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Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome *c* oxidase

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Human *SCO1* and *SCO2* are paralogous genes that code for metallochaperone proteins with essential, but poorly understood, roles in copper delivery to cytochrome *c* oxidase (COX). Mutations in these genes produce tissue-specific COX deficiencies associated with distinct clinical phenotypes, although both are ubiquitously expressed. To investigate the molecular function of the SCO proteins, we characterized the mitochondrial copper delivery pathway in *SCO1* and *SCO2* patient backgrounds. Immunoblot analysis of patient cell lines showed reduced levels of the mutant proteins, resulting in a defect in COX assembly, and the appearance of a common assembly intermediate. Overexpression of the metallochaperone COX17 rescued the COX deficiency in *SCO2* patient cells but not in *SCO1* patient cells. Overexpression of either wild-type SCO protein in the reciprocal patient background resulted in a dominant-negative phenotype, suggesting a physical interaction between SCO1 and SCO2. Chimeric proteins, constructed from the C-terminal copper-binding and N-terminal matrix domains of the two SCO proteins failed to complement the COX deficiency in either patient background, but mapped the dominant-negative phenotype in the *SCO2* background to the N-terminal domain of SCO1, the most divergent part of the two SCO proteins. Our results demonstrate that the human SCO proteins have non-overlapping, cooperative functions in mitochondrial copper delivery. Size exclusion chromatography suggests that both the proteins function as homodimers. We propose a model in which COX17 delivers copper to SCO2, which in turn transfers it directly to the Cu_A site at an early stage of COX assembly in a reaction that is facilitated by SCO1.

INTRODUCTION

Mammalian cytochrome *c* oxidase (COX) is a multimeric protein complex composed of 13 structural subunits encoded by both the nuclear and the mitochondrial genomes. Embedded in the inner mitochondrial membrane (IMM), COX catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen in the terminal step of the respiratory chain. The core of the enzyme is composed of three mitochondrially-encoded subunits (I–III). Highly conserved domains within subunits I and II contain two heme (a and a_3) and two copper (Cu_A and Cu_B) moieties essential for catalytic function (1,2). The 10 remaining subunits are of nuclear origin, and are thought to confer structural stability and modulate enzyme activity. Additional, nuclear-encoded ancillary factors are also required for the maturation and assembly of individual structural subunits into a functional holoenzyme complex (3).

COX deficiency is one of the most frequent causes of respiratory chain defects in humans. Patients can present with a wide variety of clinical phenotypes, primarily affecting those organs with high-energy demand such as the brain, skeletal

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muscle and heart (4). Although pathogenic mutations in the three mitochondrially-encoded COX subunits have been reported (5), none have yet been identified in the ten nuclear-encoded structural subunits (6,7). In contrast, mutations in six nuclear genes coding for ancillary factors required for the assembly of the COX complex have been described: *SURF1* (8), *COX10* (9,10), *COX15* (11), *SCO1* (12), *SCO2* (13,14) and *LRPPRC* (15).

Mutations in two of these genes, *SCO1* and *SCO2*, cause severe, tissue-specific COX deficiencies owing to a failure to assemble the holoenzyme: *SCO2* mutations are predominately associated with early-onset hypertrophic cardiomyopathy and encephalopathy (13,14), whereas mutations in *SCO1*, so far described in a single pedigree, result in a progressive neonatal disorder predominantly affecting the liver (12). Both genes code for closely related, ubiquitously expressed transcripts with roles in mitochondrial copper delivery that have largely been inferred from studies in yeast. Yeast *SCO1* and *SCO2* were originally identified as high-copy suppressors of mutations in *COX17*, a copper metallochaperone that localizes to both the cytosol and the mitochondria (16–18); however, only mutations in *SCO1* result in COX deficiency (19). Overexpression of Cox17p, or addition of copper to the medium fails to rescue growth of strains containing *SCO1* mutations on a non-fermentable carbon source, arguing that Cox17p functions upstream of Sco1p (19). It is not clear what role Sco2p plays in mitochondrial copper delivery to COX in yeast, as there is no discernible phenotype associated with its deletion. An analysis of amino acid similarity of the human and yeast sequences suggests that the human genes are not orthologues of the yeast genes, but rather that gene duplication has occurred separately in the two lineages (14).

Both Sco1p and human SCO2 have been shown to bind copper via conserved CxxxC domains (20–22). In contrast to yeast, supplementation of the culture media with copper rescues the COX deficiency in myoblasts and fibroblasts derived from *SCO2* patients (21,23). Studies of COX assembly in yeast implicate Sco1p in the biogenesis of the Cu_A site in Cox2p (24); however, physical evidence for a direct interaction between these two proteins has only been demonstrated in yeast overexpressing an epitope-tagged portion of the soluble domain of Sco1p (25). A fourth copper-binding protein, COX11 (26), appears to be essential for the maturation of the Cu_B site in COX I, as a Δ*COX11* strain in *Rhodobacter sphaeroides* contains a fully assembled oxidase lacking only the Cu_B moiety (27). A fifth COX assembly factor, Cox19p, is also thought to be involved in metal transport to mitochondria (28,29).

Although significant progress has been made in the biochemical characterization of the known copper-binding proteins, particularly in yeast, our current understanding of how copper is delivered to COX remains limited, and the exact function of the two human SCO proteins in COX assembly remains unknown. In particular, it is not known why two closely related proteins are essential for mitochondrial copper delivery in humans, but not in yeast, and how mutations in one or the other human proteins lead to strikingly different tissue-specific deficiencies in COX associated with different clinical phenotypes. Here, we have investigated the roles of human mitochondrial copper-binding proteins in delivering copper to COX in cell lines established from patients who have mutations in either *SCO1* or *SCO2*. Our results suggest a model in which SCO1 and SCO2 have cooperative, non-overlapping functions in Cu_A site formation.

RESULTS

Different steady-state levels of proteins involved in mitochondrial copper delivery in *SCO1* and *SCO2* patient cells

We first characterized the effects of mutations in *SCO1* and *SCO2* in immortalized patient fibroblast and myoblast cell lines using specific antibodies against each protein, and investigated the effects on COX assembly using blue-native polyacrylamide gel electrophoresis (BN-PAGE). Immunoblot analysis showed reduced levels of the respective proteins in all patient cell lines investigated (Fig. 1A), which resulted in a marked defect in COX assembly (Fig. 1C). To test whether these mutations provoked compensatory responses in the mitochondrial copper delivery system, we measured the steady-state levels of COX11, SCO1 and SCO2 in *SCO1* and *SCO2* patient fibroblasts and *SCO2* patient myoblasts (Fig. 1A and B). COX11 levels were similar in control, *SCO1* and *SCO2* fibroblasts, but were less than half that of control in *SCO2* myoblasts (Fig. 1B). SCO1 was present at control levels in both *SCO2* fibroblasts and myoblasts, despite the complete absence of immunologically detectable SCO2 in some patient cell lines. In contrast, reduced levels of SCO1 in patient S were accompanied by an ∼2-fold increase in SCO2 content. Thus, mutations in *SCO1* and *SCO2* have modest, but distinct cell-type specific effects on the levels of proteins involved in the delivery of copper to COX.

A COX assembly intermediate in *SCO1* and *SCO2* patient cells

The reduced level of fully assembled COX in *SCO1* fibroblasts was accompanied by the accumulation of a similar amount of an assembly intermediate (Fig. 1C, arrow). This subcomplex was also observed in the *SCO2* patient cells, particularly patient B myoblasts, albeit at much lower levels relative to the fully assembled holoenzyme. BN/SDS–PAGE analysis revealed that the subcomplex had a molecular weight of ∼100 kDa, similar to that of the subcomplex observed in *SURF1* patients (30), and contained COX structural subunits I and IV, but not COX II (Fig. 2). No assembly intermediates were observed in the controls (Figs 1C and 2A). The presence of the same assembly intermediate in both *SCO1* and *SCO2* patient cells suggests that both proteins are required for the delivery/insertion of copper at the same stage of COX assembly, and that a failure to do this efficiently stalls the assembly process at an early stage. Overexpression of SURF1 in *SCO1* or *SCO2* patient cells had no effect on COX activity (data not shown), arguing that the activity of SURF1 at this stage of COX assembly is independent of copper addition.

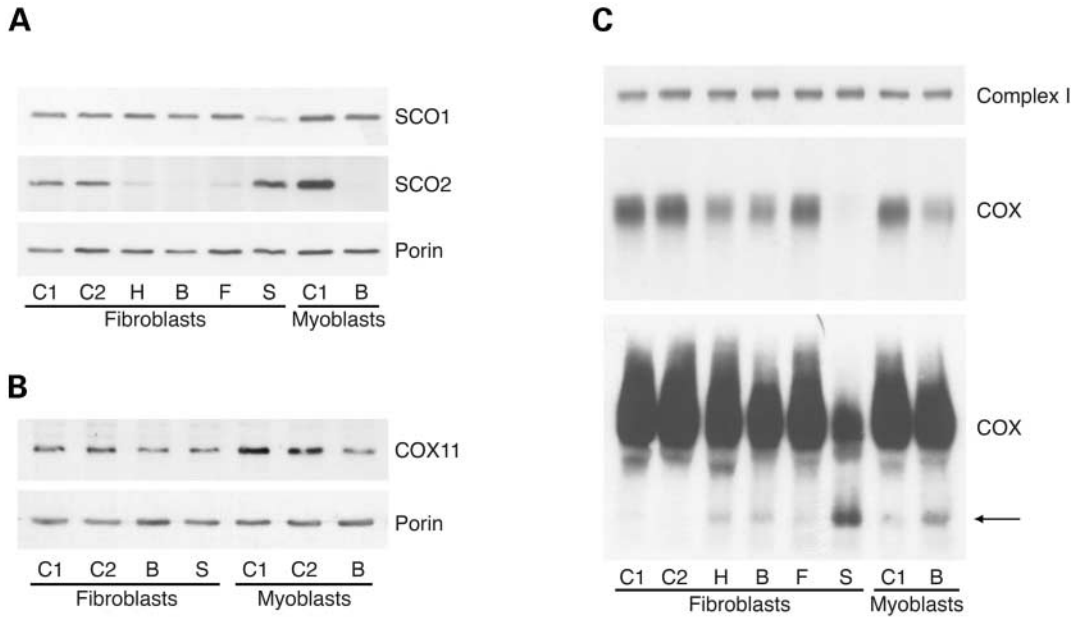

Figure 1. *SCO1* and *SCO2* patient cells contain reduced levels of fully assembled COX and different levels of mitochondrial copper delivery factors. (**A** and **B**) Mitoplasts (20 μg) from control (C), *SCO1* (patient S) and *SCO2* (patients H, B, F) cells were fractionated by SDS–PAGE. Blots were decorated with antibodies specific to COX11, SCO1 and SCO2. Porin served as an internal loading control. Patients H and B were compound heterozygotes, whereas patient F was homozygous for the E140K mutation. (**C**) Mitoplasts (20 μg) were fractionated by BN-PAGE, and blots were decorated with antibodies against complex I (ND1) and COX (COX IV). Overexposure of the COX IV immunoblot revealed the presence of an assembly intermediate in both *SCO1* and *SCO2* patient cells (arrow).

COX deficiency is partially rescued by overexpression of COX17 in *SCO2* but not in *SCO1* patient cells

The COX deficiency in *SCO2* fibroblasts and myoblasts can be rescued by prolonged proliferation in culture media supplemented with copper (13,23). We obtained similar results in *SCO1* fibroblasts (data not shown). These observations suggested that it might be possible to rescue the COX deficiency in both *SCO1* and *SCO2* patient backgrounds by overexpressing other proteins in the mitochondrial copper delivery pathway. To test this hypothesis we transduced *SCO1* and *SCO2* patient fibroblasts and myoblasts with retroviral vectors expressing COX11, COX17 and COX19 (Fig. 3A).

Overexpression of COX11 had no effect on residual COX activity in either *SCO1* or *SCO2* patient cells (Fig. 3A), even though immunoblot analysis of fibroblasts infected with the COX11 retrovirus showed a 2–3-fold increase in COX11 over control (data not shown). Similarly, overexpression of COX19, confirmed by RT-PCR analysis, did not influence COX activity in either patient background.

In contrast, *COX17* overexpression partially rescued the COX deficiency in *SCO2*, but not in *SCO1*, patient cells (Fig. 3A). COX activity in fibroblasts increased from 55.7 ± 1.8 ($n = 14$) to $80.7 \pm 8.3\%$ ($n = 5$) of control, a level similar to that seen in fibroblasts from *SCO2* patients homozygous for the common E140K mutation (31). Supplementation of the medium of COX17-overexpressing cells with 150 μM copper histidine (Cu–His) resulted in a full rescue of the COX deficiency (data not shown). These data suggest that COX17 interacts with SCO2 in mitochondrial copper delivery. Although we cannot rule out the influence of different genetic backgrounds in the *SCO1* and *SCO2* patients, the data further suggest that rescue of the SCO1 defect at high copper concentrations occurs via a COX17-independent pathway.

Overexpression of SCO1 in a *SCO2* patient background, and vice versa, has a dominant-negative effect on COX activity

The relatively high degree of relatedness of the mature human SCO proteins (40% amino acid identity, 63% similarity) prompted us to test whether overexpressing SCO1 in a *SCO2* patient background and vice versa could at least partially rescue the COX deficiency. To test this we transduced *SCO1* and *SCO2* patient cells with retroviral vectors expressing SCO1 or SCO2. COX deficiency was completely rescued in *SCO1* and *SCO2* patient cells overexpressing the respective wild-type cDNA (Fig. 3). Unexpectedly, overexpression of both SCO1 in a *SCO2* patient background, and SCO2 in a *SCO1* patient background, further decreased COX activity in the patient cell lines (Fig. 3). COX activity in control fibroblasts and myoblasts was not affected by overexpression of either factor. These dominant-negative interactions imply that the residual function of the mutated form of each SCO protein is antagonized by overexpression of the other, and that this exacerbates the assembly defect, further reducing the amount of fully assembled COX. These results not only argue strongly for essential, non-overlapping functions of both human SCO1 and SCO2, but also suggest that their functions are somehow related. This is strikingly different from the situation observed in yeast, where the respiratory

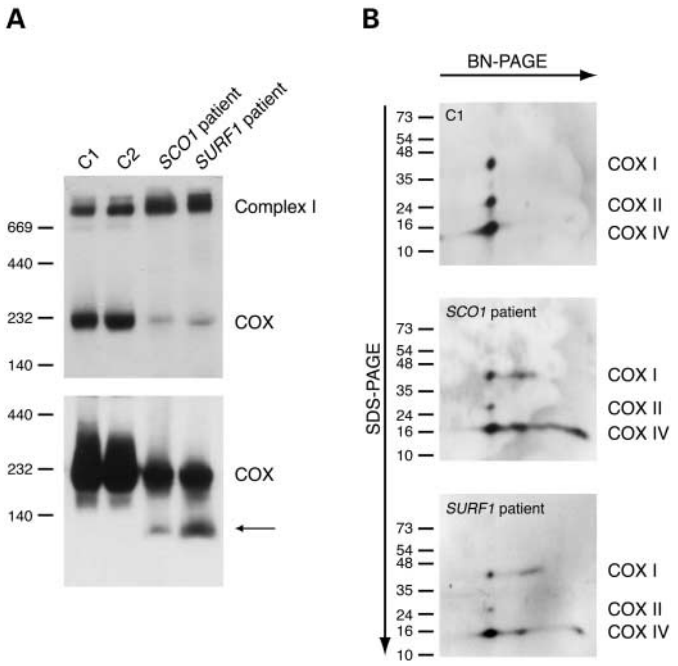

Figure 2. The assembly intermediate common to *SCO1*, *SCO2* and *SURF1* patient cells contains COX I and COX IV but not COX II. (**A**) Mitoplasts (20 μg) from control (C), *SCO1* and *SURF1* fibroblasts were fractionated by BN-PAGE, and blots were decorated with antibodies against complex I (ND1) and COX (COX IV). Overexposure of the COX IV immunoblot revealed the presence of an assembly intermediate in both *SCO1* and *SURF1* patient cells (arrow). (**B**) Mitoplasts, isolated as described in (A), were separated by 2D-BN/SDS–PAGE and immunoblotted with antibodies against COX structural subunits I, II and IV. Migration of the molecular weight markers (kDa) in the second dimension is highlighted on the left.

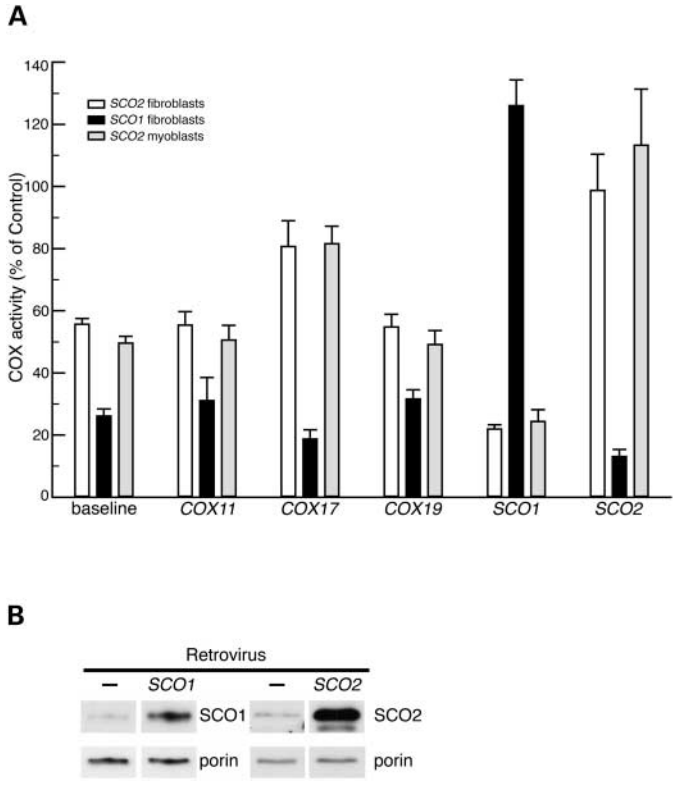

Figure 3. Complementation analysis in *SCO1* and *SCO2* patient cells transduced with a panel of retroviral expression vectors expressing mitochondrial copper delivery proteins. (**A**) Control, *SCO1* (patient S) and *SCO2* (patient B) cells were transduced with *COX11*, *COX17*, *COX19*, *SCO1* or *SCO2* retrovirus. COX and CS activities were measured as described in Materials and Methods. COX activity in patient cells was normalized to CS activity, and is expressed as a percentage of control [$n = 3–6$ for retrovirally transduced cell lines; $n = 11–14$ for control and patient cell lines (baseline)]. (**B**) Mitoplasts (20 μg) from control and retrovirally transduced contol fibroblasts were fractionated by SDS–PAGE, and immunoblotted with antibodies specific to SCO1 and SCO2. Porin served as an internal loading control. Composite images were generated from lanes on the same blot that had been exposed to film for the same length of time.

phenotype of a *SCO1* point mutant, but not the null mutant, was rescued by overexpression of Sco2p (19).

The non-overlapping functions of SCO1 and SCO2 are not based on different intramitochondrial compartmentation of their copper-binding motifs

Both SCO1 and SCO2 are predicted to have a single transmembrane (TM) domain. In yeast, the copper-binding domains are C-terminal to the TM domain in both Sco1p and Sco2p, and face the intermitochondrial membrane space (IMS) (17,20,32). If these domains in human SCO1 and SCO2 were localized to different mitochondrial compartments, this might provide a mechanism for the essential, non-overlapping role of each factor in copper delivery. To investigate this possibility, we characterized the topological organization of the copper-binding domains of SCO1 and SCO2 in the IMM, in addition to that of COX11, utilizing peptide antibodies made against sequences located C-terminal to the predicted TM domains. Mitochondria isolated from HEK293 cells were differentially permeabilized by exposure to increasing amounts of digitonin in the presence of proteinase K (SCO2, COX11) or trypsin (SCO1). Samples were denatured and immunoblotted for SCO1, SCO2 and COX11 along with protein markers of the outer mitochondrial membrane (BCL2), the IMS (TIM13) and the matrix side of the IMM (ATPase α, TFAM). The pattern of proteolytic digestion of SCO1, SCO2 and COX11 was comparable to that of TIM13, the IMS marker, with all immunologically detectable protein disappearing prior to the digestion of either matrix side marker of the IMM (Fig. 4A and B), demonstrating that the copper-binding domains of all three proteins face the IMS.

To address the nature of the association of each protein with the IMM, mitochondria were extracted with alkaline carbonate (33) or Tx-114 (34). Pellet and supernatant fractions were subsequently immunoblotted for SCO1, SCO2 and COX11, and the distribution of all three proteins was compared with that of COX I, a known integral IMM protein, and SDH70, a well established IMM-associated protein (Fig. 4C). All immunodetectable SCO2 partitioned to the pellet fraction in both treatments, whereas a small amount of SCO1 was present in the supernatant fraction. COX11 was equally distributed between the pellet and supernatant fractions in alkaline carbonate-treated mitochondria, and exclusively localized in the pellet fraction following Tx-114 treatment (Fig. 4C). This result likely reflects the fact that Tx-114 treatment

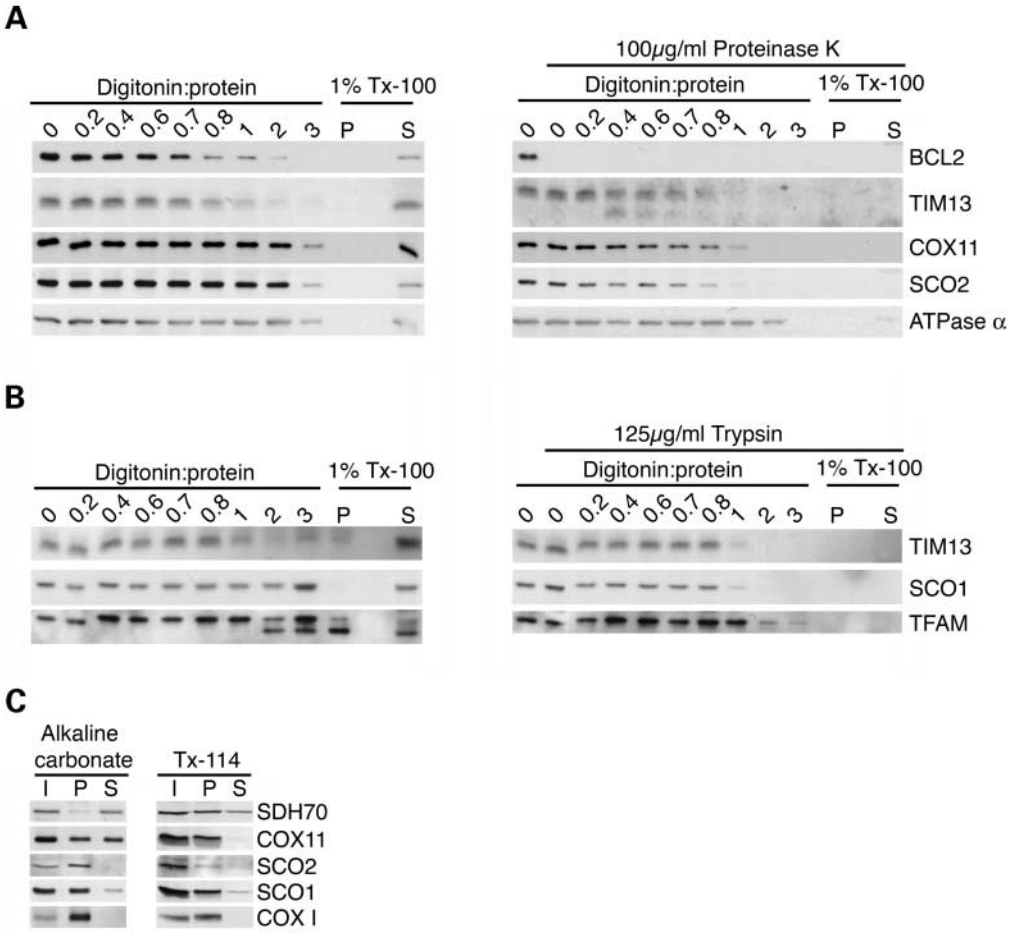

Figure 4. SCO1, SCO2 and COX11 are all integral IMM proteins whose copper-binding motifs face the IMS. (**A**) Isolated mitochondria (30 μg) from HEK293 cells were incubated in increasing amounts of digitonin (0–3 : 1 mg digitonin/mg protein), both in the absence and in the presence of 100 μg/ml PK, as described in Materials and Methods. A sample containing 1% Triton X-100 plus PK was included as a positive control to ensure that all proteins of interest were digested, with the pellet (P) and protein TCA-precipitated from the supernatant (S) being run on the relevant gels. Blots were decorated with antibodies against COX11, SCO2, BCL2, TIM13 and ATPase α. (**B**) Mitochondria were treated as described in (A), except that PK was substituted with 125 μg/ml trypsin. Blots were decorated with antibodies against SCO1, TIM13 and TFAM. The IMS localization of the copper-binding domain of SCO1 was confirmed by treating mitochondria with trypsin in the presence of osmotic swelling (data not shown). (**C**) Mitochondrial proteins were extracted with 100 mM alkaline carbonate (33) or 1% Triton X-114 (34). Equal volumes of the input (I), pellet (P) and supernatant (S) fractions were separated by SDS–PAGE, and immunoblotted with antibodies against COX11, SCO1, SCO2, COX I and SDH70.

represents a gentler manipulation than alkaline carbonate extraction, as evidenced by the observed shift in SDH70 from the supernatant to the pellet fraction under these conditions. These experiments demonstrate that all three proteins are integral to the IMM.

A SCO1/SCO2 chimeric protein acts as a dominant-negative in *SCO2* but not in *SCO1* patient cells

The shared topology of SCO1 and SCO2 suggests that regions outside the C-terminal copper-binding domains are important for their functions in mitochondrial copper delivery. To test this hypothesis, we overexpressed chimeric *SCO1(AA1–135)/SCO2(AA98–266)* and *SCO2(AA1–98)/SCO1(AA136–301)* cDNAs in *SCO1* and *SCO2* patient cells (Fig. 5A). Overexpression of SCO1(AA1–135)/SCO2(AA98–266) acted as a dominant-negative in *SCO2* patient fibroblasts and myoblasts (Fig. 5B). In contrast, residual COX activity in *SCO1* fibroblasts (Fig. 5B) and in control fibroblasts and myoblasts (data not shown) was unaffected. To ensure that these results were specific to the overexpression of the chimera and not attributable to an indirect effect, we overexpressed SCO1(AA1–135)/SCO2(AA98–266) in HEK293 cells to determine its localization and association with the IMM (Fig. 5C and D). These experiments confirmed that SCO1(AA1–135)/SCO2(AA98–266) is an integral IMM protein with its copper-binding motif facing the IMS, and suggest that SCO2 function is critically dependent on amino acids N-terminal to its copper-binding motif. Further, they argue that overexpression of the N-terminal half of SCO1 is sufficient to confer the dominant-negative phenotype in *SCO2* patient cells (Fig. 3A).

Surprisingly, overexpression of the reciprocal chimera, SCO2(AA1–98)/SCO1(136–301), had no discernible effect on residual COX activity in *SCO1* or *SCO2* patient cells (Fig. 5B) despite the fact that it was abundantly expressed in

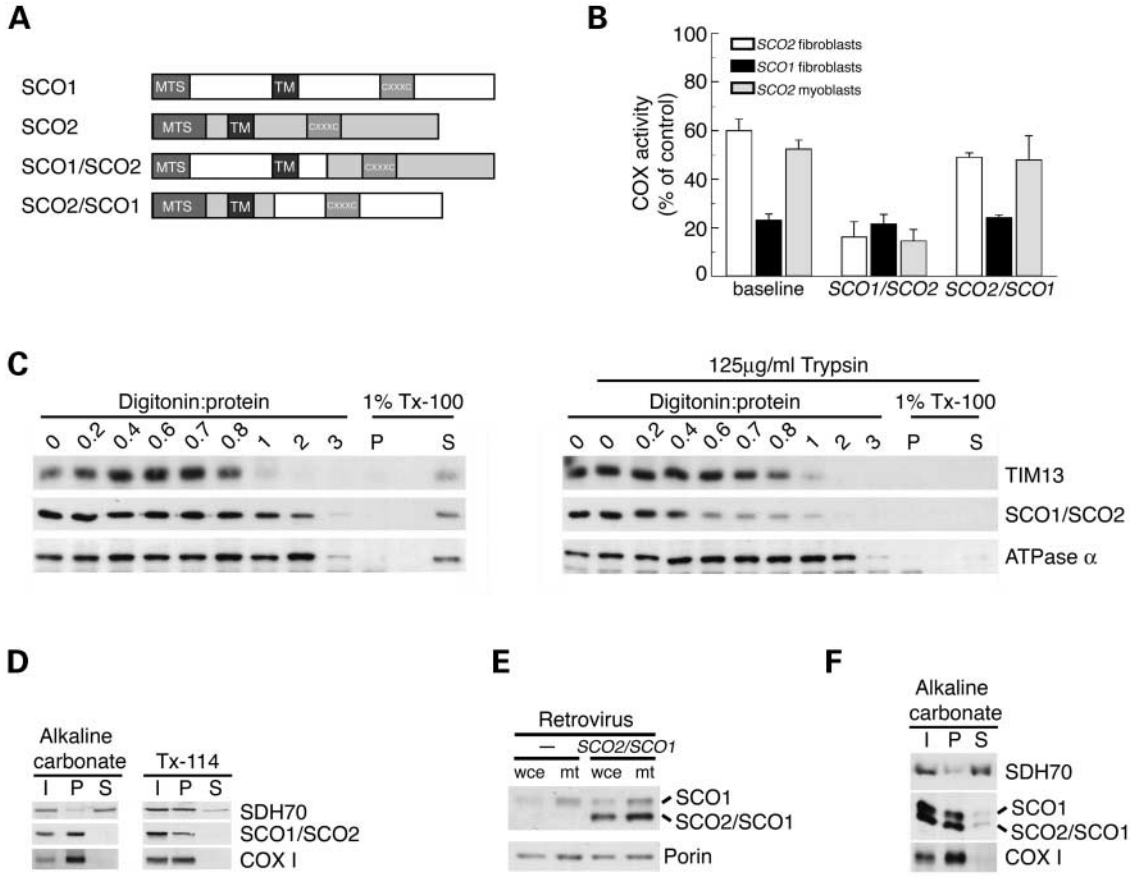

Figure 5. Overexpression of a SCO1/SCO2 chimeric protein results in a dominant-negative phenotype in a *SCO2* but not a *SCO1* patient background. (**A**) Schematic of full-length SCO1 and SCO2, and the chimeric SCO1(AA1–135)/SCO2(AA98–266) and SCO2(AA1–98)/SCO1(136–301) proteins. MTS, mitochondrial targeting sequence; TM, transmembrane domain; CXXXC, copper-binding motif. (**B**) Control, *SCO1* (patient S) and *SCO2* (patient B) cells were transduced with *SCO1(AA1–135)/SCO2(AA98–266)* or *SCO2(AA1–98)/SCO1(136–301)* retrovirus. COX and CS activities were measured as described in Materials and Methods. COX activity in patient cells was normalized to CS activity, and is expressed as a percentage of control ($n = 3–4$). (**C**) Isolated mitochondria (30 μg) from HEK293 cells overexpressing SCO1(AA1–135)/SCO2(AA98–266) were incubated in increasing amounts of digitonin (0–3 : 1 mg digitonin/mg protein) in the absence and presence of 100 μg/ml PK as described in Materials and Methods. A sample containing 1% Triton X-100 plus PK was included as a positive control to ensure that all proteins of interest were digested, with the pellet (P) and protein TCA-precipitated from the supernatant (S) being run on the relevant gels. Blots were decorated with antibodies against SCO2, TIM13 and ATPase α. (**D**) Mitochondrial proteins were extracted with 100 mM alkaline carbonate (33) or 1% Triton X-114 (34). Equal volumes of the input (I), pellet (P) and supernatant (S) fractions were separated by SDS–PAGE, and immunoblotted with antibodies against SCO2, COX I and SDH70. (**E**) Equal amounts (20 μg) of whole cell extract (wce) and isolated mitochondria (mt) from control and *SCO2(AA1–98)/SCO1(136–301)*-transduced control fibroblasts were separated by SDS–PAGE, and immunoblotted with antibodies against SCO1 and porin. (**F**) Mitochondria from fibroblasts overexpressing SCO2(AA1–98)/SCO1(AA136–301) were extracted with alkaline carbonate as described in (D) and the relevant fractions immunoblotted with antibodies against SCO1, COX I and SDH70.

isolated mitochondria, and alkaline carbonate extraction showed that it behaved as an integral IMM protein (Fig. 5E and F). These results show that the C-terminal copper-binding domains are not functionally equivalent in the two SCO proteins. The lack of an effect of either chimeric protein on residual COX activity in patient S fibroblasts further suggests that overexpression of full-length SCO2 is required to achieve a dominant-negative phenotype in a *SCO1* patient background.

SCO1, SCO2 and COX11 exist in higher order, homo-oligomeric complexes

Human SCO2 has been shown to homo-oligomerize *in vitro* (21), and studies in yeast have also reported that Sco1p and Cox11p exist in higher order oligomers, either *in vivo* or *in vitro* (20,22,26,35). To characterize the oligomeric state of SCO1, SCO2 and COX11, isolated mitochondria from HEK293 cells were solubilized in 1% deoxycholate and protein extracts were separated by size exclusion chromatography (Fig. 6). Under these conditions COX ran as a dimer with a predicted molecular mass of ~491 kDa. COX activity and COX II immunoreactivity exhibited an identical elution profile, and the preparation retained full oxidase activity (data not shown).

Immunoblot analysis of these same fractions with antibodies specific to SCO1, SCO2 and COX11 demonstrated that none of these factors co-eluted with the fully assembled holoenzyme (Fig. 6). COX11 (~24 kDa), SCO1 (~29 kDa) and SCO2 (~26 kDa) eluted in fractions corresponding to predicted molecular masses of 44.9, 59.5 and 54.1 kDa respectively, very close to those expected if each protein

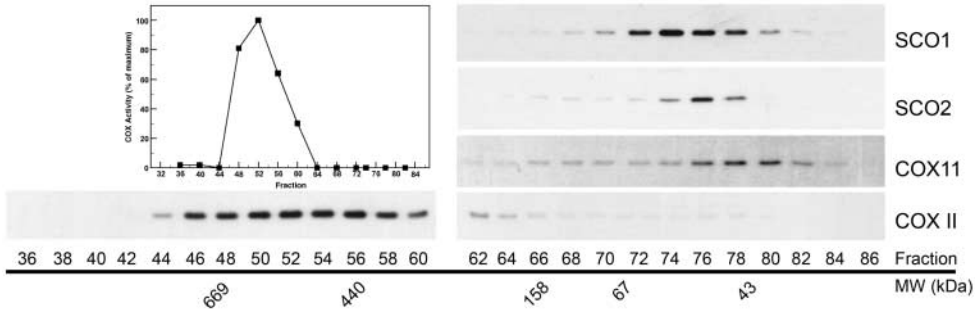

Figure 6. SCO1, SCO2 and COX11 all exist in higher order, homo-oligomeric complexes. Mitochondria (2.5 mg/ml) were extracted with 1% sodium deoxycholate as described in Materials and Methods. Extracts were loaded onto a pre-equilibrated Superdex 200 GL column (Pharmacia), and fractions were collected every 0.2 ml at a flow rate of 0.4 ml/min. COX activity was assayed in each fraction, and the elution profile depicted as a percentage of the fraction that contained maximal kinetic activity. Following TCA precipitation, samples were separated by SDS–PAGE, and immunoblotted with antibodies against COX II, COX11, SCO1 and SCO2. The elution profile of a known set of standards (Pharmacia) is shown relative to the fractions that were used for immunoblot analysis.

existed as a homodimer (Fig. 6). Although the elution profiles of COX11, SCO1 and SCO2 overlapped, maximal immunoreactivity for each protein was contained within distinct fractions, further arguing that under these extraction conditions, each of these COX assembly factors exists in a homo-oligomeric state.

DISCUSSION

This study represents the first investigation of the relative roles of human SCO1 and SCO2 in mitochondrial copper delivery and COX assembly. Several lines of evidence suggest that the functions of each protein are non-overlapping and cooperative in the maturation of the Cu_A site at an early stage of holoenzyme assembly. First, mutations in *SCO1* and *SCO2* result in a marked reduction in the amount of fully assembled COX and the accumulation of the same early assembly intermediate containing COX I and COX IV. Second, COX deficiency in *SCO2* but not in *SCO1* patient cells is partially complemented by overexpressing COX17, though increased concentrations of copper in the culture media partially rescue both. Third, despite their high degree of similarity at the amino acid level, overexpression of either factor in the reciprocal patient background acts as a dominant-negative, exacerbating the COX deficiency, an effect that is recapitulated by overexpression of a *SCO1*/*SCO2* chimeric construct in a *SCO2* patient background.

The loss of function mutations in *SCO2* severely decreased steady-state levels of SCO2 protein in the cell lines we investigated. All *SCO2* patients so far identified share a common E140K allele, and patients homozygous for this allele have a less severe COX deficiency in fibroblasts and later onset of the clinical symptoms (31). Fibroblasts derived from two *SCO2* patients, one of whom was a compound heterozygote and the other homozygous for the common E140K mutation, contained similar amounts of residual SCO2 protein, but SCO2 was immunologically undetectable in fibroblasts and myoblasts of a second compound heterozygote. Thus, there is no simple correlation between the amount of mutant SCO2 protein and the severity of the clinical phenotype, suggesting that the nature of the mutation, and hence the residual activity of the mutant protein, is also an important determinant of COX deficiency. Mutations in *SCO1* similarly reduced the steady-state amount of protein in fibroblasts, but as only a single *SCO1* pedigree has been identified, it is not possible to comment on the generality of this result.

With the exception of the mutant gene products, very modest differences in the levels of COX11, SCO1 and SCO2 were observed in the patient cells we were able to investigate. These data suggest that the genetic response to loss of function mutations in either *SCO* gene does not include compensatory increases in the levels of other known mitochondrial copper chaperones. The inability of COX11 to functionally complement the COX deficiency in either *SCO1* or *SCO2* patient cells supports the idea that COX11 functions in the delivery of copper to the Cu_B site (27), and that this is mechanistically independent of the maturation of the Cu_A site. Partial complementation of the COX deficiency in *SCO2*, but not in *SCO1*, patient fibroblasts by COX17 suggests that COX17 specifically and physically interacts with SCO2 in order to deliver copper to COX. A similar interaction has been proposed for Cox17p and Sco1p in yeast (18,24); however, overexpression of Cox17p is unable to rescue point mutations in Sco1p in yeast (18), indicating either that the nature of the interaction may be different in mammals, or that other interacting partners may be necessary in yeast.

The dominant-negative phenotypes we observed when we expressed SCO1 in a *SCO2* patient background, and vice versa, also suggest a physical interaction between the two SCO proteins. The fact that COX activity is unchanged in control cells overexpressing either SCO1 or SCO2 indicates that this phenotype can only be observed when there is a large imbalance in the relative stoichiometries of each factor. The ability of the SCO1/SCO2 chimera to recapitulate the dominant-negative phenotype in *SCO2* patient cells argues that domains N-terminal to the copper-binding motif of SCO1 mediate its effects on the function of mutant SCO2. These domains in both SCO proteins are localized within the matrix, but their function is unknown. The N-terminal tail in SCO1 (63 amino acids) is strikingly larger than that of SCO2 (19 amino acids) and represents the most divergent part of the two proteins. These domains are of similar length in yeast Sco1p and Sco2p and, in contrast to what we observed

in humans, chimeric proteins consisting of the C-terminal domains of Sco2p and the N-terminal domain of Sco1p and vice-versa were able to rescue a *SCO1* deletion (36), indicating a high degree of functional redundancy in the yeast SCO proteins. These observations suggest that a key feature in the specialization of the human SCO proteins may have involved evolution of the N-terminal matrix tails. These domains might be important to the homo-oligomerization of each factor. Alternatively, they may be involved in the stabilization of interactions between SCO1 and SCO2 homo-oligomers, or between SCO proteins and COX II. Lack of an effect of either chimera on residual COX activity in *SCO1* patient fibroblasts in our studies suggests that the effects of SCO2 on SCO1 function likely involve more complex interactions that require multiple domains contained within both the N- and C-terminal portions of the protein.

A role for SCO1 and SCO2 at the same stage of assembly is suggested by the accumulation of an assembly intermediate containing COX I and COX IV in cells from both patient backgrounds. Independent analysis of this assembly intermediate in *SCO1* patient fibroblasts also revealed the presence of COX V_a (37). It is clear from our topological analyses and localization studies that the unique function of each factor at this stage of COX assembly is not based on different intramitochondrial localization of their copper-binding motifs. The absence of COX II from the observed subcomplex also indicates that the maturation of the Cu_A site is likely a prerequisite for the stable incorporation of COX II into the assembling holoenzyme, an event that occurs relatively early on in the assembly process.

In *SCO1* patient fibroblasts, none of the known COX assembly factors that participate in mitochondrial copper delivery were able to functionally complement the observed COX deficiency. This suggests at least two possibilities for the function of SCO1. First, its function may be contingent upon physically receiving copper from an as yet unidentified interacting partner. Alternatively, it may act to facilitate the interaction between SCO2 and COX II, thereby increasing the efficiency of copper transfer to the binuclear Cu_A site. This might be particularly crucial if copper atoms were transferred to COX II in a sequential manner instead of as a pre-existing, binuclear cluster.

Taken together, our data suggest a pathway for mitochondrial copper delivery to the Cu_A site in COX II in which COX17 transfers copper to SCO2, which in turn delivers it to COX II in a reaction that is facilitated by SCO1 (Fig. 7). One possibility is that its relatively long N-terminal domain acts to recruit SCO2 to COX II, or to an assembly intermediate containing COX II. Gel filtration studies predict that SCO1 and SCO2 function as homodimers in this process. The involvement of homo-oligomeric rather than hetero-oligomeric complexes is also suggested by the fact that mutations in either *SCO* gene had very modest effects on the levels of the reciprocal SCO protein, whose stability should be reduced if both proteins formed heterodimers. The fact that overexpression of either SCO protein in the reciprocal patient background acts as a dominant-negative does, however, suggest a physical interaction between the homodimers.

It is interesting to contrast this model with the pathway in yeast, in which only one SCO protein is required to generate

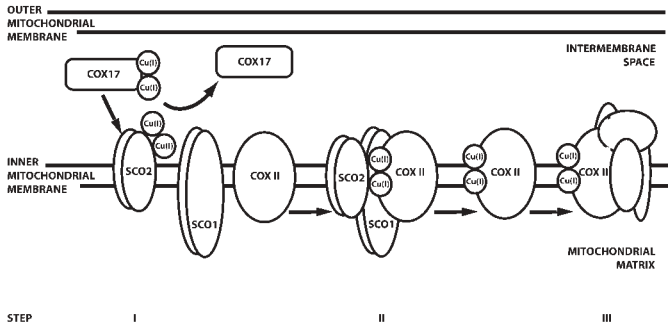

Figure 7. Proposed model of SCO1 and SCO2 function in Cu_A site assembly. COX17 transfers copper to SCO2, which in turn delivers it to COX II (I). SCO1 facilitates the latter interaction, thereby promoting the biogenesis of the Cu_A site (II). The metallation of COX II occurs at an early stage of COX assembly and is required for the incorporation of this structural subunit into the assembling holoenzyme (III).

the Cu_A site. The two yeast SCO proteins are significantly more similar to each other than the two mammalian SCO proteins (59 versus 39%). Consistent with this the yeast proteins have partially overlapping functions, whereas the human proteins have non-overlapping, cooperative functions, at least in cells in culture. These functional differences are also consistent with the idea that duplication of the *SCO* genes occurred independently in the two lineages. In mammals, these functional differences may have evolved to serve different tissue-specific requirements for the regulation of COX assembly that may be necessary to accommodate different rates of turnover of the enzyme, or different mechanisms regulating enzyme content. Additional studies will be required to clarify the tissue-specific roles of these factors.

MATERIALS AND METHODS

Cell lines

Primary cell lines from control, *SCO1* (12) and *SCO2* (13,31) patient skin fibroblasts and myoblasts were immortalized as previously described (11). HEK293 cells were obtained from the American Type Culture Collection (ATCC). All cell types were grown at 37°C in an atmosphere of 5% CO_2. Fibroblasts and HEK293 cells were grown in high glucose DMEM supplemented with 10% fetal bovine serum (FBS), whereas myoblasts were proliferated in supplemented growth medium containing 15% FBS (38).

Mitochondrial isolation

HEK293 cells were resuspended in ice-cold HIM buffer [200 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EGTA (pH 7.5)] containing 0.2% BSA and homogenized with 10 passes through a pre-chilled, zero clearance homogenizer (Kontes Glass Co.). Samples were centrifuged twice for 20 min at 600*g* to obtain a post-nuclear supernatant. Mitochondria were pelleted by centrifugation for 10 min at 10 000*g*, and washed once in HIM buffer alone prior to further analyses.

Mitochondrial localization experiments

Isolated mitochondria were resuspended in a standard buffer used for mitochondrial import assays (39), and incubated in increasing amounts of digitonin (0–3:1 mg digitonin/mg protein) in the presence of either proteinase K (PK; 100 μg/ml) or trypsin (125 μg/ml). Reactions were allowed to proceed for 20 min on ice, at which point the relevant protease inhibitor, 1 mM PMSF or 1.25 mg/ml soybean trypsin inhibitor, was added. Following a further 20 min incubation, samples were overlaid on a sucrose cushion, and spun at 16 600*g* for 10 min at 4°C. Pellets were resuspended in sample loading buffer, denatured and used for immunoblot analysis. To ensure that all proteins of interest were protease digestible, a sample containing 1% Triton X-100 plus protease was included as a positive control, with both the pellet and the TCA-precipitated supernatant being run on the relevant gels. Mitochondrial proteins were also extracted with alkaline carbonate (33) and Triton X-114 (34) as previously described, and the relevant fractions analyzed by SDS–PAGE.

Size-exclusion chromatography

Mitochondria (2.5 mg/ml) were extracted for 30 min at 4°C in 50 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1 mM DTT, 0.5 mM PMSF, complete protease inhibitor cocktail (Roche) and 1% sodium deoxycholate. Extracts were centrifuged at 200 000*g* for 30 min at 4°C, the supernatant passed through a 0.45 μm filter, and loaded onto a Superdex 200 GL column (Amersham Pharmacia) that had previously been calibrated with a known set of standards (Amersham Pharmacia). Fractions were collected every 0.2 ml at a flow rate of 0.4 ml/min. COX activity was assayed before TCA precipitation and SDS–PAGE analysis.

Electrophoresis and immunoblotting

BN-PAGE (40,41) was performed on mitoplasts following solubilization in 1% lauryl maltoside. Protocols for immunoblotting of first dimension gels and second dimension gel electrophoresis were as previously described (9). Individual structural subunits of complexes I, II and IV were detected by immunoblot analysis using commercially available, monoclonal antibodies (Molecular Probes).

Denatured mitochondrial extracts were resolved using a 15% SDS–PAGE gel (42). Blots were decorated with monoclonal antibodies raised against human porin (Calbiochem), ATPase subunit α (Molecular Probes), Bcl-2 (Santa Cruz Biotechnology) and polyclonal antibodies raised against TIM13 (kind gift of C. Koehler, UCLA), SCO2 (21), TFAM, COX11 and SCO1. Following incubation with the relevant secondary antibody, immunoreactive proteins were detected by luminol-enhanced chemiluminescence (Pierce). COX11 and SCO1 antibodies were generated by injecting rabbits with synthetic peptides specific to human COX11 (CFEEQRLNPQEEVD) and SCO1 (CASIATHMRPYRKKS) that had been conjugated to KLH via a reactive sulfhydryl group (Pierce) and emulsified in Titermax (Pierce). The mature form of mouse TFAM was expressed using a 6× His-tagged expression vector (Novagen), purified using conventional techniques and injected into rabbits upon emulsification in Titermax (Pierce). Testing of the specificity of the COX11, SCO1 and TFAM antisera revealed that affinity purification was not required, and crude serum was used in all subsequent analyses.

Miscellaneous

Chimeric *SCO1(AA1–135)/SCO2(AA98–266)* and *SCO2(AA1–98)/SCO1(AA136–301)* cDNA constructs were generated first by RT–PCR amplification of the N- and C-terminal portions of SCO1 and SCO2, followed by ligation of the relevant cDNAs by overlap extension PCR (43). The fidelity of all PCR products was confirmed by sequencing, at which point cDNAs were cloned into the Gateway-modified retroviral expression vector pLXSH (44). Protocols for generating stable virus-producing cell lines expressing individual constructs of interest and infection of fibroblast and myoblast cell lines were as previously described (11). Protein concentration (45), COX (46) and citrate synthase (CS) (47) activities were measured as described elsewhere.

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