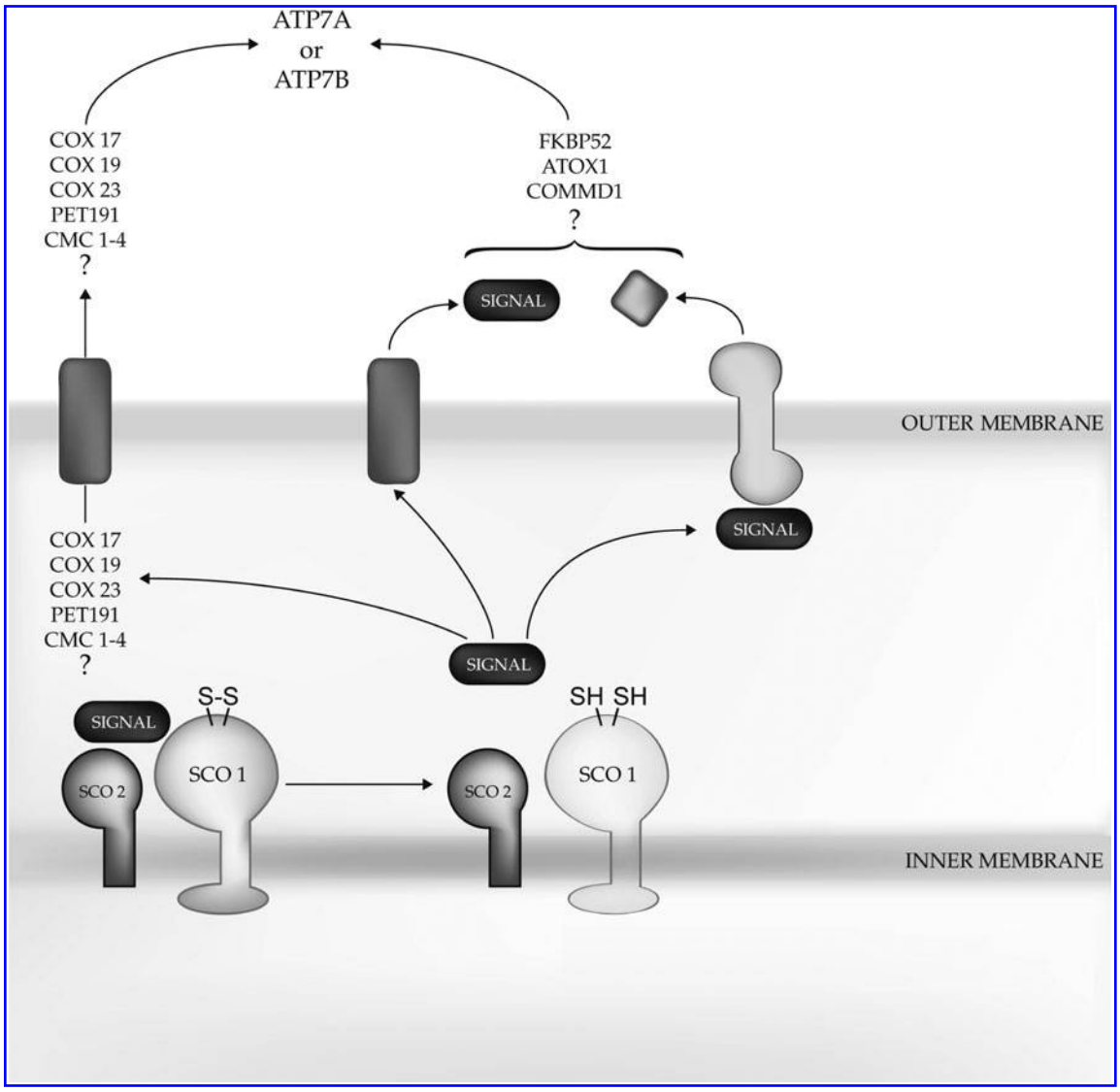

FIG. 6. Schematic illustration of potential mechanisms of SCO-dependent, mitochondrial regulation of cellular copper efflux. The redox state of the cysteines of SCO1 and SCO2 exists in a dynamic equilibrium between oxidized (S-S) and reduced (-SH) forms (47) that is sensitive to cellular and/or mitochondrial copper status (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). A shift towards a state in which the cysteines of SCO1 are disproportionately reduced, like that observed in a *SCO2* patient background or mimicked by the P174L missense mutation in the *SCO1* patient background, initiates a signaling cascade by abrogating an interaction with a molecule (*e.g.*, copper) or a sensing protein (in both cases, denoted as "signal"). Release of either factor then transduces the signal, by translocating from the IMS to the cytosol or interacting with an outer membrane protein complex to which relevant effectors are docked or recruited (depicted here as a *diamond*). The trafficking of one of several potential cytosolic effectors, including FKBP52, ATOX1, COMMD1, or an unknown factor(s), may in turn be sensitive to such a signal, and through interactions with ATP7A or ATP7B, alter the rates of copper efflux from the cell. Alternatively, one of the soluble, IMS COX assembly factors may relocalize from the IMS to the cytosol in a SCO-dependent manner and interact with either ATP7A or ATP7B to similarly regulate its activity. *N.B.* While SCO1 and SCO2 are present as homodimers in human cells (48), they are presented here in their monomeric state for ease of depiction.

amongst the yeast, mouse, and human SCO proteins in that the third amino acid is a valine as opposed to an isoleucine (Fig. 3); however, its CPDVC motif is identical to that of *T. thermophilus* SCO1 and *R. sphaeroides* PrrC, two SCO proteins known to possess thiol–disulfide oxidoreductase activity. Whether SCO1 acts as a *bona fide* thiol–disulfide oxidoreductase and whether this activity is perturbed by the redox state of its cysteines awaits the identification and characterization of candidate substrates.

Irrespective of the molecular mechanism(s) by which SCO-dependent, mitochondrial signals are generated, the regulation of an effector that ultimately catalyzes copper efflux from the cell requires soluble factors to transduce the organellar signal to extra-mitochondrial compartments (Fig. 6). This could be achieved by soluble factors that interact with the IMS side of an integral, outer membrane protein, which in turn would either recruit or release a downstream, cytosolic protein critical to signal transduction (51). Alternatively, soluble

factors themselves could be released from the organelle and traffic to extra-mitochondrial compartments to interact directly with an effector (51). In either case, such a role could easily be fulfilled by one or more of the soluble IMS COX assembly factors with twin Cx_9C motifs, with changes in the redox or metallation state of their cysteines and subcellular localization representing potential mechanisms that may contribute to signaling. With the exception of Cox17, however, very little is known about the *in vivo* redox state of the cysteines of these proteins. Equally unclear is whether their metallation state is important to protein stability or function. Calculations based on the relatively oxidizing nature of the IMS and the redox potential of Cox17 predict that the cysteines of its Cx_9C motifs exist entirely as disulfides *in vivo* (37), which we indeed observe in cultured human fibroblasts (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). Although disulfide bonds between the cysteines of the two twin Cx_9C motifs are crucial to retention of these factors within the IMS (59, 60), the maintenance of oxidized cysteines poses a potential challenge with respect to metallation of those proteins whose only cysteine residues are contained with their twin Cx_9C motifs (Fig. 1). However, the observation that copper is bound by a fraction of yeast Cox19 purified from the IMS argues that mechanisms exist to transiently reduce the cysteines of these motifs for copper-loading (79). It is therefore conceivable that transient reduction of these cysteines without metallation, or retention of reduced thiols following ligand-exchange reactions, may facilitate protein relocalization from the mitochondria to other subcellular compartments in a SCO-dependent manner (Fig. 6).

SCO-dependent, mitochondrial signaling through soluble IMS COX assembly factors may also be achieved by changes in their steady-state levels or differential expression of variants derived from alternative splicing or translation initiation at multiple sites. In fact, we now have considerable evidence that mutations in *SCO1* and *SCO2* significantly perturb the whole cell abundance of COX17, COX19, COX23, and PET191, with the overall expression profile that we observe in patient fibroblasts essentially being recapitulated in control cells after pharmacological depletion of cellular copper levels or knockdown of COX19 (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). Whether the observed changes in their steady-state levels are caused by altered rates of protein synthesis or degradation is currently being investigated. Altered signaling through SCO1 may also regulate the expression of splice variants that differentially modulate the activity of an effector that catalyzes copper efflux from the cell. Distinct variants have been detected for Cmc3 in yeast (55) and COX19 in *Arabidopsis thaliana* (6), with the expression of those for COX19 increasing upon copper supplementation and treatment with ROS-inducing agents (6). In humans, COX23 is predicted to have as many as six distinct variants, three of which completely lack the canonical Cx_9C motifs (Fig. 1). We have also detected two splice variants for CMC1, one of which lacks a twin Cx_9C motif (Leary, Nishimura, Cobine, Winge, and Shoubridge, unpublished data). While one would predict that such variants fail to localize to mitochondria, it has yet to be determined if they are expressed at the protein level.

Independent of the molecular mechanisms that transduce a SCO-dependent signal from the organelle to an extra-mitochondrial effector, ATP7A is the most obvious candidate to catalyze copper efflux from the cell in fibroblasts (Fig. 6). Although we failed to detect gross changes in the subcellular localization of ATP7A in *SCO* patient fibroblasts in our initial study (47), previous ^{64}Cu pulse-labeling studies demonstrated that redistribution of only a minor fraction of the total cellular pool of ATP7A is sufficient to effect rapid changes in efflux kinetics (70). Whether the severe copper deficiency in *SCO1* patient liver is also caused by the inappropriate regulation of ATP7B, the functional homologue of ATP7A and the predominant P-type ATPase expressed in this tissue (51), remains unknown.

Redox Redux: Teasing Apart the Tissue-Specificity of Disease in *SCO* Patients

One of the biggest challenges in the field of mitochondrial genetics is to identify mechanisms that can explain the tissue-specific etiology of diseases that result from mutations in ubiquitously expressed, housekeeping genes. The discovery of a SCO-dependent, mitochondrial signaling pathway that regulates cellular copper homeostasis is therefore significant, because it likely accounts for some of the tissue-specific clinical phenotypes observed in *SCO1* and *SCO2* patients, with those tissues that are most severely affected being both COX and copper deficient (47). While it is clear from molecular genetic manipulation of cultured fibroblasts that these two phenotypes are dissociable (47), a satisfactory mechanistic explanation that accounts for this observation is currently lacking. One possibility is that the molecular architecture of the SCO-dependent, mitochondrial signaling pathway we are currently mapping in human fibroblasts differs across cell types (Fig. 6). Such variability could be built into the system at several levels; for example, the immunophilin FKBP52 or the soluble copper metallochaperones ATOX1 and COMMD1 may have tissue-specific roles in a SCO-dependent, mitochondrial signaling pathway (51). Similarly, in some cell types, the activity of effectors such as ATP7A and perhaps ATP7B may be less responsive to SCO-dependent, mitochondrial signaling. Another possibility, however, is that the molecular organization of the archetypal, SCO-dependent copper efflux pathway is essentially conserved across all cell types, and that differences between tissues in local oxygen tension and the redox state of the IMS provide variable protection with respect to its functional integrity in a mutant *SCO* genetic background. An implicit assumption of each of these possibilities is that the relative contributions of mitochondria to the regulation of cellular copper homeostasis may not be equivalent in all tissues. Indeed, experimental findings in several model systems emphasize the fact that the cell contains the requisite machinery to monitor its total copper content and differentially regulate the activity of individual copper trafficking pathways (58, 67, 96); however, the molecular mechanisms and signaling pathways that coordinate these adaptive responses at the cellular level have yet to be identified. In this respect, well controlled, high throughput functional genomic analyses will be crucial to advancing our understanding of the dynamic regulation of cellular copper homeostasis.

Conclusion

It is now abundantly clear that redox-dependent IMS pathways regulate the localization and metallochaperone

function of a number of accessory proteins crucial to COX assembly and maturation of IMS-localized SOD1; however, our understanding of the relative importance of redox biology to copper handling at the organellar level is still very limited. Equally uncertain is whether redox reactions provide a mechanistic framework that allows pathways that handle copper within mitochondria to communicate with those localized elsewhere in the cell. Such mechanisms must exist, as copper can be prioritized to discrete trafficking pathways at the cellular, tissue, and whole organism levels when its concentration becomes limiting (58, 67). In this sense, future mechanistic investigations will benefit greatly from SXRF (100) and the availability of various fluorescent probes capable of visualizing and quantifying dynamic, *in vivo* changes in cysteine modifications (71), redox potential (16), and copper content (61), both within the organelle and elsewhere in the cell.

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Abbreviations Used

ALS = amyotrophic lateral sclerosis
ATP = adenosine triphosphate
*Bs*SCO = *Bacillus subtilis* SCO
COX = cytochrome *c* oxidase
IMS = mitochondrial intermembrane space
ROS = reactive oxygen species
SMA = spinal muscular atrophy
SOD1 = copper-zinc superoxide dismutase
SXRF = synchotron X-ray fluorescence

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