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4-2012

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### **Recommended Citation**

Strogolova, Vera; Furness, Andrew; Robb-McGrath, Micaela; Garlich, Joshua; and Stuart, Rosemary A., "Rcf1 and Rcf2, Members of the Hypoxia-induced Gene 1 Protein Family, Are Critical Components of the Mitochondrial Cytochrome bc1-cytochrome Oxidase Supercomplex" (2012). *Biological Sciences Faculty Research and Publications*. 139.

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# Rcf1 and Rcf2, Members of the Hypoxia-Induced Gene 1 Protein Family, Are Critical Components of the Mitochondrial Cytochrome *bc*<sub>1</sub>-Cytochrome *c* Oxidase Supercomplex

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We report that Rcf1 (formerly Aim31), a member of the conserved hypoxia-induced gene 1 (Hig1) protein family, represents a novel component of the yeast cytochrome *bc*<sub>1</sub>-cytochrome *c* oxidase (COX) supercomplex. Rcf1 (respiratory supercomplex factor 1) partitions with the COX complex, and evidence that it may act as a bridge to the cytochrome *bc*<sub>1</sub> complex is presented. Rcf1 interacts with the Cox3 subunit and can do so prior to their assembly into the COX complex. A close proximity of Rcf1 and members of the ADP/ATP carrier (AAC) family was also established. Rcf1 displays overlapping function with another Hig1-related protein, Rcf2 (formerly Aim38), and their joint presence is required for optimal COX enzyme activity and the correct assembly of the cytochrome *bc*<sub>1</sub>-COX supercomplex. Rcf1 and Rcf2 can independently associate with the cytochrome *bc*<sub>1</sub>-COX supercomplex, indicating that at least two forms of this supercomplex exist within mitochondria. We provide evidence that the association with the cytochrome *bc*<sub>1</sub>-COX supercomplex and regulation of the COX complex are a conserved feature of Hig1 family members. Based on our findings, we propose a model where the Hig1 proteins regulate the COX enzyme activity through Cox3 and associated Cox12 protein, in a manner that may be influenced by the neighboring AAC proteins.

Mitochondria are responsible for the aerobic production of ATP through a process termed oxidative phosphorylation (OXPHOS) (29, 35, 39). The enzyme complexes involved in the OXPHOS pathway are embedded in the mitochondrial inner membrane and include the electron transport chain complexes (ETC) and the F<sub>1</sub>F<sub>0</sub>-ATP synthase. Cytochrome *c* oxidase (COX) is the terminal member of the ETC and passes electrons from cytochrome *c* to O<sub>2</sub>, generating water (13, 30, 35, 41). The ATP generated in the mitochondrial matrix is shuttled out of the mitochondria, in exchange for ADP, through the ADP/ATP carrier (AAC) protein family (33). The ETC physically associate with each other in the membrane to form supercomplexes, e.g., the cytochrome *bc*<sub>1</sub>-COX supercomplex, which is thought to enhance the electron transfer and regulation of these complexes (8, 12, 20, 29, 35, 39, 40). Evidence for the physical association of the AAC proteins with the cytochrome *bc*<sub>1</sub>-COX supercomplex has also been reported, and this may represent a mechanism for communication between the levels of OXPHOS activity and the ATP/ADP levels in the mitochondria (7, 11, 40).

Both the cytochrome *bc*<sub>1</sub> and COX complexes are oligomeric complexes of mosaic origin. In the case of the COX complex, the three central core subunits, Cox1, Cox2, and Cox3, are encoded by the mitochondrial DNA (mtDNA; [*rho*]) and are homologous to the corresponding COX enzyme subunits in bacteria (9, 41). The remaining COX subunits are encoded in the nucleus and imported into mitochondria, where they are assembled in an organized and multistep fashion with the proteins Cox1, -2, and -3 (9, 13–15, 30, 31). A significant amount of information is known about the assembly and function of the Cox1 and Cox2 proteins, and specific assembly chaperones of these proteins have been reported (13, 30, 41). No assembly partner for Cox3 has been identified to date, in either bacterial or mitochondrial systems.

A number of independent studies in diverse organisms aimed at identifying genes whose expression is induced during hypoxia have led to the discovery of a gene family termed the hypoxia-

induced gene 1 (Hig1) family (1, 10, 17, 19, 38, 42). The Hig1 family is conserved throughout evolution, with members in the alphaproteobacteria and diverse eukaryotic taxa, including plants, fungi, nematodes, and mammals. Elevated expression of Hig1 family members was also found in response to other stress inducers, including low glucose and light and oxidative stress, and in hypoxic cervical carcinoma cells (1, 10, 17, 19, 38, 42). Preliminary studies indicate that some Hig1 isoforms may exert an antiapoptotic function, in particular in response to prolonged exposure to oxidative and/or hypoxic stress (1, 38, 42). Although one study showed a Hig1 isoform to be a mitochondrial inner membrane protein (42), the function(s) of these conserved Hig1 proteins is currently unknown.

In the yeast *Saccharomyces cerevisiae*, the Aim31 protein displays a high degree of similarity to members of the Hig1 protein family. Originally discovered in a screen designed to identify genes encoding proteins whose absence caused an altered inheritance of mtDNA (AIM) (22), the function of Aim31 was unknown. Here we report that Aim31 can be found in association with the cytochrome *bc*<sub>1</sub>-COX supercomplex, where it appears to bind to both aspects of this complex, i.e., to both the cytochrome *bc*<sub>1</sub> and COX enzyme domains. We demonstrate that Aim31 is more tightly associated with the COX complex, where it displays a close phys-

Received 29 September 2011 Returned for modification 19 December 2011

Accepted 27 January 2012

Published ahead of print 6 February 2012

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doi:10.1128/MCB.06369-11

ical relationship with the Cox3 protein. Furthermore, we show that Aim31 shares an overlapping function with a second mitochondrial protein with limited similarity to Aim31 and Hig1 proteins, the Aim38 protein. Our data indicate that Aim31 and Aim38 may independently bind to the cytochrome  $bc_1$ -COX supercomplex and that loss of both Aim31 and Aim38 (but not loss of only one of them) has a significant impact on the COX enzyme activity and assembly of the peripheral COX subunits Cox12 and Cox13. The presence of Aim31 and Aim38 proteins is required for the correct assembly of the cytochrome  $bc_1$ -COX supercomplex, and we propose that they may act as bridges to support the assembly of the supercomplex state. Given their functional relevance for the COX enzyme and their physical association with the cytochrome  $bc_1$ -COX supercomplex, we propose renaming the Aim31 and Aim38 proteins Rcf1 and Rcf2, respectively (Rcf is for respiratory supercomplex factors). Together, our findings shed light on the important role the conserved Hig1 protein family plays in the regulation and functioning of mitochondrial COX complex, the most abundant oxygen-consuming enzyme of the cell.

## MATERIALS AND METHODS

**Yeast strains and growth conditions.** The YML030w/AIM31 and the YNR018w/AIM38 genes are referred to here as the *RCF1* and *RCF2* genes, respectively. *S. cerevisiae* strains used in this study are the wild type (WT; W303-1A *mat $\alpha$  leu2 trp1 ura3 his3 ade2*), an *RCF1::HIS3* ( $\Delta$ *rcf1*) strain (W303-1A *mat $\alpha$  leu2 trp1 ura3 ade2*), an *RCF2::KAN* ( $\Delta$ *rcf2*) strain (W303-1B *mat $\alpha$  leu2 trp1 ura3 his3 ade2*), and an *RCF1::HIS3 RCF2::KAN* ( $\Delta$ *rcf1*  $\Delta$ *rcf2*) strain (W303-1A *mat $\alpha$  leu2 trp1 ura3 ade2*). The double mutant was created by mating the respective single mutant haploid strains and followed by tetrad dissection of the resulting diploid strain. Yeast strains were maintained and cultured using standard protocols at 30°C on YP medium supplemented with 2% glucose and 20 mg/liter adenine hemisulfate (YPAD). Mitochondria were isolated from cultures grown in YP-0.5% lactate medium supplemented with 2% galactose (YP-Gal).

**Expression of the His-tagged protein derivatives.** For the expression of His-tagged proteins, a DNA fragment encoding Rcf1<sub>His</sub>, Rcf1 $\Delta$ C<sub>His</sub> (residues 1 to 112), Rcf2<sub>His</sub>, M05D6.5<sub>His</sub>, Aac1<sub>His</sub>, and Aac3<sub>His</sub> (each bearing a 12-histidine tag at the C terminus) was generated through PCR using yeast genomic DNA or *Caenorhabditis elegans* cDNA as a template and was cloned as an XbaI/PstI fragment into a Yip351-based vector downstream of the galactose-inducible GAL10 promoter. The recombinant plasmids were integrated into the yeast genome of indicated strains at the *leu2* gene locus following linearization with BstEII. Expression of the His-tagged proteins was induced by the inclusion of galactose in the growth medium, as indicated in the legend to Fig. 3, and was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and immunodecoration of mitochondrial extracts from resulting transformants. The creation and expression of the HisAac2 derivative have been previously described (11).

**Affinity purification of His-tagged proteins.** Mitochondria (200  $\mu$ g protein) were solubilized in lysis buffer (100 mM KCl, 20 mM HEPES-KOH, 10 mM MgCl<sub>2</sub>, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4), containing either 2.0% digitonin or 0.25% Triton X-100, or as otherwise indicated, for 30 min on ice and then Ni-NTA purification of His-tagged proteins was essentially performed as previously described (24).

**Sucrose gradient centrifugation.** Mitochondria (200  $\mu$ g protein) were solubilized in 20 mM potassium phosphate (pH 7.4), 15% glycerol, 2 mM EDTA, 0.25% Triton X-100, 1 mM PMSF, subjected to a clarifying spin, applied to a 5 to 50% sucrose (wt/vol) gradient, and centrifuged, essentially as described in reference 6. Fractions were collected, trichloroacetic acid (TCA) precipitated, and analyzed by SDS-PAGE and Western blotting.

**Miscellaneous.** Chemical cross-linking with *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) was performed essentially as de-

scribed previously (25). NADH-cytochrome  $bc_1$  and COX enzyme activities were measured in the presence of sodium deoxycholate (0.5%) essentially as previously described (11, 24). The relative specific activities of the enzymes were calculated for both wild-type and mutant mitochondria and were averaged from three replica measurements. Mitochondrial isolation, protein determinations, carbonate extraction, blue native PAGE (BN-PAGE) and SDS-PAGE were performed according to published methods (2, 11, 24, 27). Translation *in organello* with [<sup>35</sup>S]methionine labeling was performed as described previously (24).

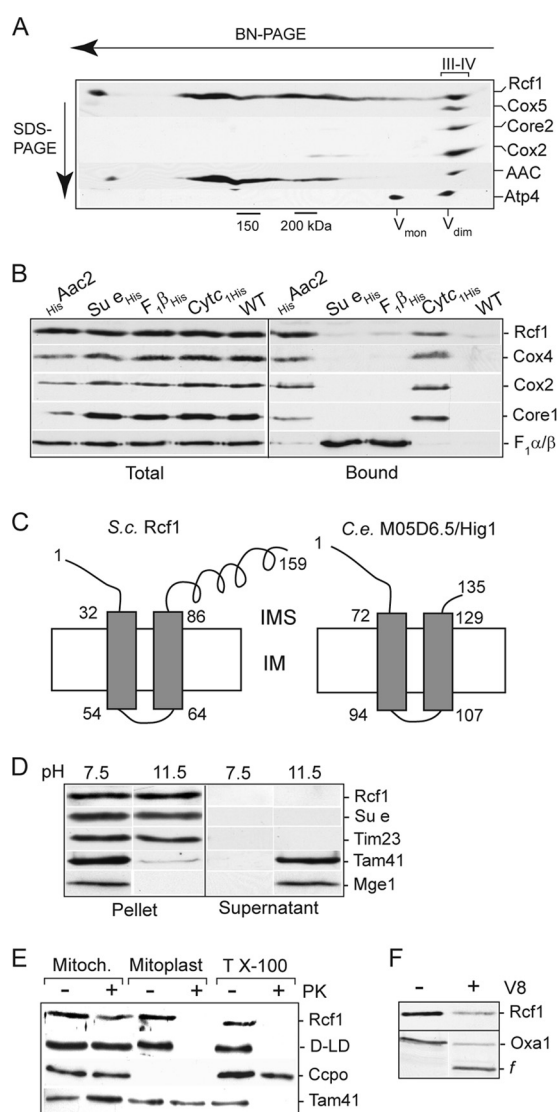
## RESULTS

### Rcf1 is associated with the cytochrome $bc_1$ -COX supercomplex.

When analyzing the association of the Aac2 protein with the cytochrome  $bc_1$ -COX supercomplex on native gels (BN-PAGE), we discovered a novel comigrating 18-kDa protein (Fig. 1A). Mass spectrometry analysis indicated it to be the protein encoded by the AIM31/YML030w gene, and it is referred to in this study as Rcf1. To directly demonstrate that the Rcf1 protein was physically associated with the cytochrome  $bc_1$ -COX-AAC supercomplex (and not with the closely migrating F<sub>1</sub>F<sub>0</sub>-ATP synthase dimeric complex), we performed an affinity purification of the cytochrome  $bc_1$ -COX-AAC supercomplex using histidine (His)-tagged cytochrome  $c_1$  and Aac2 derivatives. Mild (digitonin) solubilization conditions were used to maintain the organization of this supercomplex (Fig. 1B). Rcf1 copurified with both cytochrome  $c_{1\text{His}}$  and HisAac2 but not with His-tagged F<sub>1</sub> $\beta$  and subunit e (Su e) proteins, two components of the dimeric ATP synthase complex. We conclude therefore that Rcf1, like the AAC proteins, exists in association with the cytochrome  $bc_1$ -COX supercomplex.

The Hig1 homology domain is localized to the N-terminal region of Rcf1, a region that encompasses two putative transmembrane segments. The C-terminal hydrophilic segment with a predicted coiled-coil domain is not a conserved feature of Hig1 proteins and appears to be limited to the fungal Rcf1 proteins (Fig. 1C). Carbonate extraction experiments demonstrated that Rcf1 was resistant to the alkaline extraction and thus behaved like the integral inner membrane control proteins, Su e of the F<sub>0</sub>-ATP synthase and Tim23. In contrast, Mge1 and Tam41, which are matrix-soluble and matrix-localized peripheral inner membrane control proteins, respectively, were extracted by the carbonate treatment (Fig. 1D). Protease (proteinase K and V8 proteases) accessibility studies demonstrated that Rcf1 is localized in the inner membrane, where it became accessible to proteases only after hypotonic swelling (Fig. 1E and F). In mitoplasts, Rcf1 displayed a degree of susceptibility to the V8 protease similar to that of Oxal, a control inner membrane protein with an N<sub>out</sub>-C<sub>in</sub> orientation. As the V8 protease cleaves preferentially after negatively charged residues, found only in the N- and C-tail segments of Rcf1 (and not present in the hydrophilic loop between the two transmembrane domains of this protein), we conclude that the N and C termini of Rcf1 are exposed to the intermembrane space, where they are accessible to the V8 protease. Protease accessibility analysis of the C-terminal His epitope of Rcf1<sub>His</sub> also confirmed this membrane orientation (results not shown). In summary, we conclude that Rcf1 is an integral inner membrane protein that adopts an N<sub>out</sub>-C<sub>out</sub> orientation (Fig. 1C).

**Rcf1 associates with Cox3 and partitions with the COX complex but also can bind to the cytochrome  $bc_1$  complex.** In order to further explore the association of Rcf1 with the cytochrome  $bc_1$ -COX supercomplex, we expressed a C-terminally His-tagged Rcf1 in the wild-type and  $\Delta$ *rcf1* strains. Under digitonin solubilization



**FIG 1** Rcf1, an inner membrane protein, associates with the cytochrome *bc*<sub>1</sub>-COX supercomplex. (A) Wild-type mitochondria were solubilized with digitonin (1%) and subjected to BN-PAGE electrophoresis followed by a second-dimension SDS-PAGE. The resulting Western blot was immunodecorated with antibodies directed against subunits of the COX (Cox5 and Cox2), cytochrome *bc*<sub>1</sub> (Core2), and F<sub>1</sub>F<sub>0</sub>-ATP synthase (Atp4), AAC, and Rcf1. The positions of the dimeric and monomeric ATP synthase (*V*<sub>dim</sub> and *V*<sub>mon</sub>), cytochrome *bc*<sub>1</sub>-COX supercomplex (III-IV), and 150- and 200-kDa markers are indicated. (B) Mitochondria were isolated from strains harboring either His-tagged Aac2, subunit e (Su e), F<sub>1</sub>β, or cytochrome *c*<sub>1</sub> (Cyt *c*<sub>1</sub>) proteins or from control wild-type cells (i.e., lacking a His-tagged protein), solubilized with digitonin (0.5%), and subjected to Ni-NTA purification, Western blotting, and immunodecoration. "Total" indicates 5% of the solubilized mitochondria; "Bound" indicates the Ni-NTA-eluted material. (C) Depiction of the N<sub>out</sub>-C<sub>out</sub> orientation of *S. cerevisiae* (S.c.) Rcf1 and *C. elegans* (C.e.) M05D6.5 (Hig1 homolog) in the inner membrane (IM), with N and C tails exposed to the intermembrane space (IMS). (D) Isolated wild-type mitochondria (0.1 mg/ml) were extracted by alkaline treatment using 0.1 M carbonate buffer at pH 11.5. Control mitochondria (pH 7.5) were resuspended in 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.5. Following extraction, samples were subjected to ultracentrifugation, and the resulting membrane pellet and supernatant (after TCA precipitation) were analyzed by SDS-PAGE, Western blotting, and immunodecoration for Rcf1 and control proteins. (E) Proteinase K (20 μg/ml) treatment was performed on intact mitochondria (Mitoch.) or mitochondria which had been subjected to hypotonic swelling (Mitoplast) or Triton X-100 detergent lysis (T X-100) on ice. Samples were subjected to

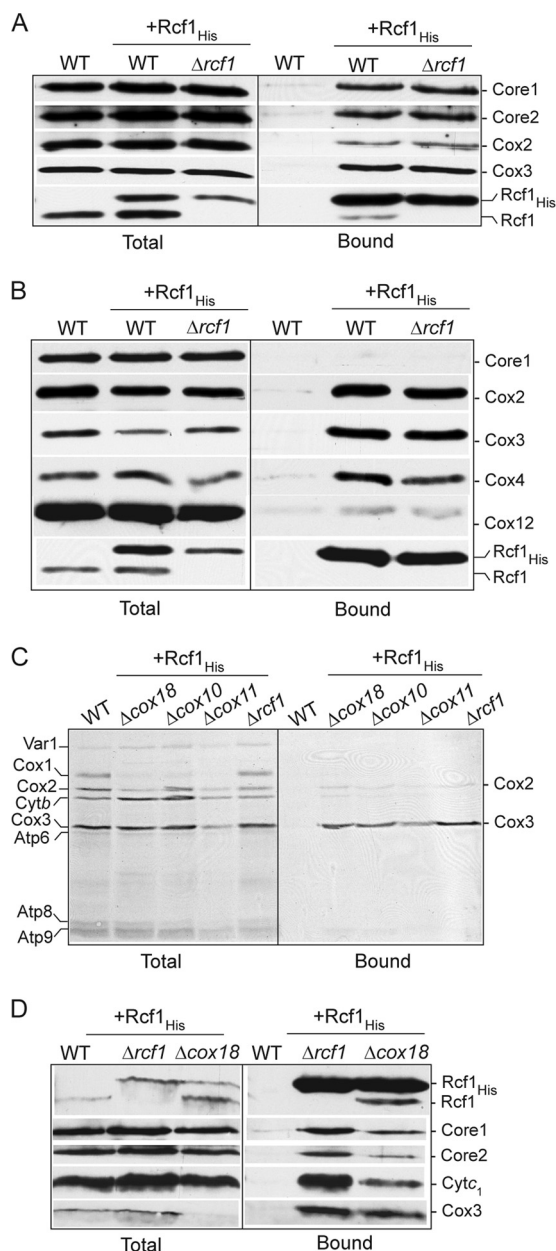
conditions, Rcf1<sub>His</sub> from both mitochondrial types was found in association with the cytochrome *bc*<sub>1</sub>-COX supercomplex, as demonstrated by the copurification of components of both the cytochrome *bc*<sub>1</sub> (Core1 and Core2) and the COX (Cox2 and Cox3) complexes (Fig. 2A). To analyze if Rcf1 preferentially associates with either the cytochrome *bc*<sub>1</sub> or COX aspect of this supercomplex, we next purified Rcf1<sub>His</sub> under Triton X-100 solubilization conditions, because this detergent causes the dissociation of the cytochrome *bc*<sub>1</sub> from the COX complex (8, 24). Under these conditions, Rcf1<sub>His</sub> was observed to fractionate with the COX and not with the cytochrome *bc*<sub>1</sub> complex, as indicated by the copurification of COX subunits and the absence of the Core1 protein (Fig. 2B). The efficiency of Cox12 copurification with Rcf1<sub>His</sub> was significantly less than that of the other COX subunits analyzed. Cox12 (equivalent to mammalian Cox6b [mCox6b]) is a peripheral COX subunit associated with the intermembrane-space side of the complex (4, 9, 23, 28, 41), which reflects the fact that the association of Cox12 with the COX complex is not as stable as other COX subunits under these Triton X-100 solubilization conditions (results not shown).

To address the question of whether Rcf1 displayed the capacity to directly interact with one of the mitochondrially encoded cytochrome *bc*<sub>1</sub> or COX subunits (cytochrome *b*, Cox1, Cox2, or Cox3), protein synthesis in the presence of [<sup>35</sup>S]methionine was performed in mitochondria isolated from the  $\Delta$ *rcf1* strain harboring Rcf1<sub>His</sub>. Following translation, mitochondria were solubilized with Triton X-100, and the Rcf1<sub>His</sub> and associated proteins were affinity purified. Radiolabeled Cox3, and to a significantly lesser extent Cox2, was found to copurify with Rcf1<sub>His</sub> (Fig. 2C). As the assembly of newly synthesized Cox3 does not proceed to completion in isolated mitochondria, we conclude that the association of the Rcf1 and Cox3 proteins occurs independently of their final assembly into the COX enzyme. This conclusion was further supported by the observation that radiolabeled Cox3 synthesized in mitochondria isolated from the  $\Delta$ *cox10*,  $\Delta$ *cox11*, and  $\Delta$ *cox18* mutants could also be affinity purified with Rcf1<sub>His</sub> (Fig. 2C). These mutant strains are defective in different stages of the COX assembly process, due to the absence of specific COX assembly factors (13, 30). In the absence of assembly into the COX complex, the Cox3 protein is susceptible to proteolytic turnover, but the low steady-state levels of Cox3 remaining in the isolated  $\Delta$ *cox18* mitochondria were efficiently recovered in association with Rcf1<sub>His</sub> under both Triton X-100 (results not shown) and digitonin (Fig. 2D) solubilization conditions.

We next took advantage of the COX assembly mutants to address the question of whether Rcf1 could associate with the cytochrome *bc*<sub>1</sub> complex in the absence of an assembled COX complex. When Rcf1<sub>His</sub> was purified from  $\Delta$ *cox18* mitochondria under digitonin solubilization conditions, Rcf1<sub>His</sub> was found in association, not only with Cox3, but also with the cytochrome *bc*<sub>1</sub> com-

plex. Cytochrome *c* peroxidase (Ccpx), a soluble IMS marker protein, displays inherent protease stability and hence is not degraded by the proteinase K treatment in the presence of Triton X-100. D-LD, D-lactate dehydrogenase, an IM protein with a large C-terminal domain exposed to the IMS. (F) Wild-type mitochondria were hypotonically swollen, subjected to treatment with V8 protease (20 μg/ml), and further processed as described for panel B. Oxa1 and the fragment (f) of Oxa1 resulting from V8 degradation of the N-tail of Oxa1 exposed to the IMS are indicated.





**FIG 2** Rcf1 can independently associate with Cox3 and the COX and cytochrome  $bc_1$  complexes. (A and B) Mitochondria isolated from wild-type (WT) cells and  $\Delta rcf1$  cells harboring Rcf1<sub>His</sub> and control wild-type mitochondria were solubilized in 2% digitonin (A) or 0.25% Triton X-100 (B), subjected to Ni-NTA purification, and further analyzed as described for Fig. 1B. (C) *In organello* protein synthesis in the presence of [<sup>35</sup>S]methionine was performed in mitochondria isolated from wild-type control cells or from Rcf1<sub>His</sub>-containing cells. Following Triton X-100 solubilization, Ni-NTA purification was performed, followed by SDS-PAGE and autoradiography. (D) Rcf1<sub>His</sub> was purified from  $\Delta rcf1$  or  $\Delta cox18$  mitochondria under digitonin solubilization conditions and further analyzed as described for Fig. 1B.

plex, as evidenced by the coassociation of Core1, Core2, and cytochrome  $c_1$  proteins (Fig. 2D).

From these results, we conclude that Rcf1 is found together with the cytochrome  $bc_1$ -COX supercomplex, where it must be supported by at least two independent associations, one contact point being with the cytochrome  $bc_1$  complex (which is digitonin

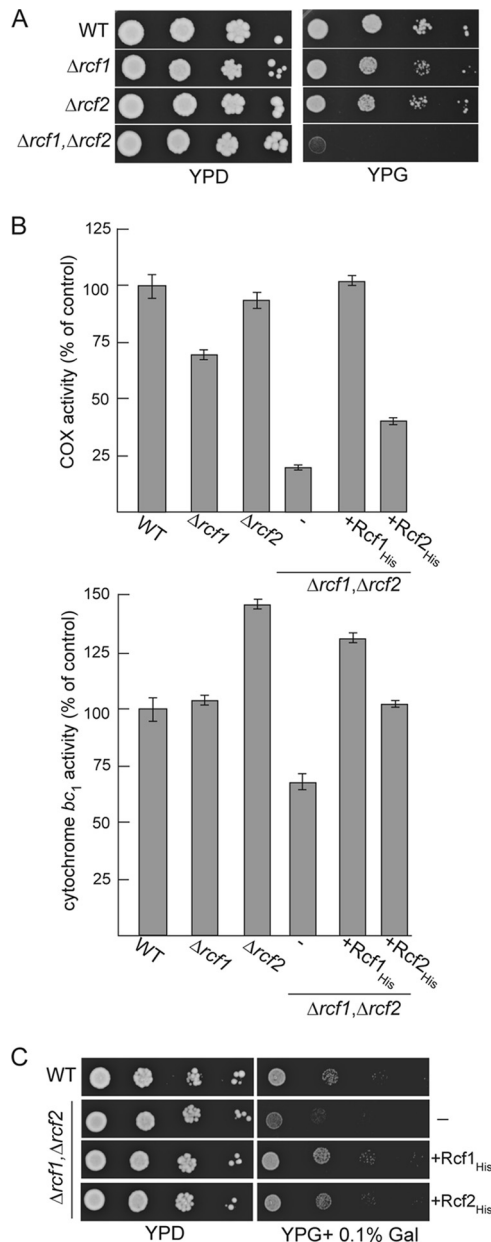
stable but Triton X-100 sensitive) and the second being with the COX complex, which is Triton X-100 stable. Rcf1 associates with Cox3 prior to its assembly into the COX complex, and presumably this strong Rcf1-Cox3 interaction may contribute to the partitioning of Rcf1 with the assembled COX complex. Importantly, Rcf1 can interact with the Cox3 protein and cytochrome  $bc_1$  complexes independently of the assembly state of the COX complex.

#### The Rcf1 and Rcf2 proteins overlap in function and support the activity of the COX enzyme.

Deletion of the *RCF1* gene in a haploid yeast strain did not significantly impact the respiration-based growth of the yeast cells, and weak growth of the  $\Delta rcf1$  strain was observed on glycerol, a nonfermentable carbon source (Fig. 3A). Thus, Rcf1 does not play an essential role in the assembly of the COX (and/or cytochrome  $bc_1$  complex), as the activity of these enzymes is required for growth on glycerol media. BLASTp analysis indicated that Rcf1 is a member of a conserved family of proteins termed the hypoxia-induced gene 1 (Hig1) family (discussed further below), which bears limited similarity to a second yeast mitochondrial membrane protein, Aim38 (YNR018w) (22), referred to in this study as Rcf2. We therefore explored the possibility of genetic interaction between *RCF1* and *RCF2*. Deletion of *RCF2* alone failed to significantly impact the respiration-based growth of the haploid yeast. However, a yeast mutant deficient in both the Rcf1 and Rcf2 proteins, the  $\Delta rcf1 \Delta rcf2$  strain, displayed a strongly impaired growth phenotype on nonfermentable media (Fig. 3A). The COX enzymatic activity levels were severely compromised in the resulting  $\Delta rcf1 \Delta rcf2$  mitochondria and were present at approximately 20% of the levels in the wild-type control mitochondria. Deletion of either *RCF1* or *RCF2* alone only partially affected the COX enzymatic activity levels, with a stronger dependence on the Rcf1 protein being observed (Fig. 3B, top). The expression of Rcf1<sub>His</sub> fully restored the COX enzyme activity levels in the  $\Delta rcf1 \Delta rcf2$  mitochondria, whereas the expression of Rcf2<sub>His</sub> resulted in only a partial recovery of the enzyme activity (Fig. 3B). As the COX levels in the  $\Delta rcf1 \Delta rcf2$ +Rcf2<sub>His</sub> mitochondria were not restored to those in the  $\Delta rcf1$  (i.e., containing endogenous Rcf2) mitochondria, we conclude that the presence of the C-terminal His tag on the Rcf2 protein may have partially compromised Rcf2's stability and/or function. The cytochrome  $bc_1$  enzymatic activity levels were unaffected in the absence of Rcf1 (Fig. 3B, bottom). In contrast, elevated cytochrome  $bc_1$  activity levels were observed in the  $\Delta rcf2$  mitochondria, suggesting that Rcf2 may function to exert control over the cytochrome  $bc_1$  enzyme. When both Rcf1 and Rcf2 were absent, the levels of cytochrome  $bc_1$  activity were partially affected suggesting that Rcf1 and Rcf2 proteins act differently to regulate this enzyme (Fig. 3B, bottom).

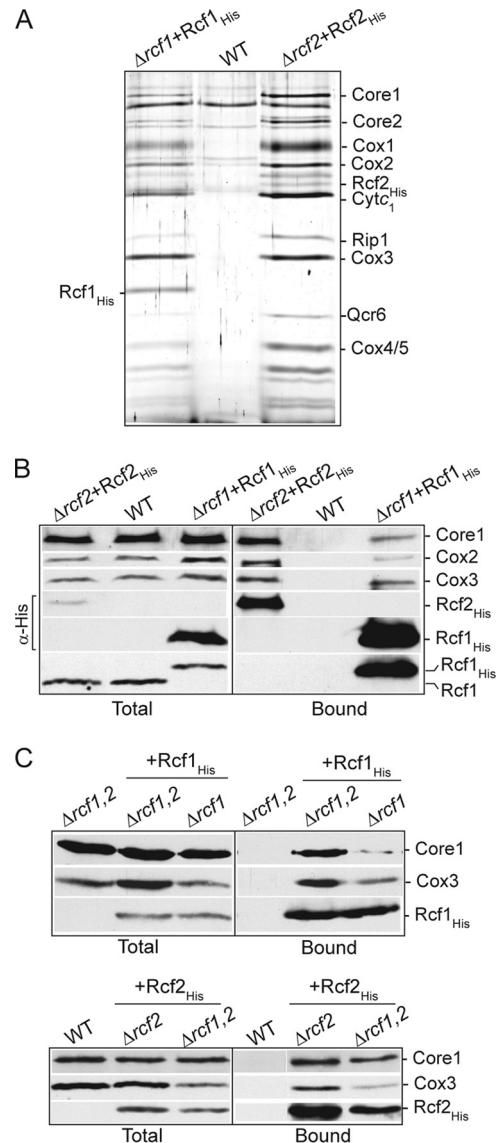
The expression of either the His-tagged Rcf1 or the Rcf2 derivative was observed to largely complement the respiratory growth defect of the  $\Delta rcf1 \Delta rcf2$  mutant, where the His-tagged Rcf1 performed slightly better (Fig. 3C). Although the His-tagged Rcf2 protein failed to fully restore the COX enzyme levels in the  $\Delta rcf1 \Delta rcf2$  mutant, the Rcf2<sub>His</sub> protein and COX activity were clearly present at levels sufficient to sustain respiration-based growth of the  $\Delta rcf1 \Delta rcf2$  strain.

To confirm that Rcf2, like Rcf1, physically associates with the cytochrome  $bc_1$ -COX supercomplex, we expressed Rcf2<sub>His</sub> in a  $\Delta rcf2$  mutant and purified it under digitonin solubilization conditions. Subsequent silver stain analysis indicated that the cytochrome  $bc_1$ -COX supercomplex copurified with Rcf2<sub>His</sub> (Fig. 4A), and was this confirmed with parallel Western blot analysis and



**FIG 3** *RCF1* and *RCF2* genetically interact and together are required to support the COX enzyme activity. (A) Serial 10-fold dilutions of wild-type (WT),  $\Delta rcf1$ ,  $\Delta rcf2$ , or  $\Delta rcf1 \Delta rcf2$  cells were spotted on YP plates containing glucose (YPD) or glycerol (YPG) and grown at 30°C. (B) The COX and cytochrome *bc*<sub>1</sub> relative specific enzyme activities were determined and expressed as a percentage of the value for the wild-type control. The averages of three replicate measurements are given. (C) Serial 10-fold dilutions of wild-type (WT) cells and  $\Delta rcf1 \Delta rcf2$  cells expressing *Rcf1*<sub>His</sub> or *Rcf2*<sub>His</sub> or not (–) were spotted on YP plates containing glucose (YPD) or glycerol supplemented with 0.1% galactose (YPG + 0.1% Gal) and grown at 30°C.

immunodecoration for Core1 of the cytochrome *bc*<sub>1</sub> complex and Cox2 and Cox3 of the COX complex (Fig. 4B). The level of cytochrome *bc*<sub>1</sub>-COX purified with *Rcf2*<sub>His</sub> was higher than with *Rcf1*<sub>His</sub>, and given that the levels of *Rcf2*<sub>His</sub> were lower than those of *Rcf1*<sub>His</sub> (as judged by the His epitope signal; as discussed above, this may indicate that the His epitope compromises that stability of *Rcf2*), it appears that the digitonin-solubilized *Rcf2* protein can



**FIG 4** *Rcf2*, like *Rcf1*, associates with the cytochrome *bc*<sub>1</sub>-COX supercomplex. (A and B) *Rcf1*<sub>His</sub> and *Rcf2*<sub>His</sub> were expressed in their respective null mutant strains ( $\Delta rcf1 + Rcf1_{His}$  and  $\Delta rcf2 + Rcf2_{His}$ ), and mitochondria were isolated and, together with wild-type control mitochondria analyzed in parallel, were solubilized in digitonin and subjected to Ni-NTA chromatography. Following SDS-PAGE of purified material, the gel was subjected to silver staining (A) or Western blotting and immunodecoration (B). (A) The identity of subunits verified by immunoblotting of parallel samples is indicated. (B) The levels of *Rcf2*<sub>His</sub> and *Rcf1*<sub>His</sub> were directly compared using an antibody against the His epitope (indicated by  $\alpha$ -His). The presence of *Rcf1* in the *Rcf2*<sub>His</sub> purified material was analyzed in a parallel sample using antibodies directed against the *Rcf1* protein. Samples were further analyzed as for Fig. 1B. (C) *Rcf1*<sub>His</sub> (top) and *Rcf2*<sub>His</sub> (bottom) were purified under digitonin solubilization conditions from the indicated isolated mitochondria.  $\Delta rcf1, \Delta rcf2$  indicates  $\Delta rcf1 \Delta rcf2$  mitochondria. Samples were further analyzed as described for Fig. 1B, and Cox3 and Core1 were immunodecorated. The recovery of *Rcf1*<sub>His</sub> and *Rcf2*<sub>His</sub> was monitored using an antibody directed against the His epitope.

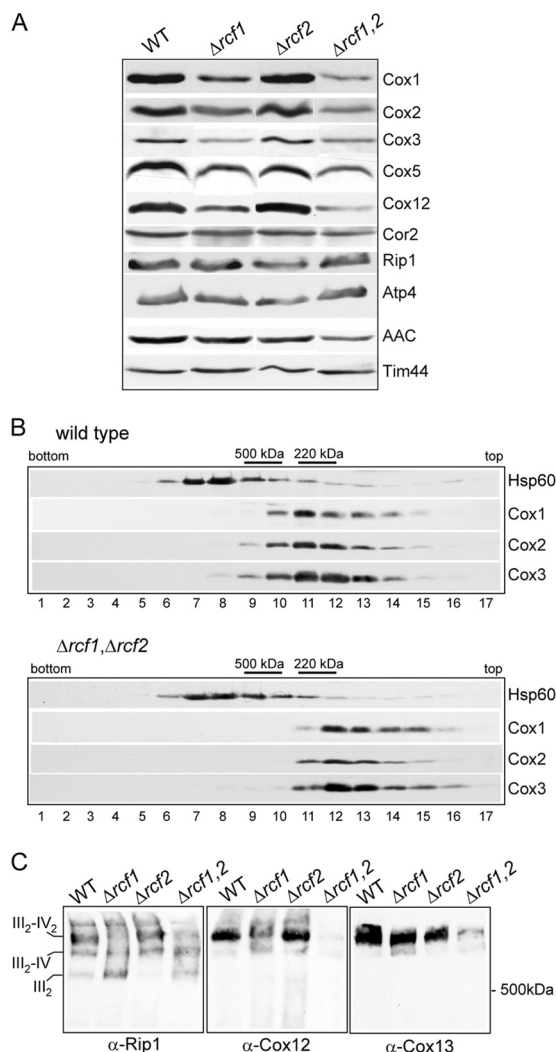
more efficiently (or more stably) associate with the cytochrome *bc*<sub>1</sub>-COX supercomplex than *Rcf1* does. When purified under Triton X-100 conditions, *Rcf2*<sub>His</sub> was not recovered in association with either the cytochrome *bc*<sub>1</sub> or the COX complex (results not

shown), suggesting that its association with the supercomplex may require elements of both complexes and thus is stable only when the supercomplex remains together, i.e., under digitonin conditions, or simply that its association with the supercomplex is not Triton X-100 stable. In contrast to results obtained with Rcf1, we could not detect an association between newly synthesized Cox3 and Rcf2<sub>His</sub> even under milder digitonin solubilization conditions (results not shown). We cannot rule out the possibility that Rcf2 may also directly bind to the COX complex, possibly to the Cox3 subunit, but in a detergent-labile fashion.

Using His-tagged derivatives expressed in the  $\Delta rcf1 \Delta rcf2$  strain, we could show that the association of either Rcf1 or Rcf2 with the cytochrome *bc*<sub>1</sub>-COX supercomplex did not depend on the presence of the other protein (Fig. 4C). These results confirm that Rcf1 and Rcf2 can independently associate with the cytochrome *bc*<sub>1</sub>-COX supercomplex. Consistently, the Rcf1 protein was not detected with the affinity-purified Rcf2<sub>His</sub> complex (Fig. 4A and B). (The lack of available antibodies against Rcf2, and the fact that Rcf2 would comigrate with the cytochrome *c*<sub>1</sub> protein, makes it difficult at present to determine if the converse is true, i.e., that Rcf2 does not copurify with Rcf1<sub>His</sub>.) The independent association of Rcf1 and Rcf2 with the cytochrome *bc*<sub>1</sub>-COX supercomplex raises the interesting possibility that two different forms of this supercomplex, an Rcf1- and an Rcf2-associated one, may exist within mitochondria.

Taking all these results together, we conclude that Rcf1 and Rcf2 together play a critical role in the activity of the COX complex. The pronounced growth defect observed in the absence of both Rcf1 and Rcf2, and the ability of either Rcf1<sub>His</sub> or Rcf2<sub>His</sub> to complement both the growth phenotype and the COX activity defect observed in the  $\Delta rcf1 \Delta rcf2$  mutant, demonstrates that Rcf1 and Rcf2 share overlapping functions. Consistent with their genetic interaction data, Rcf1 and Rcf2 can independently associate with the cytochrome *bc*<sub>1</sub>-COX supercomplex.

**The assembly of the COX complex, and its association with the cytochrome *bc*<sub>1</sub> complex, is altered in the absence of Rcf1 and Rcf2 proteins.** We next addressed the question of whether the reduced COX enzyme levels in the  $\Delta rcf1 \Delta rcf2$  mitochondria was due to an altered assembly of the COX complex. The COX subunits (Cox1, Cox2, and Cox3 proteins) are highly susceptible to proteolytic turnover when their assembly is hindered, so the steady-state levels of these subunits can be used as an indicator of their assembly state. A partial reduction in the levels of these COX subunits was observed in the  $\Delta rcf1$  mitochondria and to a similar extent in the  $\Delta rcf1 \Delta rcf2$  mitochondria, indicating that the presence of Rcf1 was required for optimal stabilization of these proteins (Fig. 5A). However, given that the steady-state levels of these and other COX subunits (e.g., Cox5 and Cox12) were not significantly different between the single  $\Delta rcf1$  mutant and the double  $\Delta rcf1 \Delta rcf2$  mutant, we conclude that a simple reduction in the level of these COX subunits cannot be the underlying reason for the severely impaired COX enzyme activity observed when both Rcf1 and Rcf2 are absent. The levels of subunits from other OXPHOS enzymes, e.g., the Rieske Fe-S protein (Rip1) from cytochrome *bc*<sub>1</sub> and Atp4 from the F<sub>1</sub>F<sub>0</sub>-ATP synthase, were unaffected by the absence of Rcf1 and/or Rcf2. A reduction in the levels of the AAC protein was observed in the  $\Delta rcf1 \Delta rcf2$  mitochondria, suggesting that the accumulation of these transport proteins may be influenced by the presence of the Rcf1 and Rcf2 proteins (Fig. 5A).



**FIG 5** The presence of Rcf1 and Rcf2 is required to secure the correct assembly of the COX complex and its association with the cytochrome *bc*<sub>1</sub> complex. (A) Steady-state levels of the OXPHOS subunits in the mitochondria (50  $\mu$ g) isolated from strains with the indicated genotypes.  $\Delta rcf1,2$  indicates  $\Delta rcf1 \Delta rcf2$  mitochondria. Tim44 was used as a loading control. Rip1, Rieske Fe-S protein. (B) Mitochondria isolated from WT and  $\Delta rcf1 \Delta rcf2$  strains were solubilized in Triton X-100 and analyzed by sucrose gradient centrifugation, as described in Materials and Methods. The fractions corresponding to the bottom and top of the gradient are indicated. Following SDS-PAGE and Western blotting, immunodecoration was performed against the indicated COX subunits and Hsp60, a control marker protein. The positions of the control marker complexes monomeric ATP synthase (500 kDa) and cytochrome *b*<sub>2</sub> (220 kDa) are indicated. (C) Mitochondria isolated from strains with the indicated genotypes were solubilized with digitonin (2%) and subjected to BN-PAGE analysis, Western blotting, and immunodecoration with Rip1, Cox12, and Cox13 antisera.

Knowing that Rcf1 can interact with newly synthesized Cox3, we next addressed the question of whether the presence of Rcf1 (and Rcf2) was required for the assembly of Cox3 with the core subunits of the COX complex. Mitochondrial proteins from the  $\Delta rcf1 \Delta rcf2$  mutant and the wild-type control were solubilized with Triton X-100 and subjected to sucrose gradient centrifugation analysis (Fig. 5B). In the wild-type control, the COX subunits cofractionated with a size consistent with the monomeric enzyme (approximately 200 kDa). Cofractionation of Cox3 with the Cox1



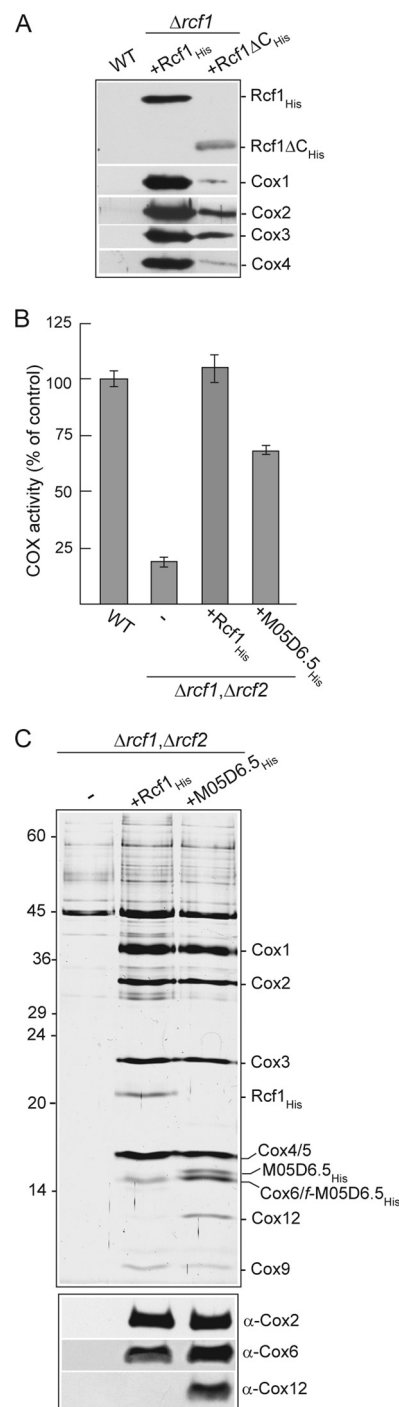
and Cox2 proteins was also observed in the  $\Delta rcf1 \Delta rcf2$  gradient, indicating that the assembly of Cox3 with the core COX subunits did occur in the absence of these Hig1 homologs. We did note that the COX complex in the  $\Delta rcf1 \Delta rcf2$  gradient appeared to be slightly smaller, eluting one fraction closer to the top of the gradient than that in the wild type, pointing to a possible assembly defect of some peripheral COX subunits in the absence of the Rcf1 and Rcf2 proteins (Fig. 5B).

The importance of Rcf1 and Rcf2 for the assembly state of the cytochrome *bc*<sub>1</sub>-COX supercomplex was directly analyzed using digitonin solubilization and BN-PAGE analysis. Using antibodies against the Rieske Fe-S protein (Rip1) of the cytochrome *bc*<sub>1</sub> complex, we observed that the assembly state of this supercomplex was adversely affected in the absence of Rcf1 (Fig. 5C, left). In wild-type mitochondria, as previously described, the majority of the cytochrome *bc*<sub>1</sub> (III)-COX (IV) complex was present in its III<sub>2</sub>-IV<sub>2</sub> form, whereas in the  $\Delta rcf1$  sample, a significant amount of complexes corresponding to III<sub>2</sub>-IV and III<sub>2</sub> forms were observed. The adverse effect on the assembly state of the cytochrome *bc*<sub>1</sub>-COX supercomplex was more pronounced in the  $\Delta rcf1 \Delta rcf2$  mutant but not in the absence of Rcf2 alone. As the sucrose gradient analysis described above indicated that the assembly of peripheral COX subunits may be affected in the absence of Rcf1 and Rcf2, we used this BN-PAGE approach to directly analyze the assembly of the Cox12 and Cox13 proteins, two late-stage-assembled peripheral COX subunits (30). In the wild-type control mitochondria, the Cox12 and Cox13 proteins were found predominantly with the III<sub>2</sub>-IV<sub>2</sub> form of the supercomplex. The assembly of Cox12 into the supercomplex was partially affected in the absence of Rcf1 alone and strongly defective in the  $\Delta rcf1 \Delta rcf2$  double mutant (Fig. 5C, middle). The assembly of the Cox13 protein was also significantly reduced in the absence of Rcf1 and Rcf2 (Fig. 5C, right).

Taken together, our data demonstrate that the COX core subunits, including Cox3, can assemble in the absence of Rcf1 and Rcf2, but these hypoxia-induced gene 1 protein homologs are required to ensure the correct assembly of the cytochrome *bc*<sub>1</sub>-COX supercomplex. Furthermore, the association of the peripheral COX subunits Cox12 and Cox13, with the COX complex was perturbed, a defect that may underlie the observed compromised activity of the COX enzyme in the absence of both the Rcf1 and Rcf2 proteins.

**The ability to support COX enzyme activity is a conserved feature of the Hig1 protein family.** The Hig1 homology domain of Rcf1 encompasses the N-terminal region of the protein, whereas the extended C-terminal tail is found only in fungal Rcf1 proteins (Fig. 1C). In order to address the question of whether the conserved Hig1 homology domain of Rcf1 was responsible for the COX association, we created a His-tagged C-terminally truncated derivative (residues 1 to 112), Rcf1 $\Delta$ C<sub>His</sub>, where the C-terminal 47 residues were deleted. Although the steady-state levels of Rcf1 were compromised through this deletion, affinity purification experiments demonstrated that the Rcf1 $\Delta$ C derivative retained the capacity to interact with the COX complex, as demonstrated by the copurification of the Cox1, Cox2, Cox3, and Cox4 subunits (Fig. 6A). From these data we conclude that the Hig1 homology domain of Rcf1 supports the COX association.

To further demonstrate that the relationship with the COX complex was a conserved feature of the Hig1 proteins, we explored the question of whether a Hig1 homolog from the nematode *C. elegans*, the M05D6.5 gene product, could functionally substitute



**FIG 6** Physical association with and supporting activity of the COX enzyme are a conserved feature of Hig1 proteins. (A) Ni-NTA purification of Rcf1<sub>His</sub> and Rcf1 $\Delta$ C<sub>His</sub> following solubilization with Triton X-100. See Fig. 1B for further details. (B) COX enzyme activity measurements in mitochondria isolated from wild-type and  $\Delta rcf1 \Delta rcf2$  strains harboring Rcf1<sub>His</sub> or the *C. elegans* Hig1 homolog M05D6.5<sub>His</sub>, as described for Fig. 3B. (C) Purification of Rcf1<sub>His</sub> and M05D6.5<sub>His</sub> from the  $\Delta rcf1 \Delta rcf2$  strain performed under Triton X-100 solubilization conditions and analyzed by SDS-PAGE and silver staining (top) or in parallel by immunodecoration for Cox2, Cox6, and Cox12 subunits (bottom). The positions of COX subunits, Rcf1<sub>His</sub>, and M05D6.5<sub>His</sub> (and a smaller proteolytic fragment of this protein, f-M05D6.5<sub>His</sub>) on the silver-stained gel, verified by immunodecoration, are indicated.

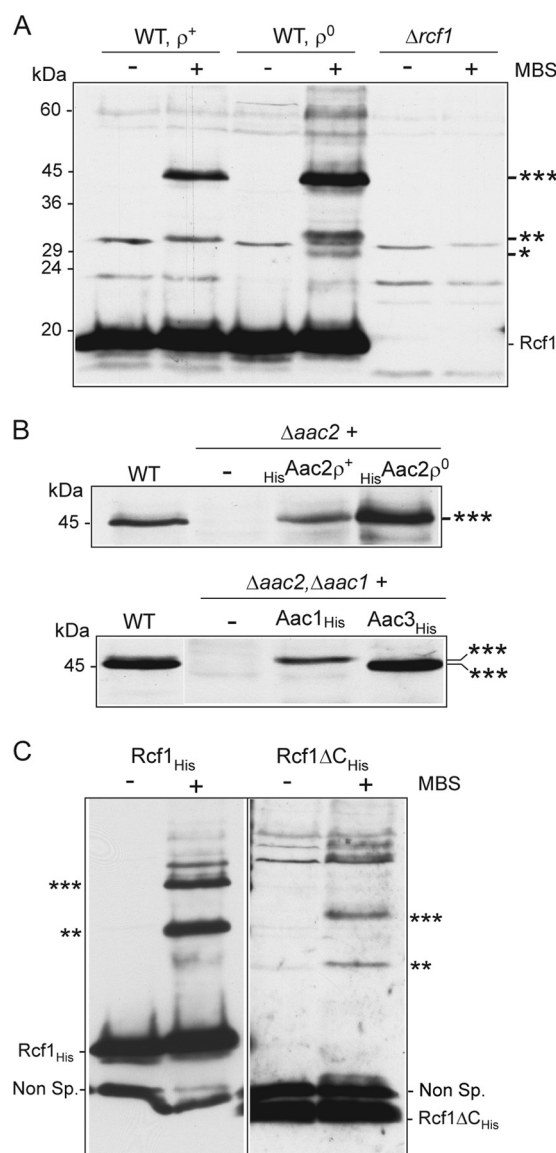


for the Rcf1 (and Rcf2) protein. The His-tagged derivative, M05D6.5<sub>His</sub>, was expressed in the  $\Delta rcf1 \Delta rcf2$  strain, where it was observed to significantly restore the COX enzyme levels in the mitochondria (Fig. 6B). Furthermore, affinity purification of the M05D6.5<sub>His</sub> protein under Triton X-100 solubilization conditions demonstrated its ability to associate with the COX complex, as evidenced by the copurification of COX subunits (Fig. 6C, top). The level of the COX subunits associated with the M05D6.5<sub>His</sub> protein was similar to that with Rcf1<sub>His</sub>, with the notable exception of the Cox12 subunit. As was observed earlier (Fig. 2B), Cox12 did not efficiently copurify with Rcf1<sub>His</sub> (Fig. 6C, bottom); however, in contrast, Cox12 was clearly recovered with the purified *C. elegans* Hig1 homolog, M05D6.5<sub>His</sub> (Fig. 6C).

In summary, these data demonstrate that the ability to associate with and support the activity of the COX complex is not unique to the yeast Rcf1 and Rcf2 proteins but instead represents a conserved feature of the Hig1 protein family, and they carry out this association in a manner that appears to modulate the association of Cox12 with the COX complex.

**The Rcf1 and AAC proteins exhibit a close physical relationship.** We used a chemical cross-linking approach to further probe the molecular environment of the Rcf1 protein. When the heterobifunctional (sulfhydryl/amino) reagent MBS was used, Rcf1 was found to form one dominant cross-link adduct of 45 kDa in wild-type mitochondria and corresponds to Rcf1 (18 kDa) and a partner protein of approximately 27 kDa. The ability of Rcf1 to form this adduct was not adversely affected and was even enhanced in [*rho*<sup>0</sup>] (i.e., mtDNA-deficient) mitochondria, demonstrating that this partnership was not dependent on the coassembly of Rcf1 with the cytochrome *bc*<sub>1</sub>-COX supercomplex, which is absent in [*rho*<sup>0</sup>] mitochondria. Furthermore, Rcf1 formed two less-abundant adducts of 33 kDa and 28 kDa, respectively, again more prominent in [*rho*<sup>0</sup>] mitochondria and corresponding to partner proteins of approximately 15 kDa and 10 kDa, whose identities are currently unknown (Fig. 7A).

Given that the protein Aac2 is known to associate with the cytochrome *bc*<sub>1</sub>-COX complex (7, 11), we addressed the possibility that Aac2 and/or the other AAC isoforms could represent the partner of Rcf1 in the 45-kDa adduct. The ability of Rcf1 to form this adduct was lost in mitochondria isolated from the  $\Delta aac2$  mutant strain. Expression of the HisAac2 derivative in the  $\Delta aac2$  mutant restored the ability of Rcf1 to form this adduct, which again was enhanced when the experiment was performed in the  $\Delta aac2$  HisAac2 [*rho*<sup>0</sup>] background (Fig. 7B, top). *S. cerevisiae* has three different AAC isoforms: the abundant Aac2 and the minor Aac1 isoform are constitutively expressed under aerobic conditions, while the Aac3 isoform is predominantly expressed under anaerobic conditions (33, 34). To test if Rcf1 could also cross-link to both the Aac1 and Aac3 isoforms, we performed cross-linking of Rcf1 in mitochondria from the  $\Delta aac2 \Delta aac1$  mutant harboring His-tagged Aac1 and Aac3 proteins and expressed from a galactose-inducible promoter, GAL10. Rcf1 failed to form the 45-kDa adduct when cross-linking was performed in a  $\Delta aac2 \Delta aac1$  mutant, but when the Aac1<sub>His</sub> or Aac3<sub>His</sub> isoform was expressed in this strain, the ability to form the adduct was restored and the size of the adduct was different: slightly larger when Aac1<sub>His</sub> was present and smaller when Aac3<sub>His</sub> was expressed (Fig. 7B, bottom). We conclude therefore that Rcf1 is physically close to the AAC isoforms within the mitochondrial membrane. The Rcf1 $\Delta$ C protein also displayed the capacity to cross-link to the AAC protein, dem-



**FIG 7** Rcf1 can be chemically cross-linked to AAC proteins. (A) Chemical cross-linking using MBS was performed on mitochondria isolated from wild-type (WT) [*rho*<sup>+</sup>] and [*rho*<sup>0</sup>] strains and the  $\Delta rcf1$  strain. Following SDS-PAGE and Western blotting, immunodecoration with Rcf1 antiserum was performed. As the Rcf1 antibody recognizes some additional protein bands, the  $\Delta rcf1$  mitochondria were analyzed in parallel to distinguish the Rcf1-specific adducts observed in WT mitochondria. \*\*\*, \*\*, and \*, 45-, 33-, and 28-kDa Rcf1-containing adducts, respectively. (B) Cross-linking of Rcf1 was performed in mitochondria isolated from the wild type and from the  $\Delta aac2$  strain or the  $\Delta aac2$  strain harboring HisAac2 protein in a [*rho*<sup>+</sup>] or [*rho*<sup>0</sup>] background (top) or from the  $\Delta aac2 \Delta aac1$  strain harboring Aac1<sub>His</sub> or Aac3<sub>His</sub> (bottom). Samples were further analyzed as described for panel A. Only the area of the gel encompassing the 45-kDa Rcf1-containing adduct (\*\*\*) is shown in both cases. (C) MBS cross-linking of Rcf1<sub>His</sub> and Rcf1 $\Delta$ C<sub>His</sub> was performed and further analyzed as described for panel A. The resulting Western blot was immunodecorated with antiserum which was directed against the His epitope but which also recognizes a nonspecific (Non Sp.) band of approximately 15 kDa. \*\*\* and \*\*, Rcf1-containing adducts.

onstrating that the association of Rcf1 with AAC proteins is a property of the Hig1 homology domain and not of the fungus-specific C-terminal domain of Rcf1 (Fig. 7C). No data to support a Rcf2-AAC interaction, however, have been obtained to date (re-

sults not shown), suggesting that the association with the AAC family members may be a specific property of the Rcf1 protein.

We conclude from these observations that the Hig1 homology domain of the Rcf1 protein interfaces with the AAC proteins and in a manner that is independent of Rcf1's or AAC's assembly with the cytochrome *bc*<sub>1</sub>-COX supercomplex.

## DISCUSSION

We report here the identification of a novel component of the cytochrome *bc*<sub>1</sub>-COX supercomplex, the Rcf1 protein, which is a member of the conserved hypoxia-induced gene 1 (Hig1) family. Rcf1 was shown to genetically interact and share overlapping functions with the related Rcf2 protein, and both proteins can independently associate with the cytochrome *bc*<sub>1</sub>-COX supercomplex. We have chosen to term these Hig1 homologs, formerly referred to as Aim31 and Aim38 (22), the Rcf1 and Rcf2 proteins, or respiratory supercomplex factors 1 and 2, respectively. Our findings here indicate that the presence of Rcf1 and Rcf2 is particularly important for the enzymatic activity and correct assembly of the COX complex and its organization with the cytochrome *bc*<sub>1</sub> supercomplex. Importantly, we demonstrate here that the *C. elegans* Hig1 homolog, M05D6.5, could substitute for the Rcf1/Rcf2 proteins in yeast, in its ability both to physically associate with the OXPHOS supercomplex and to support the enzyme activity of the COX complex. Thus, we conclude that their functional relationship with the respiratory supercomplex, and in particular with the COX enzyme, is an important and conserved feature of the Hig1 protein family members.

Although Rcf1 (formerly Aim31) and Rcf2 (formerly Aim38) were found in a genetic screen designed to identify mutants which display altered inheritance of mtDNA (22), our data indicate that the loss of COX enzyme activity in the absence of Rcf1 and Rcf2 proteins is not due to a possible loss of mtDNA and thus coding potential for the Cox1, Cox2, and Cox3 subunits. Cytochrome *b*, a key component of the cytochrome *bc*<sub>1</sub> complex, is also encoded by the mtDNA, and the levels of this enzyme were not affected to the same extent as those of the COX complex in the absence of the Rcf1 and Rcf2 proteins and were even enhanced when Rcf2 alone was absent. Furthermore, *in organello* translation experiments showed that the capacity to synthesize Cox1, Cox2, Cox3 and cytochrome *b* was unaffected by the absence of Rcf1 and Rcf2 (results not shown). Thus, we conclude that the strong COX defect in the  $\Delta rcf1 \Delta rcf2$  mutant is not indirectly due to limiting mtDNA levels but rather is directly due to the absence of Rcf1 and Rcf2 proteins and thus reflects the importance of these proteins for the correct assembly and function of the COX enzyme.

The Rcf1 and Rcf2 proteins represent novel cytochrome *bc*<sub>1</sub>-COX supercomplex components. Although both proteins are required to support the activity of the COX complex and although Rcf1, in particular, exhibits a strong affinity for the COX enzyme and Cox3 protein, we recognize that these proteins are unique as COX components because they display the ability to associate with the cytochrome *bc*<sub>1</sub> complex in the absence of an assembled COX complex. Furthermore, the assembly of the cytochrome *bc*<sub>1</sub>-COX supercomplexes was altered when Rcf1 and Rcf2 were absent, consistent with the notion that these proteins may act as bridges by supporting both enzymes of the supercomplex. It is possible that the observed association of the Rcf1 protein with the cytochrome *bc*<sub>1</sub> complex in the absence of COX complex assembly may be facilitated through Rcf1's association with Cox3, if Cox3 can as-

sociate with the cytochrome *bc*<sub>1</sub> complex, prior to the completion of the COX assembly pathway. Further experimentation using Cox3 synthesis-defective mutants will be required to explore this possibility. On a separate note, it is important to point out that, while both Rcf1 and Rcf2 could be found in association with the cytochrome *bc*<sub>1</sub>-COX supercomplex, Rcf1 did not copurify with the Rcf2 protein, and our findings indicate that both proteins could independently associate with the OXPHOS supercomplex. These observations imply that at least two distinct populations of the cytochrome *bc*<sub>1</sub>-COX exist within the mitochondrial membrane system, an Rcf1-associated population and an Rcf2-associated one. The functional significance of these two different cytochrome *bc*<sub>1</sub>-COX supercomplex populations is currently unknown; however, one could speculate that their existence enables the regulation of the OXPHOS supercomplex through alternative mechanisms.

The demonstration here that the *RCF1* and *RCF2* genes genetically interact and that the Rcf1 and Rcf2 proteins can functionally substitute for each other is consistent with the documentation in the *Saccharomyces* genome database that Rcf2 (Aim38) displays a limited similarity to the Rcf1 (Aim31) and the Hig1 proteins and also agrees with an earlier report that these proteins cofractionated with the OXPHOS supercomplex on native gels (21). Our BN-PAGE analysis here, however, indicated that not all of the abundant Rcf1 protein was recovered with the cytochrome *bc*<sub>1</sub>-COX supercomplex and that a significant fraction of the protein was found in lower-molecular-mass complexes. Whether the smaller populations of the Rcf1 protein represent a proportion of the protein whose association with the cytochrome *bc*<sub>1</sub>-COX supercomplex was labile under the detergent conditions used, or whether a non-supercomplex-associated population of Rcf1 may exist in the mitochondria, is currently unclear. In contrast to traditional COX subunits, the levels of Rcf1 are not adversely affected in the absence of COX (or cytochrome *bc*<sub>1</sub>) assembly, e.g., in [*rho*<sup>0</sup>] mitochondria, an observation which may argue that a stable non-COX-associated Rcf1 population normally exists in the mitochondria. It is therefore possible that Rcf1/Hig1 proteins exhibit a dynamic association with the OXPHOS supercomplex that may serve to regulate the COX enzyme (and possibly cytochrome *bc*<sub>1</sub>) activities in response to certain metabolic cues.

We demonstrate here that Rcf1 physically associates with newly synthesized Cox3 prior to its assembly into the COX complex, suggesting that Rcf1 and the Hig1 proteins may represent Cox3-specific chaperones. Consistently, we observed that the steady-state levels of nonassembled Cox3 in the COX assembly mutants were elevated by increasing the levels of Rcf1, indicating that Rcf1 may serve to stabilize the nonassembled Cox3 protein (results not shown). On the other hand, our findings would suggest that Rcf1 does not represent an essential Cox3 chaperone in the traditional sense. First, the assembly of the Cox3 protein (as evidenced by its steady-state levels and coassociation of the COX core subunits Cox1 and Cox2) was not severely affected in the absence of Rcf1 (and Rcf2). Second, Rcf1 is retained with the COX complex following the assembly of Cox3, a behavior untypical of chaperones, which commonly dissociate from the relationship once the assembled state has been established. We propose that the observed association between Rcf1 and the unassembled Cox3 reflects a close physical and functional partnership between these two proteins, and one that is initiated before and continued after the assembly of the COX enzyme is complete. The demonstrated

association with Cox3 enables us to map a potential site of contact between the Rcf1/Hig1 protein family members and the COX complex. The Cox3 subunit is conserved throughout prokaryotic and eukaryotic evolution, and although it does not directly contribute to the electron transfer and O<sub>2</sub> reduction events, it is thought to play a supportive or regulatory role within the COX enzyme, possibly involving conformational changes in the Cox3 protein, and may be involved in adaptive changes to oxygen levels (32, 36, 41). The observed Rcf1-Cox3 relationship possibly creates a mechanism for Rcf1 to influence and regulate the activity of the COX enzyme, perhaps communicated through conformational changes in Rcf1 and/or Cox3 (or the associated cytochrome *bc*<sub>1</sub> or AAC protein; see below). Cox3 together with Cox2 and Cox12 (mCox6b) defines the binding site for the enzyme's substrate, cytochrome *c* (9, 23, 41). In addition to Cox3, a number of our results here also point to a possible connection between Rcf1 and Cox12 subunit. First, the assembly of Cox12 into the cytochrome *bc*<sub>1</sub>-COX supercomplex was particularly affected in the  $\Delta$ rcf1  $\Delta$ rcf2 mutant. Second, although the *C. elegans* Hig1 homolog M05D6.5<sub>His</sub> displayed a capacity similar to that of Rcf1<sub>His</sub> to associate with the COX enzyme, Cox12 was efficiently recovered with the purified nematode protein Hig1 but not with the yeast protein Rcf1. This observation may indicate that the Hig1 proteins function to influence the level or the nature of Cox12's association with the COX complex, possibly in a dynamic fashion, and that the *C. elegans* M05D6.5<sub>His</sub> protein displays an altered capacity in this respect, possibly due to the heterologous nature of the system. It is interesting in this respect that the phenotype of the  $\Delta$ rcf1  $\Delta$ rcf2 mutant is reminiscent of the yeast *cox12* null mutant; both contain a high level of the core COX subunits and display strongly reduced (about 15% of wild-type levels for  $\Delta$ cox12) yet measurable COX enzyme levels (28). Taking these results altogether, we suggest that the Hig1 proteins may serve to influence the substrate (cytochrome *c*) binding site of the COX complex, as defined by the arrangement of the Cox2, Cox3, and Cox12 (and possibly Cox13) proteins.

A close relationship between Rcf1 and the members of the AAC proteins family is also reported here. Thus, it is possible that Rcf1 is centrally positioned between the cytochrome *bc*<sub>1</sub>-COX supercomplex and the AAC proteins and that it functions to communicate between these complexes to regulate the activity of the OXPHOS enzyme in response to a perceived metabolic need of the cell. The ability of the Rcf1 to covalently cross-link to the AAC proteins was found to be differentially influenced by the specific inhibitors of the AAC enzymes atractyloside and bongkreik acid (results not shown), inhibitors which each induce different conformations in the AAC proteins (33). Thus, we consider it possible that the Rcf1 protein may sense changes in the conformational states in the AAC protein and influence the activity of the COX complex through its ability to contact Cox3 and possibly the Cox2 and Cox12 proteins. Further investigation though is required to explore the proposed relationship between the AAC and Hig1 family members and their proposed influence on the cytochrome *bc*<sub>1</sub> and/or COX enzymes.

The defining members of the Hig1 protein family were described in screens measuring genes that become upregulated under hypoxic conditions (1, 10, 19, 38, 42). Most Hig1 homologs documented in the databases since, however, have been identified through sequence comparisons to the founding family members, as the Hig1 proteins display a high degree of sequence conserva-

tion. Furthermore, in many cases multiple isoforms within the same species have been documented. Based on their sequence alignments, the Hig1 family members are subdivided into two distinct but closely related subgroups, the Hig1 type 1 and Hig1 type 2 subgroups. The Hig1 family members shown in the genetic screens to be upregulated under hypoxia and stress conditions predominantly belong to the Hig1 type 1 subgroup (1, 19, 10, 42), and from genome analysis this subgroup appears to be restricted to higher eukaryotes. We therefore consider it possible that the Hig1 type 2 subgroup may represent constitutively expressed isoforms, which function under nonstress conditions to support the COX complex activity and supercomplex stability. On the other hand, the Hig1 type 1 subgroup may represent isoforms expressed largely under hypoxic conditions, when it is necessary to assemble a COX complex optimized for operation under limiting O<sub>2</sub> levels. In this respect, the regulation of the Hig1 type 2 and type 1 isoforms may be similar to that of the yeast Cox5a/5b (and mammalian Cox4-1/Cox4-2) system, where the Cox5b (mCox4-2) isoforms are expressed under hypoxic conditions and contribute to the assembly of a COX complex refined to operate under low O<sub>2</sub> tensions (5, 7, 16, 26, 37). Interestingly, the AAC proteins, demonstrated here to exhibit a close physical relationship to Rcf1, also exist as multiple isoforms, one of which (Aac3 in yeast) is upregulated under hypoxic and anaerobic conditions (3, 18, 33, 34). It is important to note that no Hig1 type 1 homolog exists in *S. cerevisiae* or in *C. elegans* and that Rcf1, Rcf2, and M05D6.5 most closely resemble the Hig1 type 2 isoforms. Consistently, our preliminary data do not indicate a role for Rcf1 (or Rcf2) in supporting yeast growth under hypoxic or anaerobic conditions (results not shown). So the presence of a hypoxic-induced (type 1) isoform of Hig1 may be a feature limited to higher eukaryotes.

In summary, our data demonstrate that the Hig1 protein family members associate with the mitochondrial cytochrome *bc*<sub>1</sub> and COX complexes, where they function to support the supercomplex organization and the activity of these enzymes, in particular the COX complex. Interestingly, putative homologs of the Hig1 proteins also exist in alphaproteobacteria, where their function is currently unknown. On the basis of our findings on the requirement of the Rcf1 and Rcf2 proteins for the assembly of an active COX enzyme, we propose that the Hig1 protein family, like the Shy1/SURF1 proteins (13, 30, 31), may represent evolutionarily important COX assembly and regulatory factors. Given that the Cox3 subunit, a primary contact point for Hig1 proteins in the mitochondrial COX, is conserved in bacteria, it is tempting to speculate that the prokaryotic Hig1 proteins also serve to interact with and modulate the activity of the COX enzyme through their Cox3 subunits.

## ACKNOWLEDGMENTS

The cDNA clone encoding the *C. elegans* M05D6.5 Hig1 homolog was a kind gift from Yuji Kohara, Center for Genetic Resource Information, National Institute of Genetics, Mishima, Japan. We acknowledge and thank Mary Dienhart, Angela Tollefson, and Ru Ya for their contributions to this project. We are grateful to Klaus Pfanner, Freiburg, Germany, for the valuable gift of the Cox6, Cox12, and Cox13 subunit antisera.

The research was supported by NSF grants MCB 0744067 and NIH GM089699 to R.A.S.

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