Key within-membrane residues and precursor dosage impact the allotopic expression of yeast subunit II of cytochrome *c* oxidase

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ABSTRACT **Experimentally relocating mitochondrial genes to the nucleus for functional expression (allotopic expression) is a challenging process. The high hydrophobicity of mitochondria-encoded proteins seems to be one of the main factors preventing this allotopic expression. We focused on subunit II of cytochrome *c* oxidase (Cox2) to study which modifications may enable or improve its allotopic expression in yeast. Cox2 can be imported from the cytosol into mitochondria in the presence of the W56R substitution, which decreases the protein hydrophobicity and allows partial respiratory rescue of a *cox2*-null strain. We show that the inclusion of a positive charge is more favorable than substitutions that only decrease the hydrophobicity. We also searched for other determinants enabling allotopic expression in yeast by examining the *COX2* gene in organisms where it was transferred to the nucleus during evolution. We found that naturally occurring variations at within-membrane residues in the legume *Glycine max* Cox2 could enable yeast *COX2* allotopic expression. We also evidence that directing high doses of allotopically synthesized Cox2 to mitochondria seems to be counterproductive because the subunit aggregates at the mitochondrial surface. Our findings are relevant to the design of allotopic expression strategies and contribute to the understanding of gene retention in organellar genomes.**

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INTRODUCTION

Most mitochondrial proteins are encoded in the nuclear genome, translated by cytosolic ribosomes, and directed to mitochondria. However, migration of mitochondrial genes to the nucleus occurs widely in nature (Adams and Palmer, 2003; Burger *et al.*, 2003; Timmis *et al.*, 2004). These relocalized nucleotide sequences acquire more complex regulation and tend to exhibit lower mutation rates (Martin and Herrmann, 1998). Mitochondrial genomes mainly encode subunits of oxidative phosphorylation (OXPHOS) complexes. In general, these proteins are embedded in membranes and are highly hydrophobic, a feature that is considered to be one of the main driving forces for the retention of a genome in mitochondria (Johnston and Williams, 2016).

Experimentally relocating mitochondrial genes to the nucleus holds promise for the development of treatments of mitochondrial diseases (De Grey, 2000; DiMauro *et al.*, 2006; Kyriakouli *et al.*, 2008). A mutation in the mitochondrial genome, yielding a defective protein, could be overcome in principle by the import of a nucleus-encoded functional version of the protein. Multiple efforts have been undertaken to express mitochondria-encoded genes allotopically using yeast or mammalian model systems, but conflicting and inconsistent results call for careful interpretation of results and warrant further experimental testing (Oca-Cossio *et al.*, 2003; Perales-Clemente *et al.*, 2011).

The *COX2* gene, encoding subunit 2 (Cox2) of cytochrome *c* oxidase (C*c*O), provides an example of natural migration of a mitochondrial gene to the nucleus. This gene is generally mitochondria-encoded, but in a few organisms it is found in the nuclear

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Abbreviations used: C*c*O, cytochrome *c* oxidase; Cox2, subunit 2 of cytochrome *c* oxidase; MTS, mitochondrial targeting sequence; TIM, translocase of the inner membrane; TMS, transmembrane segment; TOM, translocase of the outer membrane.

genome (Adams *et al.*, 1999; Pérez-Martínez *et al.*, 2001; Szafranski, 2017). For example, *COX2* was transferred to the nucleus in some members of the legume lineage, resulting in mitochondrial and nuclear versions of *COX2*. The representative species of this lineage encode Cox2 in the nuclear DNA, in the mitochondrial DNA, or in both genomes, depending on which copy was activated and/or inactivated (Adams *et al.*, 1999; Daley *et al.*, 2002a,b). Also, the *COX2* gene has been relocalized experimentally to the yeast nucleus (Supekova *et al.*, 2010), and subsequent studies have provided supporting genetic and biochemical evidence for successful allotopic expression (Cruz-Torres *et al.*, 2012; Elliott *et al.*, 2012; Rubalcava-Gracia *et al.*, 2018). The substitution of a tryptophan for an arginine in position 56 ($Cox2^{W56R}$), within the first transmembrane segment (TMS) of allotopically produced Cox2, decreases its hydrophobicity and allows the partial respiratory rescue of a *cox2*-null yeast strain, with an estimated ~60% recovery of CcO levels as compared with the wild-type strain (Cruz-Torres *et al.*, 2012). This partial complementation can be attributed solely to diminished import, since a mitochondrial *COX2* gene producing the W56R variant cannot be distinguished from wild-type (Rubalcava-Gracia *et al.*, 2018). The decrease in hydrophobicity of the first TMS in Cox2 also occurred during the natural relocation of the *COX2* gene to the nucleus in legumes (Daley *et al.*, 2002b). Other necessary modifications for successful allotopic expression include recoding the mitochondrial gene to the nuclear genetic code and fusing a sequence encoding a mitochondrial targeting sequence (MTS) to the open reading frame. Given that the W56R substitution was found in a random mutagenesis screen of 20,000 alleles (Supekova *et al.*, 2010), it is likely that this is the only permissible change that enables import from the cytosol to the mitochondria and at the same time maintains CcO functionality. This underscores the power of such an approach to uncover mutations allowing the allotopic production of other mitochondria-encoded proteins.

Here, we further explored the W56 position in Cox2 by testing whether other residues with similar properties to arginine could confer allotopic expression. We also tested whether naturally occurring changes in the legume *Glycine max* Cox2 enable yeast Cox2 to be imported from the cytosol into mitochondria and assembled into a functional CcO. Finally, we evidenced that dosage of the cytosol-synthesized Cox2 precursor is critical for the outcome of allotopic expression.

RESULTS

Other substitutions at residue 56 enable the allotopic expression of *COX2*

First, we tested whether other changes at position 56, besides the tryptophan-to-arginine substitution (Supekova *et al.*, 2010) (W56R), can restore the respiratory growth of a *cox2*-null strain expressing an allotopic version of *COX2*. We substituted W56 for two residues sharing at least one physicochemical property with arginine: lysine (W56K), which also has a positive charge, and glutamine (W56Q), which has the closest hydrophobicity score to arginine (Calado-Botelho *et al.*, 2011). Upon introduction of the allotopic constructs encoding $Cox2^{W56K}$ or $Cox2^{W56Q}$ into a *cox2*-null strain, we observed a restoration of respiratory growth. Respiratory growth of the strain with the W56K substitution was comparable to that of the strain carrying the W56R substitution, while the recovery of respiratory competence by $Cox2^{W56Q}$ was significantly less (Figure 1A). Hence, the growth behavior of yeast carrying these constructs was $Cox2^{W56R} = Cox2^{W56K} > Cox2^{W56Q}$. As a negative control, we included a strain expressing an allotopic wild-type *COX2* gene (producing the $Cox2^{WT}$ subunit). As expected, neither this construct producing $Cox2^{WT}$, nor the empty plasmid, was able to restore respiratory growth in the *cox2*-null strain (Figure 1A).

Previous work evidenced that most of the allotopically synthesized $Cox2^{W56R}$ protein accumulates as an unprocessed precursor (i.e., still carrying its MTS) detected in the mitochondrial fraction (Supekova *et al.*, 2010; Cruz-Torres *et al.*, 2012; Rubalcava-Gracia *et al.*, 2018). We thus performed cellular fractionations to test the cytosolic versus mitochondrial distribution of $Cox2^{WT}$, $Cox2^{W56K}$, and $Cox2^{W56Q}$. Most of the Cox2 precursors were found associated with mitochondria (Figure 1B), indicating that, similarly to $Cox2^{W56R}$, the limiting step occurs before their processing into mature forms in the mitochondrial matrix. Our results show that other residues at position 56 allow allotopic expression of *COX2* and that the inclusion of a positive charge in the first TMS (W56K) is favorable over a substitution that only diminishes hydrophobicity (W56Q).

Mutations that allow the nuclear expression of *COX2* in legumes also confer allotopic expression in yeast

Next, we sought to identify other changes in Cox2 that might enable its allotopic expression in the yeast system. We reasoned that in organisms that naturally encode *COX2* in the nucleus, the protein

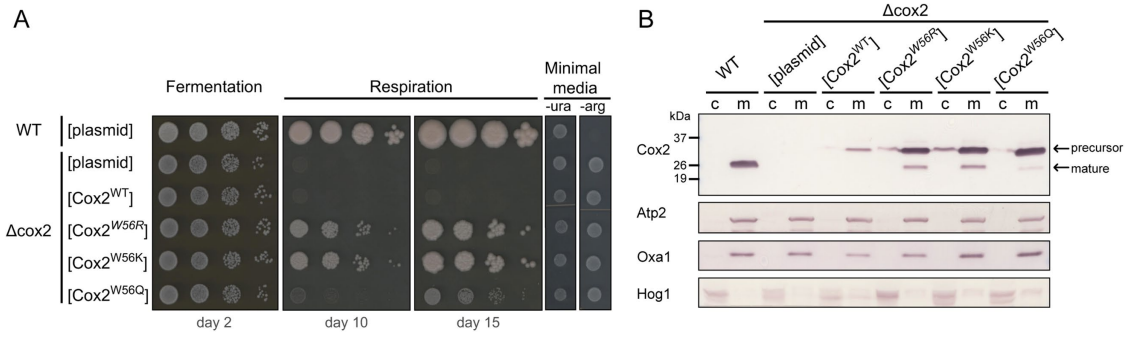

FIGURE 1: Substituting residues of properties similar to those of arginine in position 56 of allotopic Cox2 restores cellular respiration to a *cox2*-null strain. (A) Tenfold serial dilutions of yeast liquid cultures from strains transformed with the indicated constructs or with an empty vector (plasmid) were plated on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (-ura or -arg, for verifying the presence of the transforming plasmid or the mitochondrial genome, respectively). (B) Immunoblots decorated with an anti-Cox2 antibody of cytosolic (c; 100-µg) and mitochondrial (m; 50-µg) fractions of the indicated yeast strains. The Cox2 precursor and mature forms of Cox2 are indicated. Antibodies anti-Atp2 and anti-Oxa1 were used to immunodetect the corresponding mitochondrial markers and anti-Hog1 was used for the cytosolic marker. Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.

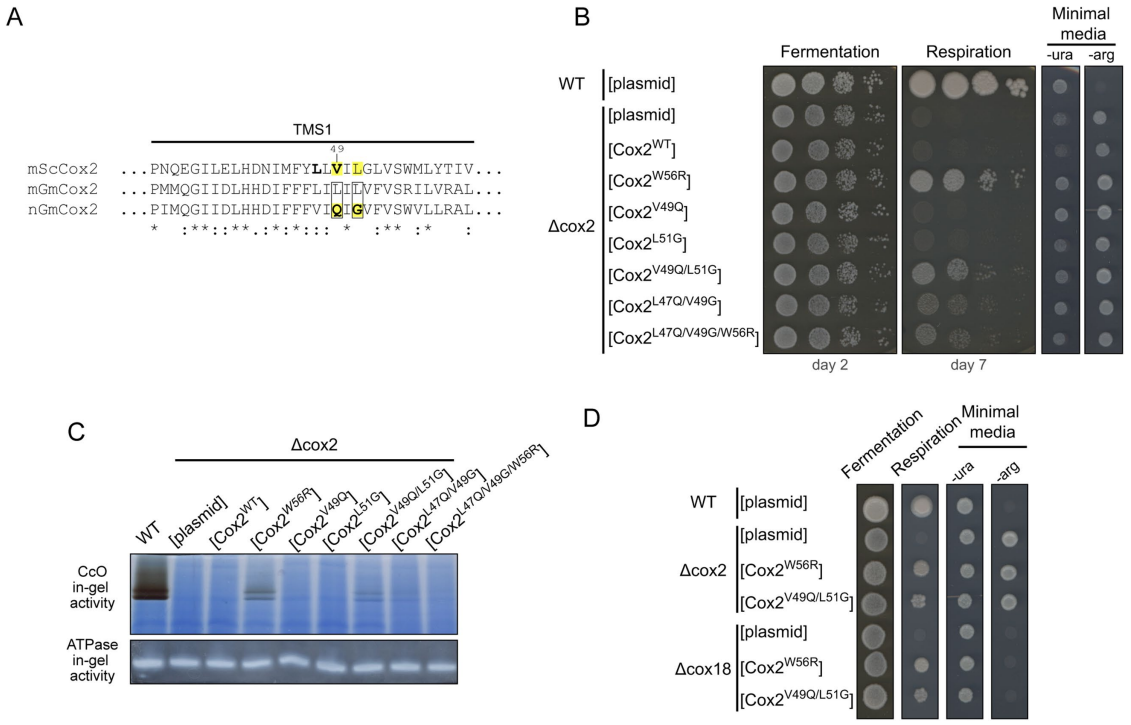

FIGURE 2: Mutations that allow the nuclear expression of *COX2* in legumes also confer allotopic expression in yeast. (A) Sequence alignments of the first transmembrane segment (TMS1) from the yeast Cox2 (mScCox2) and the mitochondrial and nuclear Cox2 versions of *Glycine max*, mGmCox2 and nGmCox2, respectively. An asterisk (*) indicates positions that have a single, fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix). A period (.) indicates conservation between groups of weakly similar properties (scoring ≤0.5 in the Gonnet PAM 250 matrix). Key residues that are critical for import of the legume Cox2 from the cytosol are boxed (see text). Two yeast Cox2 constructs were designed based on this alignment: $Cox2^{V49Q/L51G}$ and $Cox2^{L47Q/V49G}$. (B) Tenfold serial dilutions of yeast cultures from the indicated strains were spotted on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (-ura or -arg). (C) In-gel activity staining of complex IV and of complex V as a loading control. Isolated mitochondria (250 µg) from the indicated strains were solubilized with lauryl maltoside and resolved by BN-PAGE. (D) Strains (WT, Δ*cox2*, and Δ*cox18*) were plated on fermentable (glucose), respiratory (lactate), and minimal, selective media (-ura or -arg). Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.

sequence must carry determinants for import into the mitochondria and subsequent assembly into a functional holoenzyme. For example, the legume *Glycine max* expresses the *COX2* gene from both nuclear and mitochondrial genomes (Adams *et al.*, 1999). Previous research showed that a two-residue substitution within the first TMS of Cox2 is necessary and sufficient for the mitochondrial version of Cox2 to be imported into isolated mitochondria. Accordingly, the nucleus-encoded Cox2 protein could no longer be imported into the organelle when the residues (Q and G) at these two positions were modified to the ones (L and L) present in the mitochondria-encoded Cox2 (Daley *et al.*, 2002b; Figure 2A). The conclusion from this study is that this two-residue substitution was determinant in facilitating the import of Cox2 from the cytosol to the mitochondria.

To test whether this two-residue substitution could confer allotopic expression in the yeast system, we sought to reproduce the legume *COX2* mutations in the *COX2* sequence from yeast. We generated an alignment of the two legume Cox2 subunits with the one from yeast and identified that the key residues conferring import of the legume Cox2 correspond to a valine and a leucine at positions 49 and 51 in the yeast sequence (Figure 2A). We therefore engineered the mutations corresponding to the single and double substitutions V49Q and L51G in yeast *COX2*. We observed that only the $Cox2^{V49Q/L51G}$ variant was able to restore respiratory growth in a *cox2*-null strain, while the $Cox2^{V49Q}$ and $Cox2^{L51G}$ variants could not (Figure 2B), demonstrating that both changes are required for successful allotopic expression of *COX2*. However, the respiratory growth of the $Cox2^{V49Q/L51G}$ strain was less than that of the $Cox2^{W56R}$ strain. To test whether the overall decrease in hydrophobicity within the first TMS of Cox2 is sufficient for restoring respiratory growth, we substituted other aliphatic residues at other positions, resulting in the construct $Cox2^{L47Q/V49G}$. To explore any additive effect, a triple *COX2* mutant corresponding to W56R combined with a double substitution (L47Q/V49G/W56R) was also generated. All these constructs could restore the respiratory growth of a *cox2*-null strain, although to a lesser extent than the construct producing $Cox2^{V49Q/L51G}$. We also could not observe any additive effect in the strain producing the $Cox2^{V49Q/L51G/W56R}$ variant. Using *in-gel* activity staining, we verified that CcO activity correlated with the observation that the $Cox2^{W56R}$ variant exhibits increased respiratory growth compared with that in the variants containing two-residue substitutions (Figure 2C). In contrast, another OXPHOS complex, ATPase, exhibited comparable activity for all strains.

Next, we questioned whether $Cox2^{V49Q/L51G}$ follows the same biogenesis pathway as that of allotopically produced $Cox2^{W56R}$. In contrast to orthodox Cox2, synthesized in mitochondria, allotopically

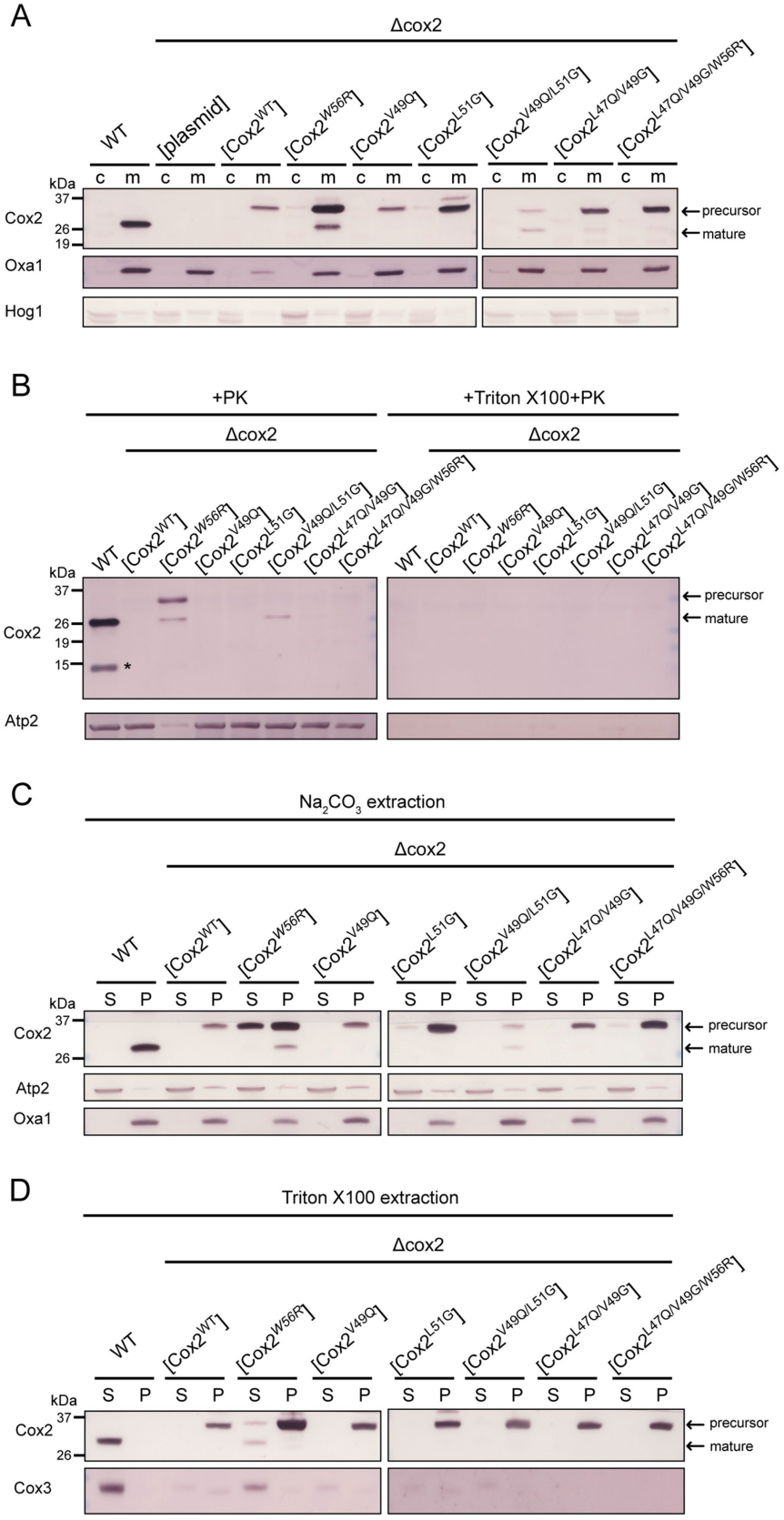

FIGURE 3: The allotopically synthesized proteins face the limiting step by aggregating at the mitochondrial periphery. (A) Immunoblots decorated with anti-Cox2 of cytosolic (c; 100-µg) and mitochondrial (m; 50-µg) fractions. Arrows indicate the precursor and mature forms of Cox2. The mitochondrial markers Oxa1 and the cytosolic marker Hog1 were decorated with their corresponding antibodies. (B) Isolated mitochondria (50 µg) were treated with proteinase K (PK) to degrade proteins external to the outer membrane. Parallel samples were preincubated with Triton X-100 to dissolve the membranes and to make all mitochondrial proteins accessible to protease degradation. The asterisk indicates partial degradation of Cox2, possibly due to imperfect mitochondrial preparation. (C) Carbonate extraction separating the membrane extrinsic proteins in the supernatant (S) from the integral membrane proteins in the pellet (P) from yeast expressing the indicated constructs. Atp2 was used as a soluble protein marker and Oxa1 as an integral membrane protein marker. (D) Triton X-100 extraction separating the detergent-solubilized proteins in the supernatant (S) from the (detergent-resistant) aggregated proteins in the pellet (P) from yeast expressing the indicated constructs. Cox3 was used as a solubilized membrane protein marker. Squared brackets indicate Cox2 variants expressed from plasmids. All the Cox2 variants contain an MTS from Oxa1.

synthesized Cox2^{W56R} does not require the Cox18 translocase for the correct insertion of its second TMS into the inner membrane (Elliott *et al.*, 2012), and it was postulated to be released laterally into this membrane directly from the TIM23 complex (Rubalcava-Gracia *et al.*, 2018). We observed that, similarly to the Cox2^{W56R} strain, the Cox2$^{V49Q/L51G}$ variant is able to restore respiration when expressed in a *cox18*-null (Figure 2D), indicating that the Cox18 translocase is completely dispensable. This suggests that the second TMS of Cox2$^{V49Q/L51G}$ is released laterally into the inner mitochondrial membrane by the TIM23 translocator.

We observed that while the mature forms of Cox2 were protected from degradation upon incubation of mitochondria-enriched fractions with proteinase K (PK), a result indicative of mitochondrial localization, the Cox2 precursors were mostly degraded by the protease (Figure 3, A and B). These results may be interpreted in two ways: either the precursors are associated with the external face of the outer mitochondrial membrane or they are retained while in transit through the TOM and TIM23 complexes with their C-termini still exposed to the cytosol (note that our anti-Cox2 antibody recognizes the C-terminus of Cox2). To distinguish between these two possibilities, we performed carbonate and Triton X-100 extractions of the mitochondrial fractions. We found that the precursors are mostly resistant to both carbonate (Figure 3C) and detergent treatments (Figure 3D). As expected, mature Cox2 proteins resist carbonate extraction but are solubilized into the supernatant upon Triton extraction (Figure 3, C and D). These observations suggest that most Cox2 precursors aggregate at the external mitochondrial surface, thus preventing their import.

Taken together, our data show that substitutions at a position different from that of W56

enable the successful allotopic expression of *COX* and that a major limiting step for importing allotopic Cox2 into mitochondria is its aggregation.

Lowering the dose of allotopic *COX2* expression enhances Cox2 import and assembly in CcO

While the use of a multicopy plasmid for allotopic expression of *COX2* to ensure successful complementation seemed justified a priori (Supekova *et al.*, 2010), we wondered whether reducing the allotopic expression of *COX2* would decrease the rescue of the respiratory defect proportionally. For this purpose, we opted to test allotopic expression under a condition where the same *COX2* construct carried in the multicopy plasmid was used as a single copy. To achieve this, we integrated the *COX2*-expressing construct (under the same phosphoglycerate kinase promoter and terminator present in the multicopy plasmid) in the nuclear genome. Surprisingly, we observed a notable increase in respiratory growth in the strains expressing the nuclear-integrated constructs producing $Cox2^{W56R}$ and $Cox2^{V49Q/L51G}$ in comparison with the strains producing the same Cox2 variants from a multicopy plasmid (Figure 4A). Growth of the yeast containing the single-copy $COX2^{W56R}$ construct was even comparable to that of a wild-type strain, and the strain containing the single-copy $COX2^{V49Q/L51G}$ exhibited more robust respiratory growth than the one with the high-copy $COX2^{W56R}$. The strain producing $Cox2^{WT}$ did not display any growth in respiratory medium, even at low doses. Immunodetection of Cox2 in mitochondria-enriched fractions of these strains showed that, at low dosage, $Cox2^{W56R}$ and $Cox2^{V49Q/L51G}$ only accumulated in their mature forms at levels similar to those of the WT strain (Figure 4B). As previously observed, at high dosage, $Cox2^{W56R}$ and $Cox2^{V49Q/L51G}$ were mainly observed as precursors, with only a fraction converted to the

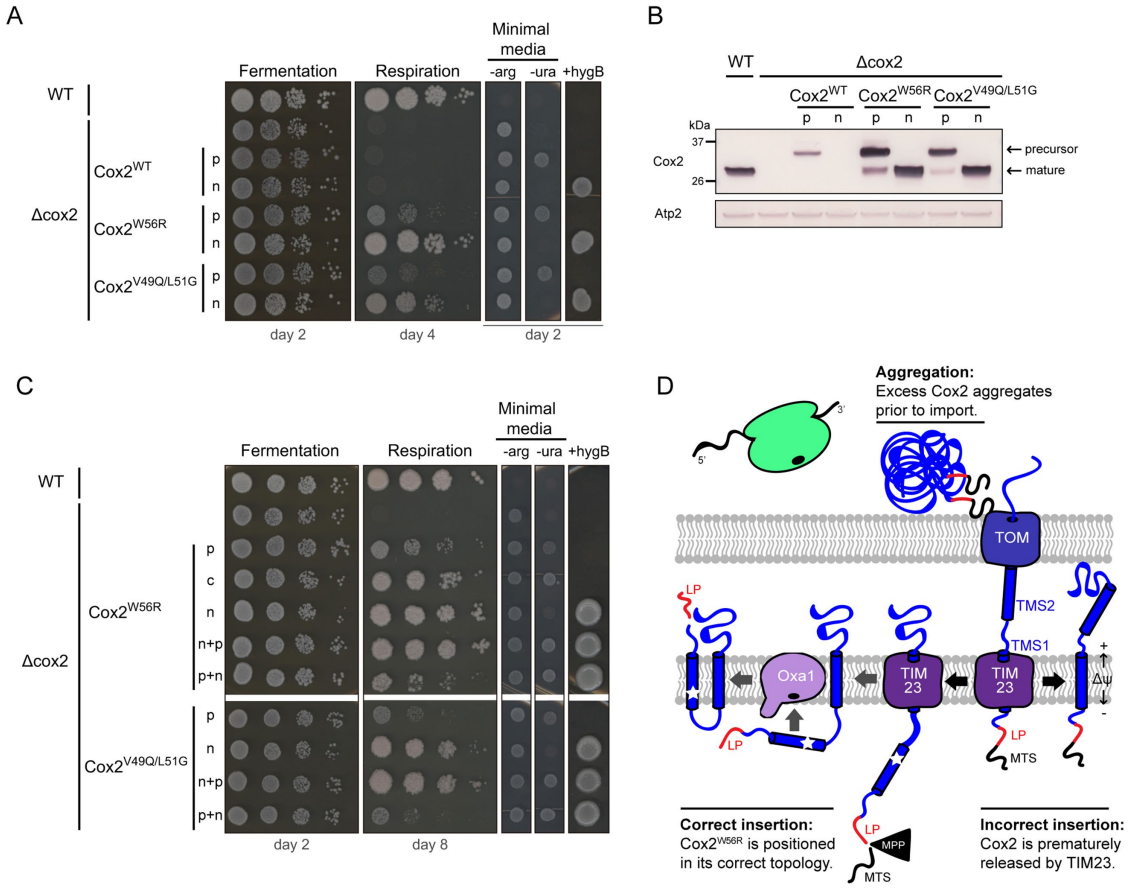

FIGURE 4: Lowering the expression of allotopic *COX2* in a *cox2*-null strain improves rescue of the respiratory growth. (A) Tenfold serial dilutions of yeast cultures from the indicated strains were plated on fermentable (glucose), respiratory (ethanol + glycerol), and minimal, selective media (-arg or -ura) and in the presence of hygromycin B (+hygB). The letter p represents expression from a multicopy plasmid, and the letter n represents expression from a single copy inserted in the nuclear genome. (B) Cox2 steady-state levels in isolated mitochondria (50 µg) from the indicated strains. Arrows indicate the precursor and mature forms of Cox2. The letters p and n indicate expression levels as in A. (C) Tenfold serial dilutions of yeast cultures from the indicated strains were plated on fermentable (glucose); respiratory (ethanol + glycerol), and minimal, selective media (-arg or -ura) and in the presence of hygromycin B (+hygB). The letter c represents expression from a centromeric plasmid. The order of the letters indicates the order in which *COX2*-expressing constructs were introduced into yeast cells. All the Cox2 variants contain an MTS from Oxa1. (D) Biogenesis model of allotopically synthesized Cox2. On the left, insertion of Cox2 results in its correct topology. On the right, the high hydrophobicity of the first TMS leads the cytosol-synthesized Cox2 to aggregate at the mitochondrial periphery or to be prematurely released by TIM23 into an incorrect topology. TOM: translocase of the outer membrane, TIM: translocase of the inner membrane, MPP: mitochondrial processing peptidase, LP: leader peptide, MTS: mitochondrial targeting sequence, TMS: transmembrane segment.

mature form. In line with the absence of respiratory growth of the strains carrying the *COX2*^{WT} construct, the Cox2^{WT} protein was observed only as a precursor and, at low dosage, its steady-state level was probably extremely low, as we could not detect it under our experimental conditions (Figure 4B). We observed the same behavior, that is, enhanced respiratory growth, lower accumulation of precursors, and higher levels of mature forms of Cox2, when the same constructs were expressed in few copies from a centromeric plasmid (Figure 4C and unpublished data). To test the importance of high versus low doses of Cox2 for the rescue of respiratory growth, we increased the dosage by producing the high-copy Cox2 variants (p) in a strain already encoding a single-copy Cox2 in the nuclear genome (n). We observed that this strain (n+p) grew in respiratory medium to levels similar to those of the strain expressing Cox2 encoded only in the nucleus (Figure 4C) and verified the presence of the precursors by immunoblotting (unpublished data). Unexpectedly, these increased levels of expression did not show the same growth defect of the high-copy Cox2 strain. However, when we inverted the order of production of allotopic Cox2 variants in the cells by integrating Cox2 into the nuclear genome in a strain already encoding a high-copy Cox2 (p+n), we observed that this strain showed the same level of respiratory growth as the strain expressing a high-copy Cox2 from a plasmid (Figure 4C).

The aforementioned results suggest that, although overexpression of allotopic *COX2* constructs is able to restore respiration to *cox2*-null strains, directing high doses of these proteins to mitochondria at the same time is counterproductive. Because the limiting step resides in the import of allotopic Cox2, low-dose expression seems to have a better outcome in terms of aggregation propensity and recovery of the respiratory growth.

DISCUSSION

The experimental relocation of mitochondrial genes to the nucleus has paved the way for the development of therapies intended to treat syndromes caused by mutations in the mitochondrial genome (González-Halphen *et al.*, 2004; Taylor and Turnbull, 2005; Kyriakouli *et al.*, 2008; Calvo and Mootha, 2010; Gorman *et al.*, 2016). Ongoing research has unveiled several constraining factors that limit allotopic expression (Claros *et al.*, 1995; Oca-Cossio *et al.*, 2003; Figueroa-Martínez *et al.*, 2011; Björkholm *et al.*, 2017). Because most proteins encoded in the mitogenome are integral to the membrane, the high hydrophobicity and the number of TMSs in these proteins seem to be major limiting factors for their import when synthesized in the cytosol (Claros *et al.*, 1995; Johnston and Williams, 2016). In this work we also found that aggregation propensity, which is directly correlated with high hydrophobicity (Chiti *et al.*, 2003; Dobson, 2004), contributes to the efficiency of importability before assembly of allotopic Cox2 into cytochrome *c* oxidase. Average hydrophobicities for the first TMSs of the different Cox2 variants used in this work are shown in Table 1.

First, we turned our attention to the Cox2 subunit to examine the importance of the residues enabling its allotopic production. Previous work demonstrated that substituting a tryptophan for an arginine decreases the hydrophobicity of the first TMS of Cox2 by adding a positive charge (Supekova *et al.*, 2010). In this study, substitution of a lysine or glutamine in the same position also allowed recovery of the respiratory growth, but residues containing a positive charge (arginine and lysine) yield better rescue than a residue decreasing the hydrophobicity (glutamine). It is possible that Cox2^{W56Q} is poorly inserted into the mitochondrial inner membrane, because the key determinant for importability is the presence of a positive charge (R^+ or K^+) and not reduction of the hydrophobicity

Cox2 variant	TMS1	H
Cox2^{WT}	IMFYLLVILGLVSWMLYTIVMTYS	0.349
Cox2^{W56K}	IMFYLLVILGLVS<u>K</u>MLYTIVMTYS	0.367
Cox2^{L51G}	IMFYLLVI<u>G</u>GLVSWMLYTIVMTYS	0.375
Cox2^{W56Q}	IMFYLLVILGLVS<u>Q</u>MLYTIVMTYS	0.395
Cox2^{W56R}	IMFYLLVILGLVS<u>R</u>MLYTIVMTYS	0.427
Cox2^{V49Q}	IMFYLL<u>Q</u>ILGLVSWMLYTIVMTYS	0.435
Cox2$^{V49Q/L51G}$	IMFYLL<u>Q</u>I<u>G</u>GLVSWMLYTIVMTYS	0.461
Cox2$^{L47Q/V49G}$	IMFY<u>Q</u>L<u>G</u>ILGLVSWMLYTIVMTYS	0.461
Cox2$^{L47Q/V49G/W56}$	IMFY<u>Q</u>L<u>G</u>ILGLVS<u>R</u>MLYTIVMTYS	0.540

Average hydrophobicity for the first transmembrane segment of each Cox2 variant used in this work. The "biological scale" was experimentally obtained and provides a hydrophobicity value for each residue present in the TMS of a model yeast mitochondrial protein (Calado-Botelho *et al.*, 2011). Cox2 variants are ordered by decreasing hydrophobicity (increasing hydrophilicity). Residue substitutions are underlined.

TABLE 1: Average hydrophobicity of the Cox2 variants.

(Q) at this position (von Heijne, 1992; Andersson and von Heijne, 1994) (see Table 1). This view is supported by the fact that while a similar number of Cox2 variant precursors accumulate in the mitochondria, the mature form of Cox2^{W56Q} is significantly less abundant than those of Cox2^{W56R} and Cox2^{W56K} (Figure 1B). This indicates that Cox2^{W56Q} is probably limited in its insertion in the inner membrane because it is more prone to aggregation. That mature Cox2^{W56K} and Cox2 ^{W56R} accumulate at the same level reinforces the view that a positive charge at this position is the key determinant of Cox2 importation into mitochondria when it originates from the cytosol.

Here, we designed allotopic Cox2 variants in yeast based on naturally evolving alterations in legumes. Notably, we observed that the double substitution reported as key for nuclear expression of Cox2 in *Glycine max* also allowed the allotopically expressed yeast *COX2* to restore respiration to a *cox2*-null mutant. Similarly to what was observed in in vitro import assays in legumes (Daley *et al.*, 2002b), each single-substitution V49Q or L51G was insufficient to restore respiration. In line with the cytosol-synthesized Cox2 biogenesis model (Daley *et al.*, 2002a; Jiménez-Suárez *et al.*, 2012; Rubalcava-Gracia *et al.*, 2018), the first TMS (TMS1) of Cox2 is transferred to the matrix through the TIM23 translocase, while the second TMS is laterally released into the inner membrane by TIM23 (Figure 4D). As a final step, TMS1 is inserted from the matrix in the inner membrane by Oxa1, positioning Cox2 in its correct topology (Figure 4D). We assume that in the presence of either substitution (V49Q or L51G), the proteins aggregate at the mitochondrial periphery before import because they are too hydrophobic (Figure 4D and Table 1). Accordingly, only the double substitution, which diminishes the overall hydrophobicity of the TMS1 of Cox2, allows its correct insertion into the inner membrane.

It should be mentioned that the legume Cox2 substitutions (V49Q/L51G) are less efficient in conferring respiratory growth than the W56R that was identified through random mutagenesis (Supekova *et al.*, 2010). Indeed, the W56R substitution meets Goldilocks conditions: besides maintaining CcO activity intact (Rubalcava-Gracia *et al.*, 2018), it balances the need to diminish the hydrophobicity of Cox2 TMS1, but at the same time, it maintains the ability of TMS1 to be recognized as a transmembrane domain by the inner membrane translocator Oxa1. Notably, in the mitochondrial Cox2 sequence from *Glycine max*, the residue corresponding

to position 56 contains an arginine, indicating that a positive charge in the first TMS of Cox2 is permissible, at least in some organisms. Production of low levels of $Cox2^{V49Q/L51G}$ results in the accumulation of mature Cox2 at a level similar to that of $Cox2^{W56R}$, suggesting that insertion in the inner membrane occurs to the same extent for both Cox2 variants (Figure 4B). However, the fact that the rescue with $Cox2^{V49Q/L51G}$ is significantly less efficient indicates that the V49Q/L51G modifications impact the biogenesis of Cox2 or the activity of the yeast enzyme. Furthermore, we observed that the combination of W56R substitution with the V49Q and L51G changes did not further improve respiratory growth, and resulted in lower C*c*O activity, as observed in Figure 2C. We assume that, despite the fact that these mutations enable respiratory growth, they may also affect C*c*O activity. Another possibility is that the hydrophobicity of the first TMS of the $Cox2^{L47Q/V49G/W56R}$ subunit is lowered to such an extent (Table 1) that it is not promptly recognized as a transmembrane domain and is therefore only partially routed for insertion into the inner membrane by the Oxa1 translocator.

Our experiments indicate that allotopically expressed proteins face a limiting step by aggregating before their import into mitochondria. When we lowered the *COX2* expression levels by integrating the *COX2* gene into the nuclear genome, we observed a notable increase in the rescue of the *cox2*-null strain, which correlated with lower levels of precursor and corresponding enhanced levels of mature Cox2. It is conceivable that excess cytosol-synthesized Cox2 aggregates at the mitochondrial surface, limiting the import of allotopic Cox2 (Figure 4D). These aggregates do not affect the import of other mitochondria-targeted proteins, as the precursors of Oxa1 and Atp2 were not observed to accumulate in our mitochondrial preparations (Figures 1B, 3A, and 4B). We noted that aggregation can also be avoided if large doses of Cox2 are produced in a strain already synthesizing small doses of allotopic Cox2. We explain this observation by the importing competence of *cox2*-null mitochondria. If large doses of the precursors of Cox2 are directly produced in a *cox2*-null mutant, most precursors aggregate and only a fraction assemble into C*c*O, resulting in difficulties in restoring respiration and in building up the electrochemical gradient required for further protein import. At small doses, aggregation of Cox2 is prevented and its assembly into C*c*O is enhanced, giving rise to an optimal transmembrane gradient that facilitates further import of cytosolic proteins. These results suggest that expression levels of the allotopic gene (from a centromeric plasmid or a high-copy plasmid) define the levels of importability versus aggregation of Cox2. It is also conceivable that cytosolic chaperones and other aggregation-response proteins might play a role in preventing the aggregation of Cox2 precursors.

Aggregation propensity is directly related to hydrophobicity and has been an underinvestigated factor in the study of allotopic expression. Highly hydrophobic proteins imported from the cytosol must avoid aggregating at the mitochondrial periphery to cross both mitochondrial membranes. All reports of allotopic expression in either yeast or cell cultures use high-level expression plasmids to ensure a high accumulation of the desired protein products inside the mitochondria (Guy *et al.*, 2002; Manfredi *et al.*, 2002; Ojaimi *et al.*, 2002; Bokori-Brown and Holt, 2006; Bonnet *et al.*, 2008; Figueroa-Martínez *et al.*, 2011; Cwerman-Thibault *et al.*, 2015; Boominathan *et al.*, 2016). Here, we found that a high level of the allotopically expressed Cox2 protein may be detrimental to the mitochondrial import system.

In summary, we provide a proof of principle that mimicking the modifications found in an organism that naturally transferred its mitochondrial gene to the nucleus can result in successful allotopic expression. This strategy includes expressing a gene with adequate mutations in a low copy number to ensure that moderate protein quantities are produced for mitochondrial import. Our results underscore that dosage, a previously unexplored important parameter, may be critical for allotopic expression in mammals, especially in human cell lines. The relevance of our finding is high, since allotopic expression still represents a promising strategy for developing treatments for patients with mitochondrial diseases. Our data also stress the relevance of studying organisms that naturally and atypically encode hydrophobic mitochondrial OXPHOS proteins in the nuclear genome.

MATERIALS AND METHODS

Strains, gene constructs, and culture conditions

The *Saccharomyces cerevisiae* strains used in this study were derived from the D273-10B parental strain. We used NB40-36A as the wild-type strain *(MATα, arg8::hisG, leu2-3, 112, lys2, ura3-52,* [rho+]), EHW154 as the Δ*cox2* strain (*MATa, arg8::hisG, his3-ΔHindIII, leu2-3, 112, lys2*, *ura3-52*, [rho+] *cox2-208::ARG8m*), and CAB116 as the Δ*cox18* strain (*MATa*, *arg8::hisG, his3-ΔHindIII, leu2-3,112, lys2, ura3-53, cox18Δ::KanMX4* [rho+]). Cells were grown on YPD as fermentable medium (1% yeast extract, 2% bactopeptone, and 2% dextrose) or YPEG as nonfermentable medium (1% yeast extract, 2% bactopeptone, 3% ethanol, and 3% vol/vol glycerol). Minimal medium was SD or SGal (0.17% yeast nitrogen base [without amino acids and $(NH_4)_2SO_4$], 0.5% $(NH_4)_2SO_4$, 2% glucose or galactose supplemented with specific amino acids and nucleotides). All strains were grown at 30°C in liquid (with shaking) or solid medium (containing 1.75% agar). We calculated duplication times by measuring the absorbance of cultures with an initial $O.D._{600}$ of 0.01 every 2 h in a Bioscreen C spectrophotometer (Growth Curves, USA). Dilution series were performed by diluting 10-fold a culture of 0.5 $O.D._{600}$ and plating 3 µl on Petri dishes with the indicated medium.

The yeast allotopic $COX2^{W56R}$ gene was recoded for expression from the nucleus and a sequence encoding the MTS of Oxa1 was added at the 5′ end, as previously described (Supekova *et al.*, 2010). The construct was chemically synthesized (GeneScript, Piscataway, NJ) and cloned at the *Not*I site in the pFL61 vector, which contains a phosphoglycerate kinase (*PGK*) promoter and terminator (Minet *et al.*, 1992).

Additional *COX2* mutations (WT, W56K, W56Q, V49Q, L51G, V49Q/L51G, L47Q/V49G, L47Q/V49G/W56R) were constructed using the pFL61 plasmid containing the $COX2^{W56R}$ insert via site-directed mutagenesis, according to the manufacturer's instructions (QuikChange II site-directed mutagenesis kit, Agilent Technologies). The presence of the site-directed mutations was confirmed by DNA sequencing. For the expression of the constructs from centromeric plasmids, the fragments containing the *COX2* constructs flanked by the *PGK* promoter and terminator were cloned from pFL61 into pRS306H upon digestion with *Hin*dIII and *Sma*I restriction enzymes. Nuclear localization was achieved by integration transformation at the *URA3* nuclear locus using the pRS306H plasmid and hygromycin B resistance as a selection (Taxis and Knop, 2006).

Yeast cells were transformed in the presence of lithium acetate and salmon sperm DNA as described (Gietz and Schiestl, 2007). At least 12 transformed individual colonies were grown in Petri dishes containing fresh SD medium and then were replica-plated into YPEG to ensure that the selected colonies had respiratory growth similar to the rest of the transformants.

Isolation of yeast mitochondria

Mitochondria were isolated from 100–200 ml of liquid SGal cultures as previously reported (Herrmann *et al.*, 1994). Briefly, cells were harvested at logarithmic growth phase ($O.D._{600}$ 0.8–1.0), washed with H_2O at 4500 rpm in a Beckman Coulter JA-25.5 rotor (2500 × *g*) for 5 min, resuspended in TD buffer (100 mM Tris, 10 mM dithiothreitol), and centrifuged at 4500 rpm (2500 × *g*) for 5 min. The cells were resuspended in 10 ml of zymoliase buffer (1.2 mM sorbitol, 20 mM KH_2PO_4 [pH 7.4], 3–5 mg zymoliase/g of wet weight), incubated for 1 h in a shaker at 30°C, and centrifuged at 4500 rpm (2500 × *g*) for 5 min at 4°C. The pellet was resuspended in 1 ml of Dounce buffer (0.6 M sorbitol, 10 mM Tris [pH 7.4], 1 mM EDTA, 0.2% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride [PMSF]) using a homogenizer and pestle (30 strokes). Samples were transferred to 1.5-ml tubes and centrifuged at 4500 rpm in a microcentrifuge (2000 × *g*) for 5 min at 4°C. The supernatants were transferred to new tubes and centrifuged at 12,000 rpm (13,500 × *g*) for 10 min at 4°C. The supernatant (cytosolic fraction) was collected in new tubes and the pellet (mitochondrial fraction) was resuspended in 100 µl of SH buffer (0.6 M sorbitol, 20 mM HEPES, pH 7.4). Protein concentration was determined by a modified Lowry method (Markwell *et al.*, 1978). Mitochondria and cytosolic fractions were frozen and stored at –70°C until use.

Proteinase K protection assays

For protease treatment of mitochondria, 50 µg of mitochondria was gently resuspended in 100 µl of SH buffer and incubated at 20°C for 30 min in the presence of 100 µg/ml Proteinase K; parallel reactions were made in the presence of 1% Triton X-100. PMSF was added to all suspensions (at 4 mM final concentration), which were then incubated on ice for 10 min and centrifuged at 14,000 rpm (18,400 × *g*) for 10 min. Most of the supernatant in each tube was discarded; the pellet was resuspended with 4 µl of 6× loading buffer (375 mM Tris-HCl [pH 6.8], 6% SDS, 48% glycerol, 9% β-mercaptoethanol) + 4 mM PMSF and incubated for 1 min in boiling water before being loaded onto 12% SDS-tricine-PAGE gels.

Sodium carbonate and Triton X-100 extractions

Integral membrane proteins were separated from peripheral or soluble proteins by carbonate extraction. Aggregated proteins were separated from membrane proteins by Triton X-100 extraction. Briefly, 50 µg of mitochondria was incubated with 100 µl of cold 0.1 M Na_2CO_3 (pH 11.0) or with 9.4 µl of 1% Triton X-100 (2 mg Triton X-100/mg protein) for 30 min on ice. The samples were centrifuged at 90,000 × *g* in a TLA 55 rotor for 30 min at 4°C. Supernatants and pellets were incubated with 500 µl of 12.5% TCA for 15 min on ice, followed by centrifugation for 15 min at 14,000 rpm (18,400 × *g*) at 4°C. Samples were washed by adding 500 µl of cold 100% acetone, centrifuged for 10 min at 14,000 rpm (18,400 × *g*) at 4°C, and dried for ~10 min at room temperature. The dried pellets were resuspended in 15 µl of 1× loading buffer and incubated for 1 min in boiling water before loading onto 12% SDS-tricine-PAGE gels.

Gel electrophoresis and in-gel enzymatic activities

Denaturing gel electrophoresis was carried out via SDS-tricine-polyacrylamide gels with 12% acrylamide (Schagger, 1994a). Sample preparation and blue native polyacrylamide gel electrophoresis (BN-PAGE) were carried out as described (Schagger, 1994b). Briefly, mitochondria were washed twice in 250 mM sorbitol, 50 mM Bis-Tris (pH 7.0), and centrifuged at 12,000 rpm (13,500 × *g*) for 10 min at 4°C. The pellet was resuspended in sample buffer (750 mM aminocaproic acid, 50 mM Bis-Tris, pH 7.0) and solubilized with 2 g of lauryl maltoside per g of protein for 30 min with gentle stirring and centrifuged at 13,200 rpm (16,400 × *g*) at 4°C for 12 min. The supernatants were loaded on 5–15% polyacrylamide gradient gels. The stacking gel contained 4% (wt/vol) polyacrylamide. In-gel activities were carried out following established procedures for C*c*O activity (Wittig *et al.*, 2007; Wittig and Schägger, 2007) and ATPase activity (Zerbetto *et al.*, 1997; Wittig and Schägger, 2005). Gels were stained with Coomassie solution (50% ethanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue G) or otherwise transferred and then subjected to immunodetection.

Immunodetection

From SDS-tricine-PAGE, proteins were electrotransferred onto a nitrocellulose Trans-Blot membrane (Bio-Rad) for immunodetection. Membranes were washed, blocked, and independently incubated for 4 h with the following antibodies: anti-Cox2 antibody at a 1:9000 dilution (Invitrogen; Molecular Probes), anti-Oxa1 antibody at a 1:1000 dilution, anti-Hog1 antibody at a 1:2000 dilution (Santa Cruz Biotechnology), and anti-Atp2 antibody at a 1:50,000 dilution. Alkaline phosphatase-conjugated IgGs (1:15,000 for 2 h) were used as secondary antibodies. Insoluble black–purple precipitates were formed upon addition of nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyl phosphate *p*-toluidine salt. Images of the immunodecorated polypeptide bands were captured in an HP Scanjet G4050. For immunodetection on previously probed membranes using a different primary antibody, membranes were stripped by incubation for 45 min at 50°C in the presence of 2% SDS, 62.5 mM Tris-HCl, pH 6.8, and 100 mM β-mercaptoethanol.

In silico protein sequence analysis

A TMS1 of 24 residues was considered for the yeast Cox2. The hydrophobicity calculations were performed using the "biological" hydrophobicity scale (Calado-Botelho *et al.*, 2011).

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